Characterisation of dicarbonyl stress-mediated hepatocyte dysfunction: Impact on hepatokines and xenobiotic metabolising enzymes

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Abstract

Type 2 diabetes mellitus (T2DM) is a disease that has been associated with hyperglycaemia-mediated complications and an increased risk of liver disease, including non-alcoholic fatty liver disease (NAFLD). However, more recently, the glucose metabolite methylglyoxal (MGO), rather than hyperglycaemia *per se,* has been implicated as the major mediator, via dicarbonyl stress resulting in cellular dysfunction. MGO is a highly reactive dicarbonyl compound and exerts its damaging effects by causing irreversible damage to proteins, lipids, and DNA. Most previous studies investigating the role of MGO in cellular toxicity and dysfunction were conducted using exogenously applied MGO in unphysiological concentrations that are significantly higher than what is observed in individuals with both type 1 and type 2 diabetes. Although providing valuable insight into the cellular effects of MGO, this approach does not accurately reflect the natural occurrence of MGO accumulation within hepatocytes in diabetes. Therefore, the overall objective of this research study was to mimic, in a more physiological manner, endogenous MGO accumulation observed in diabetes by inhibiting glyoxalase 1 (GLO1), the key detoxifying enzyme for MGO. The specific aim of this study is to examine the effects of an established GLO1 inhibitor, S-p-bromobenzylglutathione cyclopentyl diester (BBGC), on hepatocyte function and survival.

To study the effects of GLO1 inhibition in hepatocytes, the human hepatoma G2 (HepG2) cell line was used, and HepG2 cells were treated with increasing concentrations of BBGC to first characterise its effects. After the treatment duration was completed, intracellular hydroimidazolone (MG-H1) formation, which is indicative of MGO levels, was determined using confocal microscopy following immunolabelling with a human anti-MG-H1 antibody. HepG2 cell viability was assessed a using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and cell death by flow cytometric analysis following Annexin V and propidium iodide staining. Intracellular ROS generation was measured following staining with the 2′,7′-dichlorodihydrofluorescein diacetate probe by confocal microscopy and similarly, mitochondrial superoxide generation was measured following MitoSOX™ staining. HepG2 mitochondrial function was assessed by using flow cytometry to measure changes in mitochondrial membrane potential following staining with tetramethylrhodamine ethyl ester. Cellular ATP levels were also determined, by luminometry. Changes in mRNA expression of cytokines interleukin 8 (IL-8) and tumour necrosis factor-alpha, and hepatokines fibroblast growth factor 21 (FGF21) and growth differentiation factor

15 (GDF15) were also determined using reverse transcription-quantitative polymerase chain reaction (RT-PCR), and IL-8, FGF21 and GDF15 secretion was measured, using an ELISA kit. Transcription factor nuclear factor kappa B (NF-κB) activation was assessed by measuring nuclear translocation of NF-κB p65 via confocal microscopy following immunolabelling with human anti-NF-κB p65. mRNA and protein expression of key endoplasmic reticulum (ER) stress markers (CHOP and GRP78) were determined by RT-PCR and Western blotting. mRNA expression of angiotensin converting enzyme 2 was also measured by RT-PCR. Next, we investigated the mRNA and protein expression and activity of key enzymes cytochrome P450 3A4 and carboxylesterase 1 (CES1) in HepG2 cells. This was achieved using RT-PCR, Western blotting, and specific activity assay kits, respectively.

We identified that BBGC increased intracellular MG-H1 levels in hepatocytes, indicative of increased intracellular methylglyoxal concentrations and dicarbonyl stress. Our results revealed that BBGC-induced dicarbonyl stress led to decreased HepG2 cell viability, and increased ER stress and apoptosis rates, likely mediated through mitochondrial dysfunction and increased ROS generation. BBGC-mediated dicarbonyl stress increased hepatocyte inflammation, as evidenced by the induction of IL-8 expression and secretion, and NF-κB activation. In addition to corroborating previous findings linking MGO and dicarbonyl stress to these deleterious effects, this study identified new mechanisms by which MGO could instigate cellular dysfunction that could contribute to the development of the well-established liver complications of diabetes. MGO triggered hepatokines GDF15 and FGF21 expression and release, likely reflecting their dysregulated secretion during cellular stress and disease, which highlights the pathophysiological relationship between NAFLD, and other complications related to diabetes. MGO also decreases key drug metabolizing enzyme CES1, which could possibly explain altered pharmacokinetics and discrepancies in treatment outcomes in diabetic patients. CES1, having profound effects on key hepatocyte functions, such as lipid and glucose metabolism, makes this effect also particularly relevant in the context of NAFLD development in diabetes. CES1 inhibition also leads to increased IL-8, FGF21 and GDF15 mRNA expression and release, implicating CES1 in the modulation of important inflammatory mediators in HepG2 cells

We have demonstrated that inhibition of GLO1 is a more physiologically relevant model of inducing dicarbonyl stress in hepatocytes, which will allow further investigation into the role MGO and dicarbonyl stress plays in increasing the susceptibility of developing liver disease in

diabetes. In addition, we have expanded upon our knowledge of the effects of dicarbonyl stress on hepatocytes, implicating it in the disruption of xenobiotic metabolism and increased hepatokine release.

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Abbreviations

ATF4	Activating Transcription Factor 4
AGE	Advanced Glycation End Products
BBGC	S-p-Bromobenzylglutathione-Cyclopentyl-Diester
BiP/GRP78	Binding Immunoglobulin Protein/78 Kda Glucose-Regulated Protein
CES1	Carboxylesterase 1
CHOP	C/EBP Homologous Protein
CYP3A4	Cytochrome P450 3A4
DME	Drug-Metabolizing Enzyme
ER	Endoplasmic Reticulum
FFA	Free Fatty Acid
FGF 21	Fibroblast Growth Factor 21
GDF15	Growth Differentiation Factor 15
GLO1	Glyoxalase 1
IL	Interleukin
IR	Insulin Resistance
MGO	Methylglyoxal
MG-H1	MGO-Derived Hydroimidazolone
MMP	Mitochondrial Membrane Potential
NAFLD	Non-Alcoholic Fatty Liver Disease
NASH	Non-Alcoholic Steatohepatitis
NF-κB	Nuclear Factor-Kappa B
RAGE	Receptor For Ages
ROS	Reactive Oxygen Species

T2DM	Type 2 Diabetes Mellitus
TNF-α	Tumour Necrosis Factor α
UPR	Unfolded Protein Response

Key words

Dicarbonyl stress, Hepatocytes, Diabetes mellitus, NAFLD, Oxidative stress, Hepatokines, Inflammation, Carboxylesterase 1

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Author’s declaration

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree and does not incorporate any material already submitted for a degree.

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1. General Introduction

Background

Diabetes mellitus (DM) is a metabolic disorder characterised by chronic hyperglycaemia and impaired protein, carbohydrate, and lipid metabolism. DM is a complex disorder of multiple aetiology and is classified into different types, the main ones being type 1 (T1D), type 2 (T2DM), and gestational diabetes. Its pathogenesis could either involve the specific destruction of pancreatic insulin-secreting β-cells (Chen et al., 2017; Mauricio, 2016), leading to lack of insulin production in patients, or the progressive development of insulin resistance (IR), loss of β-cell sensitivity to high glucose levels and the progressive deterioration of β-cell response leading, eventually, to the loss of β-cell function. The lack of insulin secretion or loss of insulin sensitivity hinders the action of insulin on its target tissues, which in turn results in impaired carbohydrate, lipid and protein metabolism in individuals with diabetes (2014; Chen et al., 2017; Mauricio, 2016; World Health, 1999) and devastating macrovascular and microvascular complications. Diabetes has emerged as a leading cause of mortality and disability, and overall decreased quality of life in patients, with vascular complications being a significant contributor, accounting for 26.8% (Ling et al., 2020).

DM remains a substantial public health issue. As of March 2024, over 5 million people have been diagnosed with both types of diabetes in the UK, according to The British Diabetic Association's latest figures (JackW, 2024). Approximately 96% of total cases are T2DM (Ong et al., 2023) and in 2021, it was estimated that globally, there were 536.6 million people living with diabetes mellitus, and alarmingly, this number is expected to range between 783.2 million and 1.31 billion by 2050, according to current trends (Ong et al., 2023; Sun et al., 2022). According to the World Health Organisation (WHO), in 2019, the Global Burden of Disease Study reported that diabetes and diabetes-related disease caused an estimated 2 million deaths (WHO, 2023), but in 2021, as the COVID-19 pandemic was ongoing, this figure was estimated to surpass 6.7 million according to the International Diabetes Federation (IDF) (Magliano et al., 2021).

Also known as "insulin-dependent diabetes" or "juvenile-onset diabetes", T1D is the consequence of the chronic, T-cell-mediated, autoimmune destruction of the β-cells of the islets

of Langerhans (Bensellam et al., 2012; Chen et al., 2017; Mauricio, 2016), which are located in the pancreas, resulting in a loss of both β-cell function and regeneration and consequently, insulin deficiency (Mauricio, 2016; Poretsky, 2010). The most important risk factor for the prevalence of this type of diabetes is genetic as it may be hereditary. As noted above, T1D is considered, with near uniformity, to represent a disorder that is "autoimmune" in nature—meaning that patients often present features reflective of an immunological contribution to their disease pathogenesis (e.g., autoantibodies, genes associated with immune-related genetic susceptibly, etc.) (Atkinson, 2012).

The pathogenesis of T2DM, however, is more progressive and complex (Chatterjee et al., 2017; Mauricio, 2016; Poretsky, 2010). Initially, increased metabolic demand and increased peripheral IR trigger β-cell compensation mechanisms and hyperinsulinemia (Muoio & Newgard, 2008). However, the compensatory β-cell response often becomes insufficient and usually results in the deterioration of β-cell function and loss of β-cell mass, along with observable hyperglycaemia (Bensellam et al., 2012; Chen et al., 2017; Mauricio, 2016; Poretsky, 2010). Together, pancreatic β-cell dysfunction and the consequent impaired insulin secretion are fundamental to the progression of the disease to its end stage, which is characterised by β-cell failure, and ultimately β-cell death and insulin deficiency in patients (Bo et al., 2016; Chatterjee et al., 2017; Chen et al., 2017; Mauricio, 2016; Muoio & Newgard, 2008). At this stage, T2DM patients depend on exogenous insulin for blood and tissue glucose control and energy metabolism. Thus, T2DM is characterised by hyperglycaemia, receptor insensitivity to endogenous insulin (IR), and the progressive loss of β-cell insulin secretion.

Among age, ethnicity and other co-morbidities, obesity is a major risk factor for the development of T2DM as increased body fat, particularly visceral fat, contributes to the development of IR (Khan et al., 2020; Nigro et al., 2019; Tinajero & Malik, 2021). The increase in T2DM is correlated to the increase in the incidence of adults presenting with high BMI, which has been estimated to affect 42% of the global adult population in 2020, and reach 50% by 2030 (WOF, 2024). According to Taylor's analysis of the United Kingdom Prospective Study, in 2014, over 60% of T2DM patients were overweight or obese at the time of diagnosis (Taylor & Holman, 2014; Zhu et al., 2019). In addition to the excess risk of developing a wide range of vascular diseases, cardiovascular disease and renal disease notably (Morrish et al., 2001), data show that diabetes is associated with substantial premature mortality from several

cancers, infectious diseases, external causes, intentional self-harm, and degenerative disorders, independently of major risk factors (Rao Kondapally Seshasai et al., 2011).

Complications of Diabetes and the risk of liver disease

Chronic hyperglycaemia, when uncontrolled, could cause acute, life-threatening ketoacidosis or nonketotic hyperosmolar syndrome (Negera et al., 2020). Despite different aetiology, clinical presentation and disease prevalence, long-term complications of diabetes occur in both type 1 and 2 DM as a result of uncontrolled hyperglycaemia (Shamsaldeen et al., 2016). These include impairment of growth in younger patients, microvascular complications such as diabetic retinopathy, nephropathy, peripheral neuropathy, and autonomic neuropathy. DM is also associated with vascular dysfunction as part of its macrovascular complications, leading to hypertension and an increased incidence of atherosclerotic, cardiovascular, peripheral arterial, and cerebrovascular disease in patients (Lu et al., 2023; Nigro et al., 2019; Shamsaldeen et al., 2016). According to the IDF, around 72% of patients already present some degree of diabetes complications by the time the diagnosis is made (IDF, 2023). These complications may not only lead to a reduced quality of life and increased mortality in patients but have also created a large economic burden on individuals and healthcare systems around the world (Lu et al., 2023; Shamsaldeen et al., 2016). In the UK, as of 2020, the NHS spent about £10 billion – which constituted 10% of its entire budget, per year on the management of diabetes, which was mostly spent on the treatment of complications related to the disease (Whicher et al., 2020).

Furthermore, as diabetes is a metabolic disease, characterised by major biochemical and functional abnormalities in the liver (Daniel et al., 2013), and often associated with dyslipidaemia (Nigro et al., 2019), T1DM and T2DM patients manifest a higher global prevalence of hepatobiliary diseases (Daniel et al., 2013). More recently, liver complications, particularly in T2DM, have been identified as having a major impact on morbidity and mortality, as the liver is a vital organ that is responsible for many important functions that contribute to the maintenance of systemic metabolism, including glucose homeostasis, regulating blood clotting factors, production of plasma proteins, etc (Kalra et al., 2023).

Indeed, the liver plays a central role in regulating glucose, lipid and cholesterol metabolism and is essential for maintaining normal physiology (Han et al., 2016; Kim & Yang, 2021; Watt et al., 2019). Systemic energy homeostasis is highly dependent on the hepatic regulation of

metabolic activities, encompassing energy production, expenditure, storage and redistribution (Kim & Yang, 2021). In order to normalize blood glucose concentrations, hepatic glucose uptake is enhanced in the postprandial state as a result of increased insulin and plasma glucose levels. Afterwards, the absorbed glucose either gets utilised for fatty acid (FA) synthesis in a process named *de novo* lipogenesis or stored as glycogen in the liver. In response to insulin, hepatic glucose production and glycogenolysis are suppressed (Stefan & Häring, 2013; Watt et al., 2019). Conversely, during nutrient deficiency, the liver stimulates hepatic glucose production and glycogen breakdown via transcriptional and non-transcriptional mechanisms, thus optimising blood glucose concentrations and increasing the supply of glucose as fuel to peripheral tissues such as the brain, skeletal muscle and adipose tissue (Kim & Yang, 2021). Lipid homeostasis is regulated by the liver in a similar manner. When circulating FA levels start to rise, hepatic FA uptake is stimulated by insulin, and well as the hepatic synthesis and storage of triglycerides (TG) and *de novo* lipogenesis (Hodson & Gunn, 2019), to promote long-term lipid storage. Liver lipid levels are regulated by the interplay between lipid influx to the liver and their hepatic uptake, lipid synthesis, oxidation, and secretion within very low–density lipoproteins (VLDLs) (Mohamed et al., 2016). Under nutrient deficiency, the liver attempts to fulfil peripheral tissue energy demands through lipid oxidation and the production of ketone bodies, to be utilised as alternative fuel sources (Geisler et al., 2019; Hodson & Gunn, 2019; Kim & Yang, 2021). The dysregulation of these hepatic processes during metabolic dysfunction could directly lead to glycaemic dysregulation and dyslipidaemia. During IR, insulin signalling fails to adequately stimulate glucose uptake into peripheral tissues, suppress hepatic glucose production, and inhibit adipose tissue lipolysis (Samuel et al., 2004), leading to hyperglycaemia and increased levels of circulating free fatty acids (FFA). When IR starts to manifest in the liver, an excess storage of ectopic fat in the liver occurs, which promotes the onset of pathophysiological changes in the liver that can contribute to the development of non-alcoholic fatty liver disease (Kim & Yang, 2021).

Non-alcoholic fatty liver disease (NAFLD) is defined by the accumulation of fat in the liver, termed hepatic steatosis, in the absence of excessive alcohol consumption and excluding other causes of steatosis (Targher et al., 2021). Hepatic steatosis is clinically characterized by the presence of TG-rich macrovesicular and/or microvesicular lipid droplets in >5% of hepatocytes or 5% of liver weight (Nassir et al., 2015; Ress & Kaser, 2016). NALFD encompasses a spectrum of liver pathology, from the less severe hepatic steatosis, also known as non-alcoholic fatty liver (NAFL), which progresses to non-alcoholic steatohepatitis (NASH) with varying

presentations of fibrosis, cirrhosis and, finally hepatocellular carcinoma (HCC) and terminal liver failure (Barros et al., 2017; Francque et al., 2021; Mohamed et al., 2016; Regnell & Lernmark, 2011; Younossi et al., 2019). NAFLD is strongly correlated with fundamental disorders of the metabolic syndrome including obesity, dyslipidaemia, IR and T2DM (Federico et al., 2016; Giorda et al., 2017; Parry & Hodson, 2020; Yki-Järvinen, 2014), it is thus considered the hepatic manifestation of the metabolic syndrome. Metabolic dysfunction-associated fatty liver disease (MAFLD) has recently been proposed as a more appropriate umbrella term (Eslam, Newsome, et al., 2020; Eslam, Sanyal, et al., 2020). Eventually, the term metabolic dysfunction-associated steatotic liver disease (MASLD) has been proposed, which can be diagnosed based on a patient meeting one of five cardiovascular risk factors, unlike MAFLD, which required that patients meet two of seven parameters of metabolic dysfunction (Chen et al., 2024).

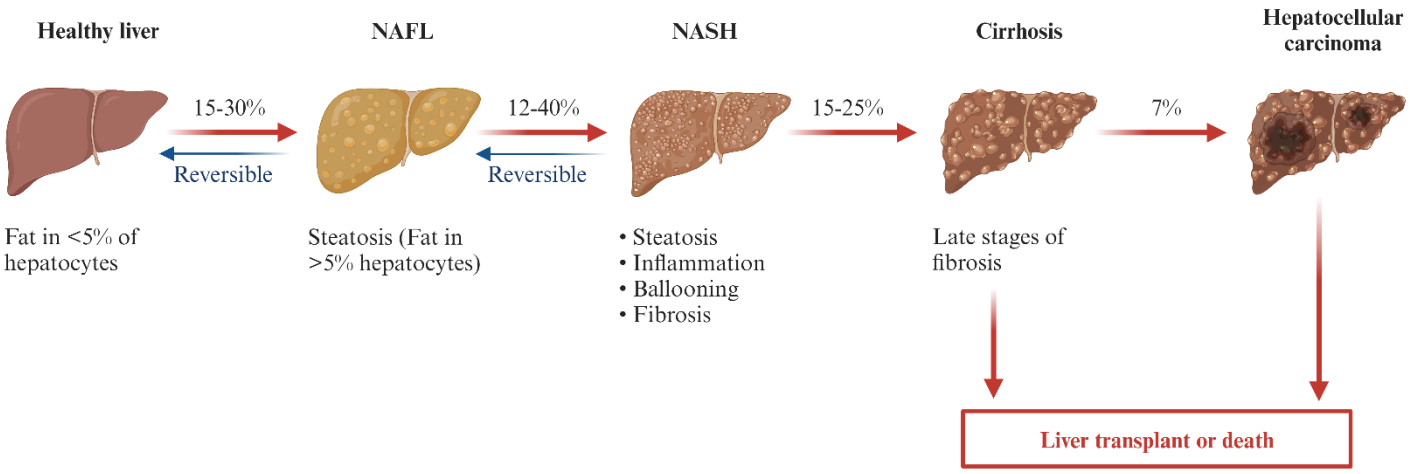


Figure 1.1. The spectrum of NAFLD. Representation of the natural progression of NAFLD pathology in stages, from simple steatosis into more severe forms NASH and Cirrhosis, the latter irremediable. Reprinted from "Non-Alcoholic Fatty Liver Disease (NAFLD) Spectrum", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

As previously discussed, the loss of glucose homeostasis in T2DM, which follows the compensatory hyperinsulinemia and progressive β-cell dysfunction, is a result of both IR and β-cell dysfunction. IR is an integral feature of the metabolic syndrome and is one of the key pathogenic factors for both NAFLD and T2DM and, thus, these two conditions commonly coexist (Targher et al., 2021). It has also long been recognized that obesity is associated with T2DM and NAFLD, as obesity, along with genetic predisposition, is the most common cause of IR (Rohm et al., 2022). In addition to being influenced by ethnic background and genetic

factors, the analysis of liver histology from human livers suggests that the prevalence rates of steatosis and steatohepatitis are approximately 15% and 3%, respectively, in non-obese persons, 65% and 20%, respectively, in persons with class I and II obesity (BMI 30.0–39.9 kg/m^2), and 85% and 40%, respectively, in extremely obese patients (BMI ≥40 kg/m^2), demonstrating the close relationship between obesity and the onset of the disease (Fabbrini et al., 2010). Data from 2017 reports that in the UK, the prevalence of hepatic steatosis was 19.9% (Wilman et al., 2017) and in 2020, a study found that 1 in 5 young adults may present with steatosis – half of these cases being severe steatosis (Abeysekera et al., 2020). However, in 2023, the NHS stated that up to 1 in 3 people now have hepatic steatosis, reflecting the rapid growth in its prevalence (NHS24, 2023).

Indeed, the prevalence of NAFLD is increasing worldwide at approximately the same rate as the epidemics of obesity and T2DM (Targher et al., 2021). It has become the largest chronic liver disorder in prevalence, estimated to afflict 38% of individuals globally as of 2023, representing an increase of >50% within the past 3 decades (Younossi et al., 2023). From the same study, the global prevalence of NASH has been estimated to be 5.27% (Younossi et al., 2023).

The link between NAFLD and T2DM remains ambiguous regarding the pathogenesis of these two interrelated conditions. It has been described as complex and bidirectional by many studies whereby the presence of one drives the progression of the other (Nigro et al., 2019; Younossi et al., 2019). NAFLD was shown to play a major role in the development of systemic and hepatic IR and altered glucose tolerance in T2DM, and the risk of developing T2DM parallels the severity of NAFLD (Younossi et al., 2019). Fasting and postprandial hyperglycaemia also correlate with the degree of hepatic steatosis in patients, and hepatic steatosis is epidemiologically associated with defects in insulin signalling and action (Loria et al., 2013; Watt et al., 2019). It has been closely associated with IR in multiple organs independently of whole body adiposity, as supported by several studies demonstrating impaired insulin action in both lean and non-diabetic obese individuals with NAFLD (Bugianesi et al., 2005; Korenblat et al., 2008; Seppälä-Lindroos et al., 2002; Vega et al., 2007; Watt et al., 2019). The development of hepatic steatosis was also suggested to precede the accumulation of lipids in skeletal muscle, macrophage-driven inflammation, extrahepatic IR, and hyperglycaemia, indicating it may serve as an early indicator of systemic metabolic dysregulation (Kim & Yang, 2021; Turner et al., 2013; Watt et al., 2019). Evidence also suggests that hepatic TG

accumulation, exacerbates hepatic IR and increases systemic levels of insulin antagonizing cytokines (Smith & Adams, 2011b). Thus, the onset of NAFLD may not only predispose individuals towards IR, but also exacerbate existing IR, initiating a vicious cycle of hepatic TG accumulation and worsening IR (Younossi et al., 2019).

The prevalence of T2DM or impaired fasting glucose (>6.0 mmol/l) is 18–33% in patients with NAFLD (Smith & Adams, 2011a), although diabetes itself is also recognised as a major risk factor for NAFLD development (Younossi et al., 2019). Although variable, epidemiological data estimates 30- to even 80% of patients with T2DM have NAFLD (Ajmera et al., 2023; Dai et al., 2017; Kosmalski et al., 2013; Kosmalski et al., 2023; Nigro et al., 2019; Rajput & Ahlawat, 2019; Stefan & Cusi, 2022; Yamane et al., 2022; Younossi et al., 2019; Younossi et al., 2023; Zhou et al., 2021). Both BMI and glycated haemoglobin, Haemoglobin A1c (HbA1c), are predictors of NAFLD development in patients with T2DM (Kosmalski et al., 2023). Research conducted by Chen and Jiang showed that not only higher BMI and IR, but also elevated fasting plasma glucose concentration and TG are important risk factors for NAFLD development (Chen & Jiang, 2022). The specific factors in T2DM that increase the risk of NAFLD remain unclear, but they share the pathogenic abnormalities of excess metabolic influx, such as glucose and adiposity, and IR (Smith & Adams, 2011b).

In T2DM, in addition to causing further distortion of glucose metabolism, IR inhibits FFA oxidation and promotes increased lipolysis, increasing the levels of circulating FFA, which subsequently accumulate in the liver due to increased uptake. Concurrently, elevated glucagon levels inhibit hepatic TG output. Furthermore, hepatic lipogenic transcription factors, sterol regulatory element-binding protein 1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP) are upregulated in response to hyperinsulinemia and hyperglycaemia, respectively (Kumar et al., 2021; Mohamed et al., 2016; Smith & Adams, 2011b). Therefore, an imbalance in the uptake, synthesis, export and oxidation of FFA in the liver all contribute to the onset of hepatic steatosis in diabetes. Hyperglycaemia was shown to worsen liver damage and promote fibrogenesis and carcinogenesis and a diet rich in fructose, which is a highly lipogenic sugar, was linked to increased IR and the development of NAFLD and NASH (Loria et al., 2013).

Thus, NAFLD and T2DM might have a synergistic association, as both T2DM and NAFLD are associated with adverse outcomes of the other (Kim et al., 2024; Loria et al., 2013). In

addition to NAFLD being and a major cause of death from both cirrhosis and HCC in diabetes patients (Loria et al., 2013), the presence of T2DM is a risk factor for severe disease and poor outcomes in patients with NAFLD (Smith & Adams, 2011a). Patients with NAFLD and T2DM are also more likely to develop NASH — According to Younossi et al., the global prevalence of NASH in patients with T2DM is estimated to be 37.3% , more than seven times higher than in people without diabetes, and the estimated prevalence of advanced fibrosis is 17% (Targher et al., 2021; Younossi et al., 2023). T2DM has been associated with an accelerated rate of hepatic fibrosis progression (Adams et al., 2005), and the prevalence of NASH increases further to 21–40% in obese patients with T2DM. Consequently, T2DM along with obesity and age have been demonstrated to be risk factors for the development of advanced fibrosis in NAFLD (Loria et al., 2013; Nassir et al., 2015; Ress & Kaser, 2016). This may be explained by findings wherein T2DM patients with NAFLD, tend to have more severe metabolic disturbances, including greater visceral adiposity and insulin resistance, more severe dyslipidaemia and higher levels of inflammatory markers such as interleukin 6 (IL-6) and tumour necrosis factor α (TNF-α), compared with those without (Banerji et al., 1995; Kelley et al., 2003; Smith & Adams, 2011b; Toledo et al., 2006).

The presence of NAFLD among individuals with T2DM also appears to be a risk factor for increased mortality; a community-based study of patients with T2DM found that those with NAFLD had a 2.2-fold increased risk of mortality compared with those without NAFLD (Adams et al., 2010). The most common causes of death were malignancy (which accounted for 33% of deaths), liver-related disease and ischemic heart disease, both of which accounted for 19% of deaths. Indeed, evidence is mounting that NAFLD may be a marker of cardiovascular risk and mortality in individuals with T2DM, which is the most common cause of death in patients with T2DM (Ling et al., 2020; Smith & Adams, 2011a; Targher et al., 2016; Targher et al., 2010). Chronic liver disease and/or HCC is the fourth most common cause of death among patients with T2DM, occurring at the rate of one in 20 deaths. The relative risk of a liver-related death is directly related to severity of diabetes mellitus, with those requiring oral hypoglycaemic medications having a 4.9-fold increased risk and those requiring insulin having a 6.8-fold increased risk compared with those on diet control alone (de Marco et al., 1999).

Xenobiotic metabolism

In addition to the metabolism of endogenous compounds, another indispensable function of the liver is xenobiotic metabolism. Due to its complexity and heterogeneity, multiple medications

from various pharmacological classes are often required in patients with T2DM to manage the existing hyperglycaemia, prevent future complications and treat comorbidities. Undoubtedly, as a therapeutic strategy, polypharmacy seems promising, but the clinical outcome of drug treatments can vary greatly between individuals and even within the same individual (Schmitt et al., 2011). Consequently, certain patients may be subject to reduced drug efficacy and/or increased risk for developing adverse drug events (ADEs) and drug-interactions (Wilkinson, 2005). T2DM patients in particular tend to show large variability in drug treatment outcomes, including glycaemic control, and in the incidence of ADEs among patients receiving the same treatment (Manolopoulos, 2007; Pacanowski et al., 2008; Pearson, 2009). The progression of the disease and the treatment itself both can disrupt the expression and activity of drug transporters and drug-metabolizing enzymes (DMEs), potentially triggering pharmacokinetics-based drug interactions due to disruptions in drug absorption, distribution, metabolism (F. Chen et al., 2018). Clinical studies examining the impact of NAFLD on pharmacotherapy are fewer, but the available evidence strongly highlights the potential of NAFLD to equally lead to alterations of drug pharmacokinetic profiles, causing variable drug response, ADEs and possibly toxicity (Cobbina & Akhlaghi, 2017).

For several medications, their pharmacokinetic and pharmacodynamic relationships are determined by the activity level and expression of the cytochrome P450 (CYP) superfamily, comprising the main group of drug-metabolising enzymes (Gravel et al., 2019b). The CYP P450 is a large superfamily of integral membrane conserved proteins present in animals, plants, and microorganisms (Nebert & Russell, 2002) and comprises 57 CYP genes and 58 pseudogenes arranged into 18 families and 43 subfamilies in man (Nelson et al., 2004). The cytochrome P450 superfamily is located primarily in liver, small intestine, and kidney (Renaud et al., 2011; Thelen & Dressman, 2009). CYPs P450 enzymes catalyse different oxidation and some reduction reactions to metabolise exogenous (xenobiotic) and endogenous compounds (Chang & Kam, 1999; Guengerich, 2017). The CYP 3A4 is the most expressed CYP in the liver and intestine and may represent about 60% of total all CYP in liver (Dostalek et al., 2011). It belongs to the CYP3A subfamily (other members include CYP 3A5, 3A7 and 3A43) that metabolise endogenous substrates, for instance, cortisol, oestradiol, progesterone, and testosterone and more that 50% of currently used medications (Dostalek et al., 2011), (Darakjian et al., 2021; Martignoni et al., 2006) including anti-diabetic drugs such as glibenlamide, glipizide, pioglitazone, alogliptin, saxaglptin, canagliflozin, nateglinide, and repaglinide.

In addition to CYP P450 enzymes playing an important role in xenobiotic metabolism, hydrolysis of ester-containing compounds in the body is accomplished in part by enzymes called carboxylesterases (CES) (Crow et al., 2012) and the hydrolytic metabolism catalysed by CES is often a detoxification reaction (Ross et al., 2012). CES subtypes include CES1, CES2, CES3, CES4A, CES5A, and a CES1-like pseudogene, CES1P1. CES1 and CES2 are the two most prevalent subtypes (Holmes et al., 2010), although CES1 is far more abundant than CES2 in the human liver (Godin et al., 2007). CES1 is considered a phase I drug-metabolizing enzyme, and a proteomics study has shown that CES1 is the most abundant drug-metabolizing enzyme in human liver microsomes, responsible for 80%–95% of total hydrolytic activity in the liver (Achour et al., 2017; Imai, 2006). Moreover, analysis of the human liver proteome indicates that CES1 is the tenth most abundant protein (out of >6000) expressed in this tissue (Sun et al., 2010). CES are responsible for in the metabolism of a variety of ester-containing drugs, prodrugs, and environmental toxins. Such compounds include the anticancer prodrugs (such as irinotecan, capecitabine), opioids and stimulants (cocaine, heroin, and meperidine), angiotensin-converting enzyme inhibitors (ramipril, enalapril, temocapril, imidapril and quinapril), prodrug-type angiotensin II type 1 receptor blocker candesartan cilexetil, and other drugs with ester moieties (oseltamivir, clopidogrel, flumazenil, procaine, oxybutynin, delapril, flutamide, and prasugrel) (F. Chen et al., 2018; Hosokawa, 2008; Imai, 2006; Ishizuka et al., 2013; Ross & Crow, 2007; Satoh & Hosokawa, 1998; Shi et al., 2016) and many other clinically important medications, such as methylphenidate and dabigatran. Thus, the potential for alterations in CES expression and activity should be fully appreciated when drugs are prescribed in diabetic patients, notably antihypertensives.

Moreover, recent studies have reported the relevance of CES to several metabolic disorders, indicating these enzymes might be potential targets for the actual treatment of these metabolic diseases (J. Liu et al., 2022; D. Wang et al., 2018). Strikingly, CES1 and CES2 protein expression in liver and adipose tissues are strongly related with disorders such as NASH and obesity (Lian et al., 2018; D. Wang et al., 2018). These findings indicate a key role for CES in endogenous lipid and glucose metabolism and inflammation, and thus CES may be significantly involved in diseases that exhibit dysregulation of these processes, such as diabetes and NAFLD (Dolinsky et al., 2004; Ghosh et al., 2010; J. Liu et al., 2022; Ross et al., 2012; Ross et al., 2010; Wei et al., 2010; Xie et al., 2010).

Collectively, with the ongoing global epidemic of T2DM and NAFLD and the fact that a subgroup of patients with both T2DM and NAFLD are at higher risk of developing advanced stages of liver disease, CVD and other cardiometabolic complications, makes these conditions an important priority for health care and research. As the liver plays a central role in lipid metabolism and glycaemic regulation, any disturbance in these processes gradually contributes to T2DM. However, a lot of evidence suggests that T2DM itself may mediate the development of progressive liver disease as a result of hyperglycaemia mediated toxicity, thereby establishing a vicious cycle. Understanding the pathways by which T2DM increases the susceptibility to developing liver disease, are critical to mitigating the progression of the disease.

Deleterious effects of chronic hyperglycaemia on cellular function

The pathogenesis of liver disease in diabetic patients is likely to be mediated by IR, compensatory hyperinsulinemia, and various hyperglycaemia-induced mechanisms that are well-known and well-established, and interconnectedly responsible for the complications in patients with DM (Bora & Shankarrao Adole, 2021; Korac et al., 2021) (Mohamed et al., 2016; Smith & Adams, 2011b). Despite biological differences between peripheral metabolic organs and tissues, substantial evidence supports that metabolic stress, such hyperglycaemia, could compel the progression of multiple fundamental cellular events that, through cellular dysfunction, drive or aggravate pathological features of T2DM and their vicious cycles, such as IR and the development of diabetic complications (Cao et al., 2023; Targher et al., 2021). According to current hypotheses, the progression of NAFLD is mediated by multiple "hits". In T2DM, following the development of steatosis, the pathological crosstalk that ensues between toxic FFAs, hyperglycaemia and detrimental cellular processes linked to the disease, establish a state of chronic low inflammation (Donath & Shoelson, 2011; Rohm et al., 2022; Wensveen et al., 2015), endoplasmic reticulum (ER) stress (Salvado et al., 2015; Xu et al., 2021), mitochondrial dysfunction (Rovira-Llopis et al., 2017), lipid peroxidation (LPO) damage (Yazıcı & Sezer, 2017), and cell death (Cao et al., 2023). Altogether, this usually leads to inflammatory attack, protein turbulence (Arunagiri et al., 2019), reactive oxygen species (ROS) accumulation i.e., oxidative stress, ATP deficiency, (Angelova & Abramov, 2016; Kumar et al., 2021) and ceramide overload (Yazıcı & Sezer, 2017), accelerating hepatocellular injury and the progression of the disease into steatohepatitis (Hijona et al., 2010; Mantena et al., 2008; Smith & Adams, 2011b).

Pathways involved in hyperglycaemia-mediated cellular oxidative stress

Multiple metabolic and signalling pathways have been implicated in the deleterious effects of chronic hyperglycaemia on cell function and recognised as major contributors to oxidative stress and the development of diabetic complications (Brownlee, 2001; Mauricio, 2016; Robertson et al., 2004; Suh et al., 2017). Oxidative stress is a state of elevated ROS levels in the cell that exceed the capacity of endogenous antioxidant defence mechanisms (Daniel et al., 2013; Gjorgjieva et al., 2019; Sena et al., 2013). This can occur when there is an excessive generation of ROS, decreased antioxidant system activity, or both. Subsequently, these free radicals lead to cellular dysfunction and apoptosis (Daniel et al., 2013; Sena et al., 2013; Zheng et al., 2016). ROS, mainly superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}HO$), can in turn result in the generation of other free radicals, nitric oxide (NO) and peroxynitrite (ONOO-) also called reactive nitrogen species (RNS) (Daniel et al., 2013; Gjorgjieva et al., 2019; Sena et al., 2013). In physiological concentrations, endogenous ROS help to maintain homeostasis - ROS/RNS are important signalling messengers for proliferation, differentiation, apoptosis, and other critical events during cellular development. However, when ROS accumulate in excess for prolonged periods of time, they lead to chronic oxidative stress which activates destructive signalling pathways and causes oxidative damage to various cellular structures such as lipid membranes, enzymes and nucleic acids, which is detrimental to cellular function (Robertson et al., 2004; Sena et al., 2013). Nevertheless, the liver is equipped with potent antioxidants systems, such as superoxide dismutase (SOD), catalase (CAT) and the glutathione (GSH) enzyme family, including glutathione-S-transferases (GSTs) and glutathione peroxidases (GPxs), that serve to protect the liver cells from oxidative damage by neutralising free radicals (Jiang & Török, 2014; Mohamed et al., 2016). There are also nonenzymatic antioxidants present, such as coenzyme Q10, and ROS binding proteins, such as thioredoxin (Jiang & Török, 2014).

The liver is the main detoxification organ of the body and plays an important role in controlling normal glucose homeostasis, and as a collection of insulin-sensitive tissues, the liver is among the primary organs susceptible to the effects of hyperglycaemia-induced oxidative stress, thereby triggering the inflammatory cascade (Mohamed et al., 2016). Both oxidative stress and inflammatory responses are recognised as predominant factors in aggravating the pathological condition of DM and NAFLD (Daniel et al., 2013). Chronic oxidative stress in diabetes is a consequence of elevated blood and intracellular glucose concentrations and its

immediate biochemical sequelae, like the by-products of mobilised compensatory pathways involved in glucose metabolism and clearance (Daniel et al., 2013; Schalkwijk & Stehouwer, 2020). The excessive formation of ROS is the result of multiple phenomena, further elaborated below, that occur when metabolic systems become overwhelmed, such as mitochondrial overproduction of ROS during oxidative phosphorylation, increased substrate flux into the polyol pathway and the hexosamine pathway, increased glucose auto-oxidation, increased glycation of proteins and thereby the formation of advanced glycation end products (AGEs) and protein cross-linking (Chang et al., 2016; Daniel et al., 2013; Gjorgjieva et al., 2019; Sena et al., 2013; Zheng et al., 2016). This in turn activates damaging pathways such as Protein kinase C (PKC) pathway and AGE - receptor for AGEs (RAGE) signalling (Sena et al., 2013), all of which have been suggested to cause tissue damage mainly through oxidative stress in the pathogenesis of diabetes and its complications (Daniel et al., 2013; Zheng et al., 2016).

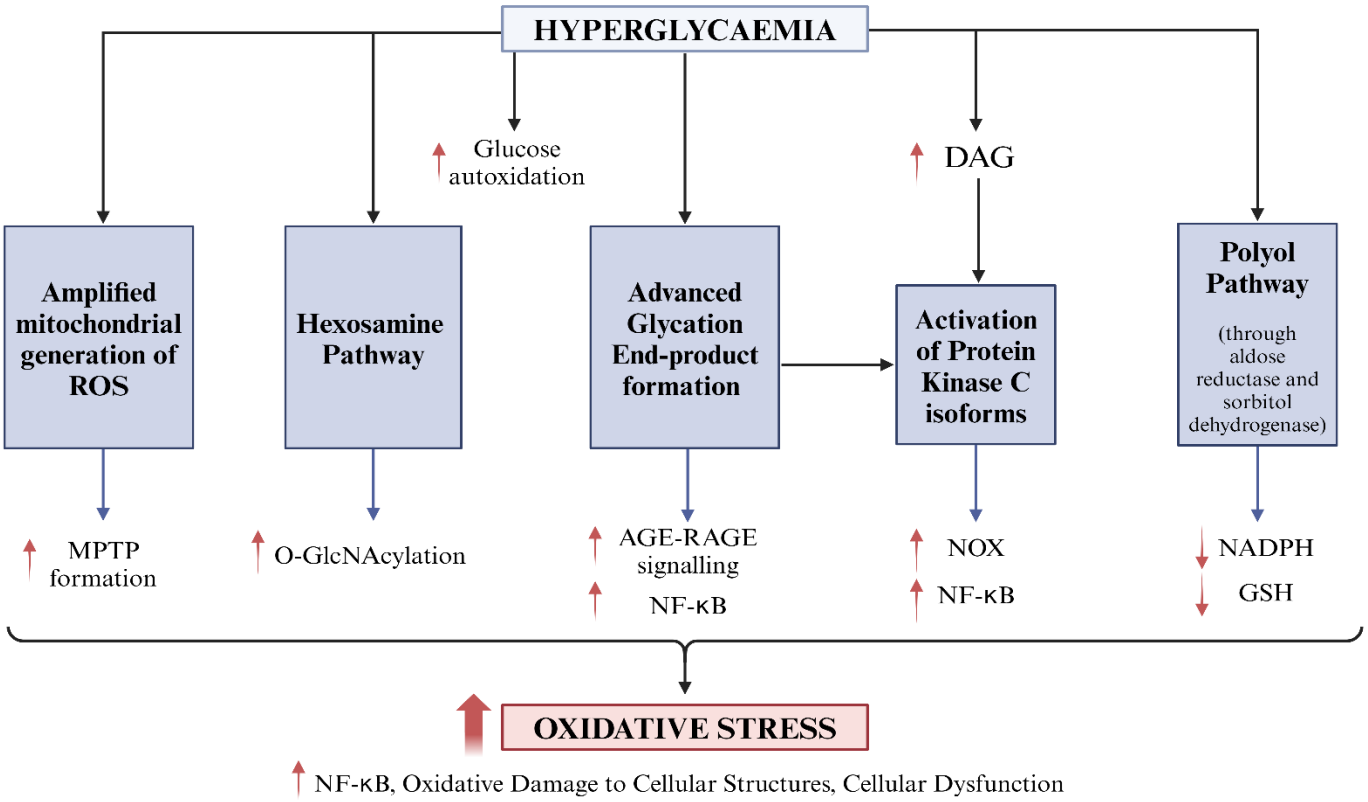


Figure 1.2. The pathways that mediate hyperglycaemia-induced cellular oxidative stress. Representation of the various pathways by which hyperglycaemia contributes to increased cellular oxidative stress. DAG, diacylglycerol, MPTP, mitochondrial permeability transition pore, NADPH, nicotinamide adenine dinucleotide phosphate, NOX, NADPH oxidase, NF-κB, nuclear factor-kappa B. Created with BioRender.com.

Increased flux of glucose and other sugars through the polyol pathway

Usually dormant, this pathway is activated in response to persistently elevated glucose levels. It can metabolize up to 30% of the glucose pool in diabetes (González et al., 2023; Zheng et al., 2016). The polyol pathway leads to the conversion of glucose to sorbitol and further to fructose, catalysed by aldose reductase and sorbitol dehydrogenase, respectively (Gjorgjieva et al., 2019). This reduces the availability of cofactors such as nicotinamide adenine dinucleotide phosphate (NADPH), hence breaching the redox balance nicotinamide dinucleotide NADH: NAD^+ ratio and increasing oxidative stress (Gjorgjieva et al., 2019; Zheng et al., 2016). Furthermore, glutathione peroxidase–glutathione reductase system uses NADPH to regenerate the reduced form of glutathione (GSH) from the oxidized form of glutathione (GSSG) (Giacco et al., 2010). Reduced NADPH can thus impair this cellular antioxidant system's efficacy, further reinforcing oxidative stress (Gjorgjieva et al., 2019; González et al., 2023; Zheng et al., 2016). Additionally, fructose metabolism bypasses glucokinase and phosphorfructokinase-1 in

the glycolytic pathway and thus is less regulated, thereby inducing further metabolic stress (Zheng et al., 2016).

Overactivation of the hexosamine pathway

The increased flux of substrates through the hexosamine pathway is another way hyperglycaemia exerts its deleterious effects (Gjorgjieva et al., 2019) where UDP-N-acetylglucosamine (UDP-GlcNAc) is generated as an end product from fructose-6-phosphate. This was found to not only increase intracellular H_2O_2 levels but also the formation of (O)-linked glycoproteins through protein O-GlcNAcylation (Gjorgjieva et al., 2019; Zheng et al., 2016). This protein modification is considered a major factor in the development of insulin resistance, diabetes and diabetic complications as it can worsen glucotoxicity in the liver through O-GlcNAcylation of FoxO1 in hepatocytes. This leads to an increase of transcriptional activity that upregulates the expression of glucose 6-phosphotase, increasing hepatic gluconeogenesis, further aggravating hyperglycaemia (Gjorgjieva et al., 2019; Zheng et al., 2016)

Activation of protein kinase C isoforms

Protein kinase C (PKC) isoforms form a family of serine and threonine kinases that regulate several major cellular responses, including cell differentiation and cell proliferation, gene expression, protein secretion and inflammatory responses (Hiramatsu et al., 2002). PKC isoforms are activated by hyperglycaemia through AGEs and diacylglycerol (DAG) (Gjorgjieva et al., 2019; Robertson et al., 2004; Sena et al., 2013). Increased DAG formation in chronic hyperglycaemia occurs via increased *de novo* synthesis (Giacco et al., 2010). Glycolytic intermediates dihydroacetone phosphate and glycerol 3-phosphate undergo stepwise acylation to lysophosphatidic acid and phosphatidic acid, which in turn forms DAG. The availability of these intermediates is increased as a result a decreased activity of the glycolytic enzyme 3-phosphate dehydrogenase (GAPDH), which is mediated by oxidative stress and commonly observed in diabetes (Giacco et al., 2010). Higher PKC activity has also been detected in tissues from diabetic patients, and *in vitro* and *in vivo* hyperglycaemic models exhibit elevated total cellular DAG content which leads to PKC overactivation (Mount & Pollak, 2007). The pathways induced by PKC overactivation are involved in the pathogenesis of the IR and the liver disease. Out of the fifteen isoforms in humans, PKC-β1 and PKC-δ activation are of particular interest in this context as they have been associated with increases

in transforming growth factor beta (TGF-β) (Xia et al., 2008), hence increasing collagen IV (Xia et al., 2006), vascular endothelial growth factor and fibronectin production, promoting fibrogenesis in cells, NADPH oxidase (NOX) activity, nuclear factor-kappa B (NF-κB) activation and ROS generation (O_2- or H_2O_2) (Gjorgjieva et al., 2019; Jiang & Török, 2014; Robertson et al., 2004). It was also discovered high glucose-induced ROS generation in mesangial cells was linked to a PKC-dependent induced NOX activity (Kitada et al., 2003; Xia et al., 2007; Xia et al., 2008).

While enzymes such uncoupled nitric oxide synthase (NOS), xanthine oxidase, cytochrome P4502E1 (CYP2E1), lipoxygenase, peroxidases and other haemoproteins are recognised sources of ROS, (Gjorgjieva et al., 2019; Jiang & Török, 2014; Sena et al., 2013) the NOX, which are membrane-bound specialized enzymes, have emerged as being one of the main sources of cellular ROS (Daniel et al., 2013). Among the seven NOX homologues found in mammals (NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2), the main ROS-producing NOXs in the liver are NOX1, NOX2, and NOX4 (Jiang & Török, 2014). In chronic liver diseases, such as alcoholic and non-alcoholic steatohepatitis (ASH and NASH), which are characterised by inflammation, fibrosis and cirrhosis, oxidative stress plays a key role, wherein NOXs are believed to be major contributors. NOX1 was found to be upregulated in fibrotic livers, and increased NOX activity was observed in high fat diet (HFD) fed rats, models of obesity, which positively correlated with an increase in LPO (Jiang & Török, 2014). Interestingly, in glomerular podocytes, the inhibition of either NOX or mitochondrial metabolism suppressed high glucose-induced ROS formation (Susztak et al., 2006) and in human aortic endothelial cells exposed to hyperglycaemic conditions, increased expression of NOX1, oxidative stress, and proinflammatory markers, were all abrogated with the use of a NOX inhibitor (Gray et al., 2013).

Amplified mitochondrial generation of ROS

Thought of as the powerhouse of the cell, the mitochondrion is a vital organelle that regulates apoptosis, buffers calcium levels, and manages the cell cycle, in addition to providing energy to the cell via oxidative phosphorylation (OXPHOS) (Casanova et al., 2023; Clemente-Suárez et al., 2023). OXPHOS is a process wherein mitochondrial enzymatic complexes that make up the electron transport chain (ETC) mediate the oxidation of sugars, lipids, and proteins to produce adenosine triphosphate (ATP), which provides readily releasable energy to the cell (Clemente-Suárez et al., 2023; Dunn & Grider, 2024). ROS are constantly produced through

this process by the ETC, in both pathological and non-pathological conditions (Gjorgjieva et al., 2019; Qian et al., 2019), making the mitochondria one of the main sources of cellular oxidative stress.

Acetyl-CoA, generated by β-oxidation of FFA within the mitochondria and glucose-derived pyruvate oxidation, enters the tricarboxylic acid cycle (TCA) cycle, where energy from its chemical bonds is released as electrons that are taken up by flavin adenine dinucleotide (FAD) and NAD+. These molecules — NADH and FADH2 in their reduced forms, transmit high-energy electrons to the ETC to ultimately produce ATP (Dornas & Schuppan, 2020; Nascè et al., 2022; Prasun et al., 2021). In the presence of elevated substrate influx, such as glucose and FFAs, cellular respiration and β-oxidation are increased, respectively, which increases the levels of metabolites within the mitochondria, electron donors from the TCA such as NADH, and the leakage of electrons from mitochondrial complexes I and III of the ETC (Cao et al., 2023; Pun et al., 2014). These are able to react with molecular oxygen, generating excess ROS such as superoxide O^{-}_{2} and H_2O_2 from the mitochondrial respiratory chain (Gjorgjieva et al., 2019; Jiang & Török, 2014; Nascè et al., 2022; Wang et al., 2020). Changes in the mitochondrial electrochemical gradient directly affect the formation of ROS, as small increases in the electrochemical gradient result in large elevations of ROS production (Miwa & Brand, 2003). ROS and ATP imbalances can usually be rectified by the mitochondria owing to their robust structural plasticity, and hepatocytes are particularly highly enriched with mitochondria (Shum et al., 2021). Maintaining the function of mitochondria is critical in metabolic disorders (Ren et al., 2010). However, as metabolic pathology proceeds and mitochondrial ROS generation is sustained, self-regulating mechanisms might deteriorate, therefore promoting hepatocyte dysfunction, IR and the progression of T2DM (Cao et al., 2023). Mitochondrial dysfunction and mitochondrial oxidative stress have indeed been highly implicated in T2DM and NAFLD progression (Morris et al., 2011; Rovira-Llopis et al., 2017; Shum et al., 2021).

Excessive ROS may not only lead mitochondrial dysfunction via the loss of mitochondrial oxidative function, and thus mitochondrial membrane potential, but are also capable of damaging other cellular organelles (Nascè et al., 2022). The inner mitochondrial membrane's mitochondrial permeability transition pores (MPTP) are formed as a result of ROS overload, which sets off a vicious cycle through inducing MPTP creation and sustained ROS production (Nascè et al., 2022). Increased ROS can lower mitochondria content through depletion of mitochondrial DNA (mtDNA) (Matthew Morris et al., 2013), and it is also important to note

that mtDNA is extremely susceptible to damage from ROS, which can lead to mutations and the formation of dysfunctional ETC proteins. As ROS are highly reactive, mitochondrial complexes can also be targets of oxidative damage. Another feed forward mechanism then emerges as reduced mitochondrial oxidative capacity and elevated ROS generation would be sustained by ETC protein dysfunction, exacerbating hepatocyte vulnerability to oxidative stress and injury, especially during metabolic overload (Nascè et al., 2022; Qian et al., 2019).

Comprehensive reviews by Morris et al. Zheng et al. cover in depth the potential role of mitochondrial dysfunction in the onset and progression of NAFLD (Matthew Morris et al., 2013; Zheng et al., 2023), wherein studies implicate several mitochondrial pathways, including impaired FA oxidation, oxidative phosphorylation, mitochondrial DNA replication, antioxidant status, and apoptosis. Disturbances in oxidative capacity and mtDNA integrity have been observed in rodent models and patients with NAFLD (Gao et al., 2004; Haque & Sanyal, 2002; Matthew Morris et al., 2013; Pérez-Carreras et al., 2003). These detrimental effects on the mitochondria altogether promote the onset of hepatic steatosis and aggravate IR, (Kumar et al., 2021; Matthew Morris et al., 2013; Mohamed et al., 2016), increase hepatic oxidative stress , chronic inflammation, ATP depletion and cellular death via the activation of caspase-mediated apoptotic pathways (Muriel et al., 2021; Nascè et al., 2022; Softic et al., 2019; Softic et al., 2020). Patients with T2DM also exhibit reductions in hepatocellular ATP concentrations and ATP synthase flux (Kumar et al., 2021; Schmid et al., 2011; Szendroedi et al., 2009; Tilg et al., 2017). Hepatic ATP synthesis correlates with both peripheral and hepatic insulin sensitivity, but negatively with body fat content (Schmid et al., 2011).

Increased intracellular formation of advanced glycation end products (AGEs)

Glucose, in its reduced form, can directly, through the Maillard reaction, form covalent adducts with proteins and lipids through a non-enzymatic process known as glycation (Gjorgjieva et al., 2019). Glucose's aldehyde group and the side chain of lysine residues and the N-terminal amino groups of proteins can react to form Schiff base and Amadori products, which in turn form irreversible AGEs (Gjorgjieva et al., 2019; Robertson et al., 2004; Zheng et al., 2016). Moreover, glucose can undergo autoxidation through a metal-catalysed reaction to form both ketoaldehyde and H_2O_2, generating superoxide throughout (Gjorgjieva et al., 2019; Zheng et al., 2016). Ketoaldehyde can further react with amino groups in proteins to form ketoimine via Schiff's base, which also elicits the formation of protein-linked AGEs. Fructose is also of interest as it can also induce protein and lipid glycation (Zheng et al., 2016). During chronic

hyperglycaemia, as glucose and fructose levels are increased, glycation is naturally increased. Glycation is widely observed in various tissues and organs of diabetic animals, yielding various kinds of glycated proteins including albumin (Gjorgjieva et al., 2019; Khan et al., 2015; Robertson et al., 2004). One of many identifiable AGEs, HbA1c, which is generated from the nonenzymatic glycation of haemoglobin, is a well-established indicator of glycaemic management as well as the degree and duration of hyperglycaemia, and higher HbA1c levels correlate with the development of diabetic complications (Bae et al., 2021; Hoffmann & Honigberg, 2022; Mohamed et al., 2016).

In general, the measurement of tissue or serum AGEs has been suggested to serve as a promising biomarker and predictor of diabetic complications and a significant association between AGE levels and both the prevalence and severity of diabetic complications can be observed (Beisswenger et al., 2013; Kopytek et al., 2020; Mengstie et al., 2022; Papachristou et al., 2021; Ying et al., 2021). Noteworthy, it was shown that in conditions of chronic liver disease, AGE accumulation is increased, and AGE levels have been found to correlate positively with the severity of steatosis in NAFLD patients (Pereira et al., 2021). As the liver plays a major role in the clearance of plasma AGEs, liver cells would be heavily exposed to the elevated levels of AGEs (Svistounov & Smedsrød, 2004). Moreover, exposed hepatic stellate cells and myofibroblasts, responsible for fibrogenesis in chronic liver disease, were found to express the receptor for AGEs (RAGE) (Mohamed et al., 2016). AGE binding with RAGE, leads to impaired extracellular matrix structure and elicits, through ligand activation, altered intracellular signalling and gene expression (Gjorgjieva et al., 2019; Sena et al., 2013); The activation of NF-κB by RAGE stimulates transcription of genes for cytokines and growth factors (i.e. TNF-α), inducing the release of pro-inflammatory cytokines and consequently, increased expression of adhesive molecules, vascular permeability, and enzymatic ROS production, notably superoxide (Gjorgjieva et al., 2019; Jiang & Török, 2014; Mohamed et al., 2016; Sena et al., 2013). It was shown that RAGE activation is directly linked to the activation of NOX 2 in the liver causing a downregulation of TIMP metallopeptidase inhibitor 3 levels and an increase in TNFα activity promoting oxidative stress, injury and fibrosis in two different dietary models of NASH (Jiang & Török, 2014). Therefore, AGEs with RAGE may serve as markers of inflammation, and their levels increase in chronic metabolic-inflammatory disorders (Lee et al., 2022).

It can be inferred that glycation modifies the structure of the molecules thereby yielding dysfunctional proteins, enzymatic activity and receptor recognition mechanisms. Their accumulation alters many cellular structures, mediating loss of cellular function and stability, making cells even more vulnerable to stress (Gjorgjieva et al., 2019; Sena et al., 2013). A highly reactive by-product of glycolysis, methylglyoxal (MGO), is a carbonyl-containing compound that has been recognised as an important precursor of AGEs (Rabbani et al., 2021). It is of particular interest as it is considered to be up to 10 000 times more reactive than glucose, and has been reported to be play a major role in the detrimental processes mediating hyperglycaemia-induced damage seen in diabetes (Beisswenger et al., 2003; Beisswenger et al., 2013), which will be discussed later on. Therefore, chronic hyperglycaemia induced damage may be mediated by glucose metabolites and not only glucose per se (Kold-Christensen & Johannsen, 2020).

Mechanisms of Oxidative Stress-Mediated Damage

These pathways have been all shown to contribute to increased cellular ROS generation further aggravating cellular redox imbalance and oxidative stress (Zheng et al., 2016). Increased ROS levels can result in deleterious irreversible oxidative modifications of cellular structures, i.e. lipids, proteins, DNA, RNA and carbohydrates (Gjorgjieva et al., 2019; Mohamed et al., 2016). This leads to the dysfunction of organelles in the cell, such as the mitochondria and the ER, establishing a pathological state in various cells including hepatocytes (Gjorgjieva et al., 2019) favouring the release of inflammatory cytokines thereby increasing the expression of adhesion molecules and the infiltration of leukocytes, and apoptosis. A combination of all of these processes causes massive tissue destruction in the liver (Mohamed et al., 2016).

Many studies have proven that patients with T2DM are exposed to chronic oxidative stress, and hyperglycaemia is associated with decreased hepatic antioxidant enzyme activities (Beisswenger et al., 2003; Han et al., 2006; Parveen et al., 2010; Robertson et al., 2004). Oxidative damage markers, such as 8-hydroxy-deoxyguanosine, 4-hydroxy-2-nonenal (HNE) proteins, 8-epi-prostaglandin F2, and oxidation of DNA bases, have been reported to be elevated in serum, plasma, and various tissues of both type 1 and type 2 diabetes patients (Daniel et al., 2013), approximately fivefold above physiological levels (Robertson et al., 2004). These results were also reflected in animal models such as and Goto-Kakizaki (GK) rats and Zucker diabetic fatty (ZDF) rats.

The liver has the highest GSH concentrations (approximately 10–15 mM) among all organs, reflecting the importance of GSH in diverse hepatic antioxidant, metabolic and detoxification processes (Yuan & Kaplowitz, 2009). Besides to being essential in preserving the cellular redox equilibrium and metabolic processes, GSH also influences the regulation of apoptosis and necrosis. Hepatocytes are particularly key to the homeostasis of the GSH enzyme family. Its synthesis is modulated by various pathways, among them transcriptional upregulation through redox-regulated transcription factors, e.g., the nuclear factor-(erythroid factor 2)-related factor 2 (Nrf2) and NF-κB (Yuan & Kaplowitz, 2009). Under physiological conditions, the ratio between reduced GSH and its oxidised state, is maintained at approximately 100:1, but under oxidative duress, it can decrease to levels as low as 10:1 (Jones, 2010).

The ER, mitochondria and nucleus all contain distinct stores of GSH, mitochondrial GSH being the most crucial to cell viability. The thiol group of GSH is usually on the front line of defence against the oxidation process (Mohamed et al., 2016). When oxidative stress is detected, there is initially an effective, swift activation of antioxidant signalling, consisting of the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) which, via the antioxidant responsive element (ARE), regulates the transcription of a range of genes related to GSH metabolism (González et al., 2023). The Forkhead box O (FOXO) transcription factor regulates the expression of SOD and CAT (Jiang & Török, 2014; Li et al., 2018). Notably, the depletion of SOD2 and GPx antioxidant abilities in mice exacerbated IR and the progression of NAFLD to steatohepatitis and fibrosis (Nascè et al., 2022; Saponaro et al., 2015; Tilg et al., 2017).

The hyperglycaemic state and prolonged chronic injury have been associated with deficits in hepatic antioxidant defence enzymes, such as CAT and SOD1, leading to an increase in readily available ROS (Daniel et al., 2013; Gjorgjieva et al., 2019; Mohamed et al., 2016; Robertson et al., 2004), which also decreases the concentration of intracellular GSH, overwhelming the cell and making it more prone to oxidation-induced damage, inflammation and fibrogenesis (Jiang & Török, 2014; Sena et al., 2013). Reports of lower levels of the reduced form of GSH and higher levels of GSSG in erythrocytes from diabetic patients reflects a state of low antioxidant activity/availability (Beisswenger et al., 2003). More importantly, observations in diabetic rat livers corroborate reduced levels of GSH decreased GST, GPx and glutathione reductase activity, leading to an accumulation of oxidative stress products, such as AGEs, protein oxidation products (POPs) and LPO (Mohamed et al., 2016).

Oxidative damage to cellular structures

Protein oxidation

Hyperglycaemia and oxidative stress give rise to several different types of posttranslational protein modifications that manifest glucotoxicity. These are all covered in the following review by Zheng et al. (Zheng et al., 2016). Irreversible protein modifications include carbonylation, nitration, and glycation, and reversible protein modifications include hydroxylation, nitrosylation, acetylation, sumoylation, O-GlcNAcylation, ADP-ribosylation, and succination (Gjorgjieva et al., 2019). Hunt et al. demonstrated that H_2O_2 and superoxide anion generated in glucose autoxidation was likely to promote further oxidation of proteins (Robertson et al., 2004). Protein oxidation can be caused directly by ROS or indirectly by secondary by-products of oxidative stress, resulting in a covalent modification of a protein (Mohamed et al., 2016). Proteins exposed to RNS can also undergo the similar modification, resulting in protein nitration, known to disturb signalling pathways such as the insulin signalling pathway promote hepatic injury by through the release of proinflammatory cytokines and chemokines by Kupffer cells, thereby resulting in neutrophil infiltration and causing inflammatory tissue injury (Abdelmegeed & Song, 2014).

ROS target mainly sulphur containing amino acids, cysteine and methionine being the most susceptible amino acids. Oxidative modification of cysteine comes with a profound impact on not only protein structure and function but also enzymatic activity as cysteine is a key compound in many metabolic enzymes, kinases, phosphatases and transcription factors (Gjorgjieva et al., 2019; Mohamed et al., 2016). Moreover, ROS can, through protein modification, yield the formation of protein-protein and protein-lipid cross-links (Mohamed et al., 2016). Protein oxidation or nitration induced alterations in protein structure and function can result in several events promoting cellular dysfunction (Gjorgjieva et al., 2019). These include impaired/increased protein aggregation, resistance to proteolysis, protein fragmentation, obstruction of the amino acid side chains hence impaired enzymatic and receptor activities, disturbing signalling pathways such as the insulin signalling pathway, increased or decreased cellular uptake, altered immunogenicity and last but not least impaired ER function and ER stress (Gjorgjieva et al., 2019; Mohamed et al., 2016).

As with AGEs, POPs are biomarkers that can be used to assess the extent of oxidative damage in the cells (Gjorgjieva et al., 2019; Witko-Sarsat et al., 1998) and thus quantify oxidative stress, and the liver plays an important role in the elimination of POPs (Iwao et al., 2006; Svistounov

& Smedsrød, 2004). In this context, serum concentrations of POP were found to increase as chronic diseases progress and are particularly exacerbated in DM (Nazratun et al., 2006; Šebeková et al., 2002). Furthermore, in a group of cirrhotic patients POP levels were found to be significantly elevated and positively correlated with the severity of the disease (Mohamed et al., 2016; Zuwała-Jagiełło et al., 2009).

Lipid peroxidation

LPO is the result of the interaction between ROS and the electrons of a lipid, such as PUFAs, altering its structure and characteristics. Considering that hepatic lipid accumulation is marked in T2DM, LPO could play a major in liver pathology in these patients. In attempt to adapt to chronic higher levels of FFAs, hepatic lipid droplets can only enlarge to a limited extent and once their storage capacity is surpassed, FFAs start accumulating in the cell and present at elevated concentrations for prolonged periods of time (Gjorgjieva et al., 2019; Robertson et al., 2004), thus initiating steatosis.

Elevated readily available FFAs in the cell can induce the production of ROS through their oxidation by mitochondria, as previously discussed (Sena et al., 2013), peroxisomes and microsomes, mediated in part by CYP2E1, CYP4A10, and CYP4A14, increasing cellular oxidative stress. ROS in turn react with cellular lipids to induce LPO (Gjorgjieva et al., 2019). LPO products include reactive aldehydes malondialdehyde (MDA) and 4-hydroxy-trans-2-nonenal (HNE) among others (Gjorgjieva et al., 2019; Robertson et al., 2004). Readily detectable, these compounds are often used as a marker of tissue and cellular oxidative stress and reflect the extent of tissue damage, and are found to be significantly increased in the livers of individuals with DM (McAnuff et al., 2003; Mohamed et al., 2016). These aldehydes can be highly damaging due to their high reactivity and longer half-lives compared to ROS, and their ability to bind to lipoproteins and enter systemic circulation, exerting their damaging effects on various other target tissues (McAnuff et al., 2003; Mohamed et al., 2016). They are known to be notorious in atherogenesis and vascular disease and are considered to play a major role in cell injury driving in lipid-related liver pathologies (Gjorgjieva et al., 2019).

LPO derived disturbances in the bi-layer lipid membranes of the cell leads to profound impairment of cell homeostasis and membrane-bound enzyme signalling and receptor activity, compromising cell survival (Gjorgjieva et al., 2019). The deleterious effects of lipotoxicity are

mediated by the same mechanisms induced by glucotoxicity, and those include oxidative stress, increased AGE formation, activation of PKC, the hexosamine pathway, and promoting a proinflammatory state (Robertson et al., 2004; Sena et al., 2013). Because T2DM is usually associated with obesity and hyperlipidaemia as well as hyperglycaemia, there have been observations that lipotoxicity is significantly marked and aggravated by chronic hyperglycaemia (Robertson et al., 2004). In the case of NAFLD, FFA-induced ROS and LPO were found to activate NF-κB and increase TNF-α expression, via the activation of adipocytokines and PKC signalling among other pathways, further inducing proinflammatory cytokine production (Mahmoud et al., 2012; Sena et al., 2013), hence favouring inflammation and cell necrosis (Han et al., 2006; Mohamed et al., 2016; Šebeková et al., 2002). This in turn triggers hepatic stellate cells to proliferate and secrete collagen producing factors, thereby increasing fibrogenesis; this forms a vicious cycle in the pathogenesis of chronic liver disease (Mohamed et al., 2016; Moreira, 2007).

Activation of PKC by increased lipids like FFAs, ceramides, DAG and phosphatidic acid, as previously discussed, is implicated in the development of IR, by increasing serine phosphorylation of insulin receptor substrate 1(IRS1), leading to reduced insulin-stimulated activation of PI-3 kinase, phosphoinositide-dependent kinase-1 and Akt (Gjorgjieva et al., 2019; Sena et al., 2013), thus worsening the diabetic phenotype. Furthermore, protein aggregation in lipotoxic conditions and dysfunctional hepatic lipid metabolism, which increases ER phospholipid composition, were found to disturb protein synthesis and induce ER stress (Gjorgjieva et al., 2019).

Oxidative DNA damage

Oxidative stress-induced DNA damage has also been reported in diabetes, which occurs through structural modifications of the bases, inter- and intra-strand crosslinks by ROS. These modifications usually lead to strand breaks and yield detectable DNA-protein crosslinks and DNA oxidation products 8-hydroxydeoxyguanosine and dihydropropidium iodide8-OxodG (Gjorgjieva et al., 2019; Sena et al., 2013). DNA damage leads to the activation nuclear poly(ADP-ribose) polymerase (PARP), an enzyme that takes on the role of repairing damaged DNA molecules. PARP uses NAD^+ as its substrate and its overactivation can deplete cellular NAD^+ and ATP, causing redox imbalance, disturbed energy homeostasis and ultimately, cell death (Gjorgjieva et al., 2019; Sena et al., 2013; Zheng et al., 2016).

Poly (ADP-ribosyl)ation of GAPDH by PARP, through the production of ADP-ribose polymers, results in the reduction of GAPDH activity (Robertson et al., 2004; Sena et al., 2013). Oxidation of sorbitol by NAD^+, disturbing the NADH: NAD^+ ratio, was also found to reduce GAPDH activity. Furthermore, exposure to high glucose concentrations decreases GAPDH activity in islets and endothelial cells. The presence of excess glucose concentrations, coupled with impaired GAPDH-mediated catabolism of glyceraldehyde-3-P, favours the accumulation of glyceraldehyde-3-P and dihydroxyacetone which in turn increases the levels of triose phosphates, MGO, and DAG, thereby simultaneously exacerbating both glycative and oxidative stress. These may act in synergy in the pathogenesis of development of diabetic complications (Robertson et al., 2004).

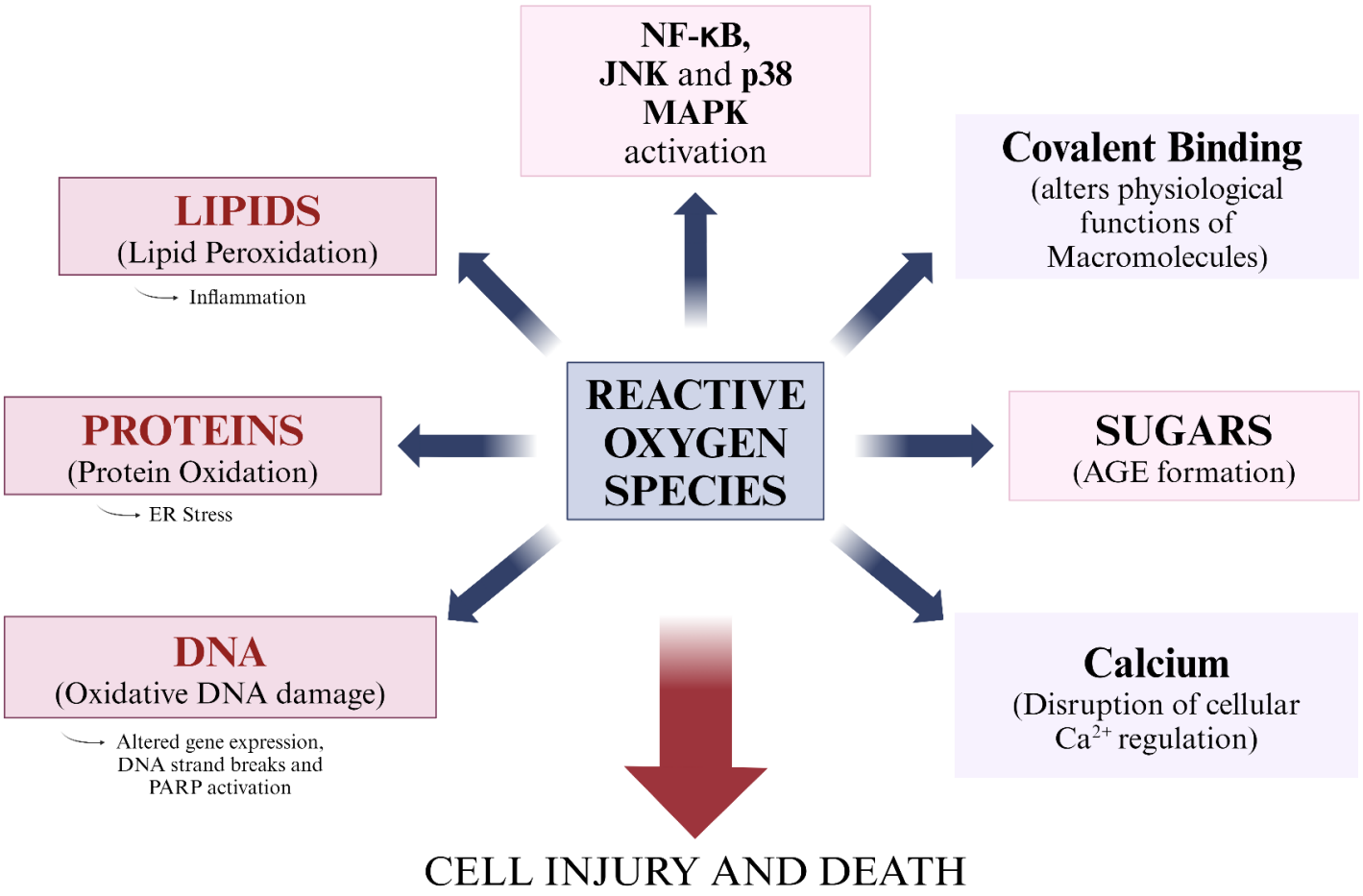


Figure 1.3. Various cellular structures and functions affected by increased cellular reactive oxygen species. In addition to causing oxidative damage to important cellular structures and molecules, impairing their function, increased ROS disturbs cellular calcium regulation, triggers ER stress and inflammation, and activates several stress signalling pathways to promote cellular dysfunction and cell death. JNK, c-JUN N-terminal kinase, MAPK, mitogen-activated protein kinases. Created with BioRender.com.

Signalling Pathways Contributing to Liver Damage in diabetes

Nuclear Factor-Kappa B (NF-κB)

The hepatic NF-κB pathway is a central mediator of immune and stress responses, and it is critical for liver tissue homeostasis as it takes on the role of proliferation and survival regulation during cell regeneration and development (Gehrke & Schattenberg, 2020; Mohamed et al., 2016). It is considered a major transcription factor in the mechanisms underlying inflammation as it is involved in the transcription of a number of genes encoding pro-inflammatory cytokines, and it has been widely found to be activated in the liver of diabetic patients (Daniel et al., 2013).

NF-κB is activated by inflammatory molecules such as IL-1β, IL-6, TNF-α and C-reactive protein (CRP) (Daniel et al., 2013; Manna et al., 2010; Martínez-Flórez et al., 2005), which are commonly observed in the serum of T2DM and NAFLD patients (Gaens et al., 2012; Sena et al., 2013). It is also activated by oxidative stress, hyperglycaemia, FFAs via increased expression of TNF-α, and PKC activation, which are also increased in these patients (Manna et al., 2010; Martínez-Flórez et al., 2005; Mohamed et al., 2016; Nakagawa & Maeda, 2012).

Activation of NF-κB in liver and appears to contribute to obesity-induced IR (Wellen & Hotamisligil, 2005) and inappropriate and persistent activation of NF-κB in T2DM patients with liver damage may play a pivotal role in the progression of hepatic steatosis to cirrhosis (Arsura & Cavin, 2005; Mohamed et al., 2016). Increased liver tissue damage can be through direct and indirect production of ROS and RNS, yielding additional LPO, AGEs and POPs, and further NF-κB-induced elevation of pro-inflammatory molecules, such as TNF-α and interleukins, cell adhesion molecules, chemokine receptors and inducible enzymes such as COX2 and iNOS (Arsura & Cavin, 2005; Han et al., 2006; Martínez-Flórez et al., 2005). It was also reported that the activation of NFκB may play a part in hyperglycaemia induced apoptosis, (Daniel et al., 2013; Mohamed et al., 2016).

Mitogen-Activated Protein Kinases

The Mitogen-activated protein kinases (MAPKs) families consist of three different signalling cascades extracellular signal-regulated kinases (ERK), p38 MAPKs and c-JUN N-terminal kinase (JNK), and each exert different types of reactions upon stimulation (Nakagawa and Maeda 2012. Activated by cytokines and growth factors, ERK play a central role in regulating cell growth and differentiation (Mohamed et al., 2016). Meanwhile, JNK and p38 MAPKs, also known as stress-activated MAPKs, are triggered by stressors such as ER stress, oxidative stress, osmotic perturbation, AGEs and pro-inflammatory cytokines such as TNF-α and IL-1β, which are all increased during hyperglycaemia found in T2DM (D. Liu et al., 2022; Manna et al., 2010; Nakagawa & Maeda, 2012; T. Shen et al., 2019; Son et al., 2011). ERK, p38MAPK and JNK signalling pathways aberrant activation may cause cellular oxidative stress, apoptosis, inflammation, or delayed tissue repair in various cells (Lai et al., 2022; D. Liu et al., 2022). The livers of mice and humans under metabolic stress show activated ERK1/2, JNKs, and p38s pathways, which lead to decreased hepatic insulin sensitivity and glucose metabolism and exacerbate the development of T2DM (Cazanave et al., 2009; Xia et al., 2020).

The 3 members of the JNK group of serine/threonine kinases, JNK-1, -2, and -3, regulate multiple activities in development and cell function, in large part through their ability to control transcription by phosphorylating activator protein 1 (AP-1) proteins, including c-Jun and JunB (Davis, 2000; Wellen & Hotamisligil, 2005). The stress activated JNK, and its downstream signalling cascade are abnormally activated in patients with NAFLD and, in particular, NASH (Cazanave et al., 2009; Xia et al., 2020). Many murine models of NASH have proven the mechanistic involvement of JNK in NASH pathogenesis (Cazanave et al., 2009; Gehrke & Schattenberg, 2020; Jörn M. Schattenberg et al., 2006). The activation of JNK signalling leads not only to pro-inflammatory cytokine production, but also to cell death via intrinsic/extrinsic apoptotic pathways (Wang et al., 2012). The resulting cellular events include apoptosis, necrosis, proliferation, and inflammation (Mohamed et al., 2016), which altogether accelerate the progression of the disease. Studies have shown that prolonged exposure to oxidative stress because of high levels of liver TNF-α will activate the pro-apoptotic function of JNKs; detrimentally affecting the hepatocytes (Nakagawa & Maeda, 2012). Furthermore, it has been suggested that hepatic JNK activation lowers the expression of target genes for PPARα, such as FGF21, which inhibits FA oxidation and exacerbates IR (Vernia et al., 2014).

Inflammation in T2DM and the development of NAFLD

Inflammation is an essential physiological response in the host defence against infections and tissue injury, through the release of pro-inflammatory cytokines such as TNF-α, IL-1β and IL-6 (L. Chen et al., 2018). Initially limited to local responses, as the inflammatory process is persistently triggered, it progresses into an acute-phase response which expands systemically and is characterised by a variety of inflammatory plasma proteins and pro-inflammatory cytokines, which become in turn responsive to inflammation. This can lead to tissue damage and fibrosis as demonstrated in a variety of inflammatory disorders (Mohamed et al., 2016). In the liver and extrahepatic organs, this can contribute cellular dysfunction, cell death, and detrimental tissue remodelling in an effort to preserve structural and functional organ integrity, (Gehrke & Schattenberg, 2020). In states of metabolic overload i.e. diabetes, obesity, there is aberrant immune activity and chronic hyperglycaemia, has been established to coexist with a chronic low-grade inflammatory state in Type 1 and Type 2 DM patients (Daniel et al., 2013; Sena et al., 2013; Wellen & Hotamisligil, 2005). It is not only considered an associated risk factor for associated inflammatory complications, but also a major contributory factor, particularly in liver disease (Mohamed et al., 2016; Wellen & Hotamisligil, 2005). As

previously discussed, emerging evidence suggests NAFLD and T2DM share pathogenetic features, and notably, NAFLD is also associated with chronic, low-grade inflammation in the liver that leads to systemic effects (Gehrke & Schattenberg, 2020; Targher et al., 2021).

Furthermore, an array of evidence has emerged revealing a strong correlation between metabolism and immunity, and scientists have coined the terms immunometabolic disorder, chronic metabolic inflammation, or "metaflammation", to describe this phenomenon that is highly implicated in the development of metabolic disorders such as T2DM and NAFLD (Gehrke & Schattenberg, 2020; Wellen & Hotamisligil, 2005). Hotamışlıgil presented an interesting theory linking the two – not only do the liver, adipose and hematopoietic tissue share developmental heritage and evolutionary underpinnings, there are overlapping pathways regulating both metabolic and immune functions through key regulatory molecules and signalling pathways such as JNK and NF-kB. According to this notion, the immune response and metabolic regulation are highly integrated and dependent on each other (Hotamisligil, 2006; Wellen & Hotamisligil, 2005). In tissues characterised by high metabolic activity, such as the liver, brain, pancreas and adipose tissue, inflammatory cell action and interactions within the stromal components are significant factors in the maintenance of tissue homeostasis as well as metabolic disease pathogenesis (Hotamisligil, 2006), thereby linking inflammatory organ injury directly to metabolic disturbances. T2DM and NAFLD are chronic metabolic disorders that might result from impaired central homeostatic mechanisms (Gehrke & Schattenberg, 2020) and these diseases can also trigger inflammatory responses through metabolic excess, further exacerbating stress and inflammation (Hotamisligil, 2006). Thus, inflammation might be considered both a major causal factor and a consequential effect of T2DM and NAFLD.

Crosstalk between tissues by means of autocrine, paracrine, and endocrine signalling is critical to energy and nutrient homeostasis. As previously mentioned, the liver is indispensable in the regulation of normal physiology and metabolism via the production and secretion of various plasma proteins, including albumin, complement factors, transport proteins, and other factors (Watt et al., 2019). The role of liver as a major secretory organ has long been appreciated, and recent proteomic advances have enabled researchers to identify ~10,200 proteins produced in the human liver (M.-S. Kim et al., 2014), and with up to 40% of hepatic transcripts encoding secretory proteins (Uhlén et al., 2015), there is clearly a large scope for significant and varied protein secretion from the liver. Plasma concentrations of acute-phase proteins are largely dependent on their hepatic biosynthesis and are influenced by pro-inflammatory cytokines

(Daniel et al., 2013). Given the functional characteristics of hepatocytes, which account for about 80% of the total liver volume and about 70% of the total liver cell number (Meex & Watt, 2017), emerging evidence has demonstrated that factors secreted from hepatocytes actively mediate metabolic regulation between the liver and other organs, and more specifically, hepatokines have drawn increasing attention due to their capacity for metabolic regulation, making them novel topics of interest in the context of metabolic disorders (Jensen-Cody & Potthoff, 2021; Kim & Yang, 2021). Similarly to adipose tissue or skeletal muscle where the secretion of respective organokines exerts their endocrine function, the liver has more recently been identified to contribute to the endocrine control of metabolism by producing liver-derived factors – hepatokines, including fetuin-A, angiopoietin-related growth factor (AGF), fibroblast growth factor 21 (FGF 21), growth differentiation factor 15 (GDF15) , insulin-like growth factors (IGF), selenoprotein P (SeP), leukocyte derived chemotaxin 2 (LECT2), etc (Ke et al., 2019; Dariusz M Lebensztejn et al., 2016; Oh et al., 2017; Watt et al., 2019).

As previously mentioned, the initial stage of NAFLD is characterised by steatosis, which is associated with a variety of disturbances in glucose, fatty acid and lipoprotein metabolism, lipotoxicity from high levels of FFAs, cholesterol, and other lipid metabolites (LPO), leading to leading to NF-κB activation, cytokine release, oxidative stress, ER stress, and mitochondrial dysfunction which suppress hepatic insulin sensitivity and promote de novo lipogenesis, and this, according to current hypotheses, in a 'multi-hits' fashion, collectively causes further injury and the progression of the disease. In this later stage, inflammation becomes overt as lipid metabolites, pro- inflammatory cytokines and hepatokines (for example, fetuin A, fetuin B, AGF, FGF21, GDF15 or selenoprotein) are observed (Targher et al., 2021).

DM patients present significantly increased serum levels of IL-6, IL-18, IL-1 and TNF-α which are products of inflammatory pathways and markers of chronic inflammation (Daniel et al., 2013). Numerous cross-sectional studies have associated IR and T2DM with higher serum levels of CRP, IL-6 and TNF-α, and Farhan et al. reported that the serum IL-8 levels in T2DM patients were markedly increased compared to healthy subjects, which also positively correlated with higher HbA1C (Farhan Mohammed et al., 2018). In obese subjects with impaired glucose tolerance, plasma IL-8 concentrations after glucose load are also increased in comparison to normoglycemic weight-matched individuals (Straczkowski et al., 2003). T2DM patients with NAFLD, tend to have higher levels of inflammatory markers such as IL-6 and TNF-α compared with those without (Banerji et al., 1995; Kelley et al., 2003; Smith & Adams,

2011b; Toledo et al., 2006). In patients with NAFLD, patients with abnormal ALT were found to have higher serum levels of IL-6 and statistically higher levels of serum IL-8, indicating that some degree of liver damage is correlated with increasing levels of circulating proinflammatory cytokines (Chu et al., 2007). Furthermore, key hepatokines FGF21 and GDF15 are both strongly linked with the occurrence of diabetes, (Adela & Banerjee, 2015; Chavez et al., 2009; Chen et al., 2008; Mraz et al., 2009), NAFLD (Dushay et al., 2010; Valenzuela-Vallejo et al., 2023; Yilmaz et al., 2010) and obesity (Asrih et al., 2023; Berti et al., 2015; Mraz et al., 2009; Zhang et al., 2008) with their elevated levels positively correlating with the presence of steatosis, hyperglycaemia, IR and inflammation (Dolegowska et al., 2019; Eddy & Trask, 2021; Meex & Watt, 2017; Mutanen et al., 2014; Vila et al., 2011).

In the liver and extrahepatic organs, chronic inflammatory responses have been linked to loss of cellular function, cell death, tissue damage and fibrosis, resulting in an increased risk of more severe stages, including NASH and HCC (Hijona et al., 2010; Mantena et al., 2008; Marchesini et al., 2003; Starley et al., 2010). As inflammation persists, increased proliferation of T and B cells and neutrophil and leukocyte infiltration into the tissue is enhanced as a result of chemokines, such as ligands 2 and 5 chemokines CCL2, CCL5 and CX3CL1, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) production (Daniel et al., 2013; Sena et al., 2013). These effects are mediated by the activation of the nuclear translocation of NF-κB and the JNK pathway (Mohamed et al., 2016), which was found to promote the development of NASH in a mice model (J. M. Schattenberg et al., 2006). Furthermore, inflammatory cytokines were found to induce the production of free radicals, thus initiating a vicious cycle (Elmarakby & Sullivan, 2012).

In addition to tissue injury and fibrosis, chronic low-grade inflammation contributes to the pathogenesis of insulin resistance, and thereby could further exacerbate the progression of T2DM, hepatic steatosis, and NAFLD. Important determinants of IR are inflammatory mediators released from a fatty liver (Gehrke & Schattenberg, 2020). The connection between hepatic steatosis and IR is supported by mechanistic studies that link hepatic fat and inflammation causally to IR (Korenblat et al., 2008; Vega et al., 2007). Circulatory cytokines have been linked to impaired insulin sensitivity through inhibitory phosphorylation of serine residues of IRS-1, which reduces both tyrosine phosphorylation of IRS-1 in response to insulin and the ability of IRS-1 to associate with the insulin receptor, and thereby inhibits downstream signalling and insulin action (Qian et al., 2019; Wellen & Hotamisligil, 2005). This is observed

as a result of exposure of cells to both TNF-α or elevated levels of FFA (Aguirre et al., 2000; Hotamisligil et al., 1996; Yin et al., 1998), which further highlights the potential synergy between hyperglycaemia and FFA. Moreover, IL-1β and TNF-α exposure was found to lead to the inhibition of glucose-induced insulin secretion and apoptotic pancreatic beta cell death in both types of DM (Daniel et al., 2013; Mohamed et al., 2016)

Two inflammatory kinases that play a large role in counteracting insulin action, particularly in response to lipid metabolites, are inhibitory-κB kinase (IKK) IKK and PKC-θ. IKKβ can impact on insulin signalling through at least 2 pathways. It can directly phosphorylate IRS-1 on serine residues (Gao et al., 2002; Yin et al., 1998) as well as phosphorylate inhibitor of NF-κB (IκB), thus activating NF-κB and stimulating production pro-inflammatory cytokines (Shoelson et al., 2003). Hepatic overexpression of B-cell leukaemia 3, a coactivator of NF-κB, in mice exacerbates the dysmetabolic and inflammatory phenotype in NAFLD, triggering metabolic inflammation in the liver and adipose tissue and whole-body IR (Gehrke & Schattenberg, 2020). A rise in levels of intracellular FA metabolites, such as diacylglycerol (DAG) and fatty acyl CoAs was correlated with activation of PKC-θ and increased Ser307 phosphorylation of IRS-1 (Smith & Adams, 2011b; Yu et al., 2002). PKC-θ may impair insulin action by activation of other serine/threonine kinases, IKKβ, or JNK (Kim et al., 2004; Perseghin et al., 2003). Other PKC isoforms have also been reported to be activated by lipids and may also participate in inhibition of insulin signalling (Schmitz-Peiffer, 2002; Serra et al., 2003). JNK appears to negatively influence insulin sensitivity by promoting inflammation (Hirosumi et al., 2002), but when JNK is activated, it can also associate with and phosphorylate IRS-1 on serine residues, impairing insulin action (Aguirre et al., 2000; Gao et al., 2002; Ozcan et al., 2004). It is worth nothing liver-derived IL6 overproduction in mice with hepatocellular overactivation of NF-kB causes both local hepatic and systemic IR (Cai et al., 2005). Although it was discovered that IL-6 can inhibit insulin receptor signal transduction and insulin action in both primary mouse hepatocytes and human hepatocellular carcinoma HepG2 cells (Senn et al., 2002), and additional studies emphasised the inhibitory effects of IL6 in liver and peripheral insulin signalling, most available evidence indicates that IL6 promotes insulin sensitivity rather than resistance, and that its negative impact is largely due to its capacity to enhance hepatic inflammatory signalling.

Endoplasmic Reticulum stress in T2DM and the development of NAFLD

In addition to inflammation, mitochondrial dysfunction and excessive ROS generation causing oxidative stress within the cell, ER stress is also highly observed in diabetes and NAFLD (Cao et al., 2023; Xia et al., 2020). The ER is responsible for a number of essential cellular processes, such as the synthesis and folding of proteins, their transportation, and the storage of calcium. The ER is home to several chaperone molecules, which have high affinity for unfolded proteins to serve as quality control and facilitate their proper folding. The main ER chaperones are the Binding immunoglobulin Protein/78 kDa glucose-regulated protein (BiP/GRP78), the 94 kDa glucose-regulated protein (GRP94), P58IPK, calreticulin and the protein disulfide isomerase. By inhibiting protein aggregation and ensuring proper protein conformation and disulfide bond formation, these chaperones preserve ER homeostasis under physiological circumstances (Nascè et al., 2022). Incorrectly folded proteins are excluded from the ER and degraded. However, protein folding is an extremely error-prone process, especially under the interference of various stimuli, such as ROS and reactive toxic metabolites such as oxidation products and AGEs (Xia et al., 2020). Increasing evidence shows that ER engagement plays a vital role in regulating hepatic protein and lipid homeostasis, and ER homeostasis may be disturbed by many factors, such as hyperglycaemia, free fatty acids, gene mutations, and environmental pathogens (Lebeaupin, Vallée, Hazari, et al., 2018).

High substrate influx in metabolic disorders can indeed impair chaperone function, resulting in the accumulation of misfolded or unfolded proteins triggering ER stress and the unfolded protein response (UPR) (Bihl et al., 2015; Nascè et al., 2022). It is considered to be a “quality control system” within the cell, relieving ER stress and restoring ER function via the limitation of protein translation and increasing the expression of ER folding enzymes and molecular chaperones, preventing further accumulation of unfolded proteins (Xia et al., 2020). This is mainly mediated through three signalling protein axes named inositol-requiring 1 alpha (IRE1a)/spliced X-box binding protein 1 (XBP1), double-strand RNA activated kinase-like ER kinase (PERK)/eukaryotic translation initiation factor 2 alpha (eIF2a)/activating transcription factor 4 (ATF4) and ATF6 (Cao et al., 2023). BiP/GRP78 works as a sensor of unfolded proteins in the ER and regulates the activation of these three ER stress transducers (Bertolotti et al., 2000; Sommer & Jarosch, 2002). Under normal conditions, BiP/GRP78 binds to the lumenal domains of Ire1α, Ire1β and PERK, and prevents their homodimerization and transport to the Golgi apparatus. Under ER stress conditions, BiP/GRP78 binds to unfolded proteins and

thereby allows each transducer to activate (Oyadomari & Mori, 2004). Their downstream signal cascade plays a vital role in either restoring ER function or inducing apoptosis signalling (Behnke et al., 2015; Xia et al., 2020). Accordingly, when ER stress persists and the initial stages of the UPR become insufficient, genes encoding ER chaperone proteins such as BiP/GRP78 and GRP94 are upregulated (Oyadomari & Mori, 2004). In particular, BiP/GRP78 expression is elevated in the liver of NAFLD patients (Rodriguez-Suarez et al., 2012). In addition to these genes, recent studies revealed that genes involved in translational recovery, amino-acid import, glutathione biosynthesis and antioxidant response are also upregulated during ER stress (Harding et al., 2003; Oyadomari & Mori, 2004).

In a later phase, the elimination of misfolded proteins in the ER by the ubiquitin-proteasome system (UPS) is induced (Oyadomari & Mori, 2004) or through autophagy. UPR regulates UPS and autophagy mainly by activating ubiquitination-related and autophagy-related genes (Xia et al., 2020). In most cases, UPS mainly specifically degrades most macromolecules or short-lived soluble proteins in cells, and autophagy mainly degrades excess or damaged intracellular organelles and macromolecule or relatively stable proteins (Nam et al., 2017). In cells, when a protein needs to be degraded, its tyrosine residues are labelled with ubiquitin, which is then specifically recognized and rapidly degraded. Protein ubiquitination involves three consecutive processes, involving ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2 and ubiquitin protein ligase E3 (Gundogdu & Walden, 2019; Xia et al., 2020). If the stress is too severe or functions of the ER are severely impaired, the UPR-mediated salvage processes are insufficient and multiple apoptotic and inflammatory pathways are engaged (Cao & Kaufman, 2014). At least three apoptosis pathways are known to be involved in this apoptotic event. The first is transcriptional activation of the gene for C/EBP homologous protein (CHOP) (Oyadomari & Mori, 2004). Another signalling pathway that operates in parallel with CHOP is mediated by JNK, which is mediated by the formation of the ‘inositol requiring 1 (Ire1)-TNF receptor-associated factor 2 (TRAF2)-apoptosis signal-regulating kinase1 (ASK1)’ complex (Merksamer & Papa, 2010; Nishitoh et al., 2002; Urano et al., 2000).The third is activation of ER-associated caspase-12 which is activated by ER stress, but apparently not by death receptor-mediated or mitochondria-targeted apoptotic signals (Nakagawa et al., 2000). All the three apoptosis pathways eventually lead to the activation of caspase-3, suggesting that ER stress signals are finally transmitted to the mitochondria (Oyadomari & Mori, 2004). Microarray studies revealed that CHOP is one of highest inducible genes during ER stress and its

expression is mainly regulated at the transcriptional level by ATF4, functional spliced XBP1 and cleaved ATF6 (p50) (Okada et al., 2002).

Notably, we now know that at least in liver, dysfunction of the ER and mitochondria are linked phenomena, and when metabolic excess occurs, the resultant ER stress leads to the activation of inflammatory signalling pathways and oxidative stress (Wellen & Hotamisligil, 2005). Elements of the UPR converge on many inflammatory pathways including JNK and IKK (Hung et al., 2004), and the ER is a critical site for inflammasome activation (Yang et al., 2015). Indeed, increased low grade inflammation has been suggested to be a consequence of activation of the UPR (Rabbani et al., 2021) as all three major UPR pathways, ATF6, PERK, and XBP-1, have been implicated in inflammatory signalling (Janssens et al., 2014; Yamazaki et al., 2009). For instance, the histone-lysine N-methyltransferase SETD7 pathway, which is activated in response to XBP-1, increases methylation of lysine 4 of histone 3 on NF-kB p65 promoter, p65 expression and binding activity. This was associated with the upregulation of the NF-kB inflammatory cascade and subsequent transcription of inflammatory genes such as IL-8, cell adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) as well as RAGE and GRP78 (Rabbani et al., 2021). It was reported that this proinflammatory signalling mediated by SETD7 was activated in a transient and persistent model of hyperglycaemia (El-Osta et al., 2008). Furthermore, NFκB may be activated in response to the accumulation of membrane proteins in the ER and ROS generation (Oyadomari & Mori, 2004), and studies suggest that eIF2α phosphorylation is required for induction of NFκB (Jiang et al., 2003). It is also believed that the stress leads to Ca^{2+} release from ER and subsequent production of ROS via opening of MPTP (Nascè et al., 2022). In turn, oxidative stress leads to further accumulation of unfolded proteins in the ER lumen. As a result, between each organelle, a vicious cycle is initiated. The mitochondria and the ER are indeed tightly interconnected, as evidenced by the existence of contact sites between both organelles, known as mitochondria-associated ER membranes (MAMs) (Rieusset, 2018). Interactions through MAMs help to maintain cell homeostasis and allow the exchange of metabolites and ions between the two organelles, and therefore each one is influenced by the oxidative state of the other. They are believed to play an important role in hepatic oxidative stress and IR (Burgos-Morón et al., 2019; Cheng et al., 2020).

To date, the importance of ER stress in T2DM progression has inspired a consensus that activation of UPR pathway is an emblematic phenomenon in T2DM-associated dysmetabolic

outcomes, which would contribute to peripheral IR and hepatocyte dysfunction via various mechanisms (Cao et al., 2023). Most UPR components, induced by increased ER stress, have been observed to be upregulated in peripheral tissues during obesity and T2DM (Cao et al., 2023; Xu et al., 2021). The UPR pathway is able to modulate IR in the liver, (Herrema et al., 2022) among other tissues, as well as adipogenesis, (Basseri et al., 2009) and its aberrant activation could lead to detrimental effects in hepatocytes. When IRE1α becomes over-activated due to unresolved ER stress, it can oligomerize, leading to the degradation of hundreds of mRNAs located within the ER, thereby reducing the folding load on the ER, or trigger cell death, through the degradation of specific miRNAs that target proapoptotic genes and by the activation of apoptosis signal-regulating kinases (Agrawal et al., 2021; Lemmer et al., 2021). In addition to increased XBP1 splicing and nuclear localization, in IR/hyperinsulinemia states, the liver also exhibits aberrant activation of the IRE1α branch (Ning et al., 2011). Moreover, activated PERK can phosphorylate eukaryotic translation initiation factor 2 (eIF2α) to attempt to reduce protein translation (Agrawal et al., 2021; Lemmer et al., 2021). PERK can also selectively upregulate the expression of ATF4, which enhances the transcription of growth arrest, to negatively regulate itself, and CHOP to induce cell death (Cao et al., 2023; Oyadomari & Mori, 2004).

Cellular modulation of apoptosis

In DM, in addition to ER stress, the role of increased oxidative stress and inflammation in the induction of apoptosis in cells is a major mechanism underlying the damaging effects of hyperglycaemia. Apoptotic of cell death is critical to hepatic cell damage and lesion formation in liver diseases (Daniel et al., 2013)

As previously discussed, damaging effects induced by oxidative stress to various cellular structures may compromise cell stability and hence favour pro-apoptotic pathways. However, apoptosis can also be directly triggered by oxidative stress. Apoptosis can be defined as a ‘cellular suicide’, a tightly regulated and controlled physiological process in which a cell programs its own death. The induction of apoptosis is characterized by bio-chemical and morphological changes in the cell. It is vital process for normal development and maintenance of tissue homeostasis, but abnormally increased rates of apoptosis can be a sign of tissue pathology, as detected in many chronic inflammatory and autoimmune diseases (Daniel et al., 2013). An increase in molecular weight, DNA fragmentation and/or fragmentation into an oligonucleosomal ladder, phosphatidyl serine externalization, proteolytic cleavage of

intracellular substrates and MPTP assembly are all processes that occur during apoptosis, which leads to blebbing, chromatin condensation, nuclear fragmentation, loss of adhesion and rounding and finally, cell shrinkage which are characteristic of apoptotic cell death (Daniel et al., 2013).

Several studies have identified ROS mediated damage as a major factor in apoptosis induction, an observation that was reversed by antioxidant treatments (Daniel et al., 2013). As mitochondria play a pivotal role in liver cell function and maintenance, their damage inevitably leads to liver dysfunction (Wang et al., 2020). Excessive cellular ROS can lead to damage of the mitochondrial inner membrane compounds, leading to mitochondrial fission, and increased apoptosis rates in several cell types (Chen et al., 2023; Qian et al., 2019; R. J. Youle & A. M. van der Bliek, 2012). Post-translational modifications of components in complex I were also identified and may play a role in mitochondrial injury (Wang et al., 2020). In hepatocytes, mitochondrial damage, including mitochondrial respiratory chain injury and abnormal mitochondrial fusion, due to altered structure and function, is observed in a variety of chronic liver diseases, including hepatic steatosis, NAFLD, and viral hepatitis (Fletcher et al., 2019, Ogrodnik et al., 2017).

Mitochondrial damage and an abrupt disruption in mitochondrial membrane potential may trigger MPTP formation. This leads to the release of cytochrome c from the mitochondrial membrane into the cytoplasm, which induces apoptosis by forming complexes with Apaf-1, activating procaspase 9, and subsequently, downstream effector caspases 3, 6 and 7(Daniel et al., 2013). Moreover, MPTP formation can be dictated by the presence of pro- apoptotic (Bad, Bax, Bcl-2) or anti-apoptotic (Bcl-2, Bcl-XL) factors, and the translocation of pro-apoptotic Bax protein into the mitochondrial membrane was found to induce an increase in caspase-3 and caspase-9 activities (Daniel et al., 2013). Interestingly, it was reported that exposure of endothelial cells to high D-glucose undergo apoptosis through the Bax-caspase activation pathway. Studies also report marked apoptosis in the diabetic liver, and the hydroxyl radical is believed to play a part in the activation of the Bax-caspase pathway which sheds light on the mechanisms underlying hyperglycaemia-induced liver cell apoptosis (Daniel et al., 2013).

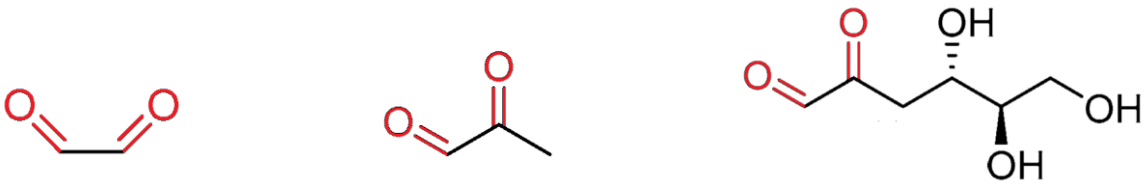
Simultaneously, increased inflammation and hepatic TNF-α in diabetes have been strongly linked to apoptosis in liver cells, via the activation of NFκB, caspase-8 and JNK (Frances et al., 2013). TNF-α exerts its effects by binding the receptors tumour necrosis factor receptor

(TNF-R)1 - which was found to be upregulated in the diabetic liver (Ingaramo et al., 2011) and is thought to play an important role in development of steatohepatitis (Bluemel et al., 2020; Wandrer et al., 2020), and TNF-R2, both contributing to cell death. While only TNF-R1 contains a death domain, TNF-R2 enhances the cytotoxic effects of TNF-R1. TNF-R1, when bound to TNF-α, stimulates the activation of NFκB and can stimulate the caspase activation pathway, which as previously discussed leads to apoptosis (Frances et al., 2013). In the liver of STZ-induced diabetic rats, where the modulation of TNF-R1 by TNF-α lead to an increased expression and activity of caspase-3, caspase-8 and mitochondrial t-Bid (Ingaramo et al., 2011). Exposure to elevated levels of TNF-α in the liver also activates the pro-apoptotic function of JNKs, which is inherently detrimental to hepatocyte function and survival (Nakagawa & Maeda, 2012; Rex et al., 2019). This was corroborated by findings suggesting that the activation of JNK in diabetes and the subsequent apoptotic cell death was nullified by the reduction of TNF-α levels by treatment with etanercept (Daniel et al., 2013).

In conclusion, the pathogenesis of diabetic complications is likely mediated by the presence of chronic and uncontrolled hyperglycaemia, possibly leading to an accumulation of toxic glucose by-products, such as methylglyoxal, leading to a state of oxidative and dicarbonyl stress. It is important to understand the mechanisms underlying the onset of these pathologies, and fully identify the links between T2DM and NAFLD development.

Dicarbonyl stress

Dicarbonyl stress is defined as a dysfunctional metabolic state characterised by the abnormal accumulation of intracellularly formed dicarbonyl α-oxoaldehyde metabolites such as methylglyoxal (MGO), glyoxal (GO) and 3-deoxyglucosone (3-DG) that are derived from various metabolic pathways, but especially glucose metabolism, (Seo, Ki, et al., 2014) and/or as a result of a decreased activity of their detoxifying systems (Nigro et al., 2019; Shamsaldeen et al., 2016), and this creates a state where dicarbonyl glycation is increased contributing, to cell and tissue dysfunction in aging and disease (Nigro et al., 2019; Rabbani et al., 2016b; Rabbani et al., 2018).



Glyoxal Methylglyoxal 3-deoxyglucosone

Figure 1.4. Chemical structure 1,2-dicarbonyl compounds glyoxal, methylglyoxal and 3-deoxyglucosone. The carbonyl group in each molecule is shown in red.

Among the α-oxoaldehydes metabolites, the notorious MGO is of primary concern as it is the most reactive and the most produced from the endogenous metabolite flux (ca. 3 mmol per day (Nigro et al., 2019)) (Rabbani & Thornalley, 2022). MGO is derived from glucose, FA, and amino acid metabolism and reacts with DNA nucleotides, proteins, lipids, and other molecules, forming stable adducts that alter their structure and/or function (Shamsaldeen et al., 2016).

Dicarbonyl stress is commonly observed in the diabetic phenotype and is recognised as identified as a major driver of the progression of diabetes and its complications (Moraru et al., 2018; Nigro et al., 2019). Increased MGO levels were found in newly diagnosed type 2 diabetic patients (Xiang Kong et al., 2014) and Maessen and co-workers reported increased α-dicarbonyl levels in patients with impaired glucose metabolism and T2DM (Maessen et al., 2015). Its deleterious effects encompass an array of mechanisms commonly observed during metabolic stress, including impairment of mitochondrial function via modifications of mitochondrial proteins (Zheng et al., 2016), oxidative stress, impairment of insulin signalling via glycation of the insulin receptor substrate, thus leading to conformational changes that hinder protein tyrosine phosphorylation and its docking function, and inflammation (Afridi et al., 2016; W.-C. Chang et al., 2015; Cheng et al., 2012; Mohamed et al., 2016; Ng et al., 2022). Hyperglycaemia-induced dicarbonyl stress has also been linked to vascular complications in diabetes such as hypertension, dyslipidaemia, and obesity (Schalkwijk & Stehouwer, 2020).

Methylglyoxal formation

MGO can be introduced into the body through the consumption of food and beverages, such as coffee, whiskies, heated and processed fats and proteins, as well as sugars and tobacco

(Shamsaldeen et al., 2016). Although these contain variable amounts of MGO, their contribution to dicarbonyl stress in disease remains contended as it is almost negligible (<1% of total MGO exposure) and it is suggested that, due to its high reactivity, it is rapidly metabolised and reacts in the intestinal lumen before adsorption to reach systemic circulation (Nigro et al., 2019). However, it has been suggested that chronic consumption of products containing large amounts of MGO-could contribute to mild liver inflammation in rats and fat deposition in parenchymal cells which could impair insulin sensitivity and glucose tolerance (Shamsaldeen et al., 2016).

Besides the negligible amount of exogenous MGO, it is endogenously formed into the cells mainly through three metabolic pathways, elaborated below.

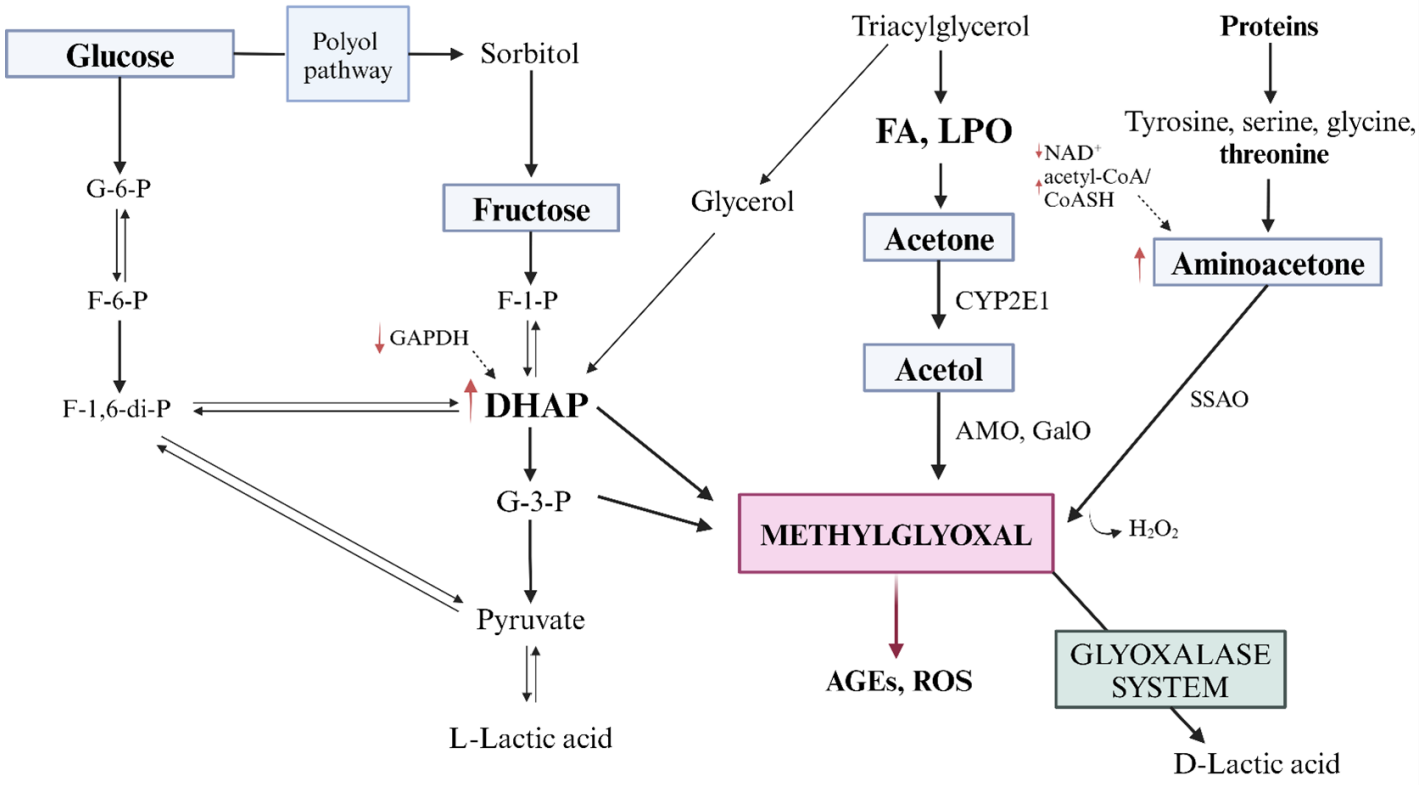


Figure 1.5. Description of the precursors and the respective pathways that yield methylglyoxal. CoA, coenzyme A, G-6-P, glucose 6-phosphate, F-6-P, fructose 6-phosphate, F-1,6-di-P, Fructose 1,6-bisphosphate, F-1-P, fructose 1-phosphate, G-3-P, glyceraldehyde-3-phosphate, DHAP, dihydroxyacetone phosphate, SSAO, semicarbazide sensitive amine oxidase, GalO, galactose oxidase, AMO, acetol mono-oxygenase. Created with BioRender.com.

Carbohydrate metabolism

Glucose is physiologically metabolised via glycolysis in the cytosol, resulting in the synthesis of pyruvate. The main intermediate metabolites of glycolysis, but also of gluconeogenesis and glyceroneogenesis (Nigro et al., 2019), glyceraldehyde-3-phosphate (G3P), also known as triose phosphate, and dihydroxyacetone phosphate (DHAP), are direct precursors of MGO (Ramachandra Bhat et al., 2019). MGO is yielded from the non-enzymatic degradation of these intermediates. They undergo enolization to form 3-phospho 1, 2-eno-diol, which further fragments, through the removal of phosphate, to form MGO. In-vitro studies report that MGO formation in this pathway is mainly from G3P rather than DHAP (Shamsaldeen et al., 2016). It is estimated that 0.1–0.4% of the glycolytic flux ends up as MGO (Allaman et al., 2015b; Angeloni et al., 2014; Dhar et al., 2008; Dornadula et al., 2015). MGO formation though glycolysis is automatically elevated when glucose metabolism is increased, which is commonly observed during hyperglycaemia, but also when the disposal of G3P is hindered by a decreased activity of the reductive pentose phosphate pathway, and when the metabolic pathways shift towards an increased dependence on anaerobic glycolysis and increased metabolic flux during hypoxia (Nigro et al., 2019). In addition to hyperglycaemia, inflammation may also drive increased MGO formation, as glycolysis is also increased under this condition ((Haik Jr et al., 1994)).

Increased levels of oxidative stress also lead to increased production of MGO due to GAPDH inhibition, as previously discussed (Giacco et al., 2010). MGO generation is enhanced as glycolytic intermediate DHAP accumulates (Dhar et al., 2008; Pacher et al., 2004), due the reduced GAPDH- mediated oxidation of DHAP into d-glycerate 1, 3-bisphosphate that is further converted to pyruvate and then L-lactate (Shamsaldeen et al., 2016)(Singh et al., 2014). Reduced GAPDH activity is detected in RBC from diabetic patients, along with a 3 fold increase in DHAP (Shamsaldeen et al., 2016). MG synthase (MGS) is also highly specific for DHAP and also catalyses the conversion of DHAP to MGO and orthophosphate (Shamsaldeen et al., 2016). Another major source of dicarbonyl generation in obesity is glyceroneogenesis, where pyruvate is the principal carbon source. In contrast to glucose, pyruvate entry and metabolism is unregulated, which constitutes an alternative constant source of triosephosphates. This could be problematic in cells susceptible to insulin-resistance, such as adipocytes and hepatocytes (Nigro et al., 2019) where diverse alterations in various intracellular insulin signalling pathways impair their normal function, playing a central role in

metabolic dysregulation (Oliveira et al., 2018). Also, auto-oxidation of glucose (Bellahcène et al., 2018) and the degradation of glycated proteins (Jagt, 2008) are also considered a minor source for the production of MGO.

Sucrose from the diet is converted into glucose and fructose and increased influx into the polyol pathway during hyperglycaemia is another source of MGO generation. Numerous studies have suggested that fructose and its metabolites operate as endogenous toxins (Feng et al., 2009; Lee et al., 2009) and fructose has been established to be an important precursor in MGO formation as it yields DHAP (Ramachandra Bhat et al., 2019). Intracellular fructose is elevated in a number of tissues of diabetic patients and has been proven to yield equivalent amounts of MGO to those of glucose (Dhar et al., 2008). This was confirmed by a study that reported that the serum level of MGO is increased in fructose-fed rats (Jia & Wu, 2007). Prolonged high fructose intake also results in decreased CAT and SOD activity, as well as oxidative changes in liver cells and diminished antioxidant capacity of mitochondria due to fructose consumption has been associated with elevated oxidative stress, which results in LPO of polyunsaturated fatty acids (PUFAs) (Muriel et al., 2021).

Protein metabolism

Tyrosine, serine, threonine and glycine rich proteins are particularly vulnerable to catabolism (Shamsaldeen et al., 2016). Aminoacetone (AA), the product of the NAD+ dependent L-threonine dehydrogenase catabolism of threonine has been proven to be a significant precursor of MGO (Dhar et al., 2008; Nigro et al., 2019; Sartori et al., 2008; Shamsaldeen et al., 2016; Thornalley, 2007). Threonine dehydrogenase catalyses the oxidation of threonine to glycine (and partially tyrosine) and acetyl-CoA through NAD+ (Dhar et al., 2008) contributing to ca. 3% of total MGO exposure (Angeloni et al., 2014; Nigro et al., 2019). In diabetes, reduced NAD+ and increased acetyl-CoA/CoASH ratio, leads to increased AA accumulation (Nigro et al., 2019). Moreover, semicarbazide sensitive amine oxidase (SSAO), catalyses oxidative deamination of aminoacetone into MGO and H_2O_2 in hyperglycaemic conditions (Kalapos, 2008; Lyles & Chalmers, 1992; Shamsaldeen et al., 2016). Increased SSAO levels have been reported in hypertension and in both T1DM and T2DM, which positively correlate with increased MGO concentrations (Dornadula et al., 2015; Pacher et al., 2004; Peter et al., 2003). Protein catabolism is increased by approximately 50% in diabetic rats and was associated with IR and increased glucocorticoids production (Shamsaldeen et al., 2016).

Lipid metabolism

Other sources of MGO may emerge through Maillard reaction, from FA metabolism and LPO (Angeloni et al., 2014; Dhar et al., 2008). Ketone body metabolism consists of acetoacetate decarboxylase generating acetone from the degradation of the major ketone body acetoacetate (Nigro et al., 2019; Shamsaldeen et al., 2016). Both acetoacetate and acetone are found to be elevated in diabetic patients' plasma (Shamsaldeen et al., 2016). Acetone is hydroxylated through the monooxygenase CYP2E1, which is mainly found in the liver, into acetol which is further oxidized to MGO and H_2O_2 through galactose oxidase (GalO) and NAPDH, and through acetol mono-oxygenase (AMO) (Dhar et al., 2008; Kalapos, 2008; Nigro et al., 2019; Shamsaldeen et al., 2016). Acetol has been reported to accumulate in some diabetic patients and produces IR in experimental animals (Vander Jagt et al., 2001). This pathway may contribute largely to the production of MGO in conditions where ketone bodies' are elevated and tend to accumulate, such as in diabetic ketoacidosis or as a result of a diet rich in fats (Angeloni et al., 2014; Nigro et al., 2019). Interestingly, CYP2E1 has been implicated in the development of NAFLD (Wang et al., 2021). In addition to generating free radicals and ROS to promote oxidative stress and LPO (Guan et al., 2019), it may be involved in excessive hepatic fat accumulation and inflammation (Jian et al., 2018). Experimental CYP2E1 inhibition showed favourable outcomes in rodent NASH models, by improving oxidative stress, liver steatosis, LPO, inflammation and fibrosis (Zhong & Liu, 2018).

LPO products, such as 4-hydroxy-2-nonenal (HNE), and ketoaldehydes are important sources of MGO generation, through nonenzymatic and enzymatic metabolism of acetoacetate or acetone intermediates (but also glucose autooxidation as previously mentioned), respectively (Angeloni et al., 2014; Nigro et al., 2019; Shamsaldeen et al., 2016). Moreover, in lipolysis, triacylglycerol hydrolysis produces glycerol which can be transformed into MGO via glycerol-3-phosphate dehydrogenase (Angeloni et al., 2014; Shamsaldeen et al., 2016). Studies report increased lipolysis in diabetes and its suppression can improve insulin sensitivity and glucose uptake (Shamsaldeen et al., 2016).

The glyoxalase system

The glyoxalase system is an enzyme system that consists of glyoxalase 1 (GLO1), glyoxalase 2 (GLO2) and GSH as a catalyst (Nigro et al., 2019). This system is found ubiquitously in all living organisms and mainly operates in the cytosol, but also, to a lesser extent, in the

mitochondria of the cell (Hollenbach, 2017). It is the most important endogenous defence system against dicarbonyl stress (Nigro et al., 2019) as it efficiently catalyses the detoxification of MGO, among other α-oxo-aldehydes, to form the inert d-lactate in normal conditions through of a GSH dependent pathway (Shamsaldeen et al., 2016). GLO1 catalyses the isomerisation of hemithioacetal, formed spontaneously from MGO and GSH to S-d-lactoylglutathione (Allaman et al., 2015b). GLO2 catalyses the conversion of S-d-lactoylglutathione to inert H2O and d-lactate and recycles the GSH consumed in the GLO1-catalysed reaction (Allaman et al., 2015b; Hollenbach, 2017; Schalkwijk & Stehouwer, 2020). Other substrates of GLO1 include glutathione-derived hemithioacetals, which are formed from different α-dicarbonyls, such as glyoxal, hydroxypyruvaldehyde, or phenylglyoxal (Schalkwijk & Stehouwer, 2020).

Noteworthy, a third glyoxalase enzyme, glyoxalase 3, was recently discovered in Escherichia coli which is able to convert MGO to D-lactate in the absence of any cofactor (Hollenbach, 2017). Furthermore, other enzymes can, to a lesser extent, contribute to the detoxification of MGO. Aldoketo reductases (AKRs) and aldehyde dehydrogenases (ADHs) catalyse the NADPH-dependent reduction of MGO to yield hydroxyacetone, lactaldehyde, and pyruvate (Hollenbach, 2017; Nigro et al., 2019). They are utilised as minor compensatory systems when the glyoxalase system is overwhelmed (Kold-Christensen & Johannsen, 2020). However, a study found that for MGO metabolism in human tissue, the glyoxalase system activity exceeds AKR activity by approximately over 30-fold and ADH activity was almost undetectable (Nigro et al., 2019).

MGO reactivity is 20,000-fold higher than that of glucose, but in health, efficient detoxification of MGO by the glyoxalase system maintains the concentration of MGO in plasma approximately 50,000-fold lower than that of glucose (Rabbani & Thornalley, 2014a). Some studies have demonstrated that small increases of MGO, within the physiological range, and in contrast to high concentrations, are beneficial and play a role in modulating physiology (Ravichandran et al., 2018; Zemva et al., 2017; X. Zhang et al., 2022).

Optimal glyoxalase system activity is essential for the effective detoxification of MGO, which in the presence of a high glycolytic flux characteristic of certain cell types, including β-cells and hepatocytes, may accumulate rapidly (Hollenbach, 2017). Adequate levels of GSH are crucial for the efficient functioning of the glyoxalase system. Although GLO2 activity can

directly affect GSH availability, under conditions of NADPH depletion, such as hyperglycaemia, reduced GSH would hinder the efficacy the GSH-dependent glyoxalase system (Dornadula et al., 2015; Giacco et al., 2010; González et al., 2023). This impairs the detoxification of MGO and further increases its half-life, favouring dicarbonyl stress (Amicarelli et al., 2003; Di Loreto et al., 2004; Nigro et al., 2019).

More importantly, the GLO1 catalysed reaction represents a crucial step in MGO detoxification as GLO1 activity directly determines the rate of MGO and MGO-derived AGEs formation (Allaman et al., 2015b; Yang et al., 2022). Cellular GLO1 concentrations are around ~0.2 μg per gram of total protein and is in the top 13% of protein abundancy (Thornalley, 2003) and studies indicate that the retina and liver display the highest GLO1 activity (Aragonès et al., 2021). The widespread distribution of GLO1 is also suggestive of its evolutionarily conserved and fundamental biological importance (Schalkwijk & Stehouwer, 2020). Interestingly, skeletal GLO1 protein expression in is lower in T2DM patients, and inversely correlates with carbonyl stress, BMI, percent body fat, and Homeostatic Model Assessment for Insulin Resistance measurements (HOMA-IR), while positively correlating with glucose disposal (Mey et al., 2018). Hepatic GLO1 expression was also found to be also decreased in paediatric NAFLD patients and in a murine NAFLD model (Spanos et al., 2018), and a rat cirrhosis model, which was accompanied by elevated levels of MGO (Hollenbach et al., 2017). GLO1 expression and activity decreases also with age, as observed in arterial, human lens and brain tissues in ageing phenotypes (Aragonès et al., 2021; Nigro et al., 2019).

Studies of levels of expression, and possible polymorphisms may elucidate the role of GLO1 in the pathogenesis of diabetes and its complications. Glo1 has a functionally operative regulatory insulin-response element (IRE), antioxidant responsive element (ARE) and a metal-response element (MRE) in its promoter (Ranganathan et al., 1999; Schalkwijk & Stehouwer, 2020; Xue et al., 2012). Glo1 expression, regulated by insulin, zinc and under stress-responsive control by transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2) (Jyotiska Chaudhuri et al., 2016; Schalkwijk & Stehouwer, 2020; Xue et al., 2012). Nrf2 binding to AREs regulates basal and inducible expression of a range of antioxidant genes including those for GLO1 as well as AKRs and ADH and the upregulation of GSH synthesis (González et al., 2023; Kold-Christensen & Johannsen, 2020; Nigro et al., 2019). In response to stress, whether oxidative or dicarbonyl, small electrophilic compounds bind to the Kelch-like ECH-associated protein (KEAP1), a negative regulator, and induce its dimerization that allows the nuclear

translocation of Nrf2, and these altogether constitute the regulatory KEAP1–Nrf2–ARE axis (Kold-Christensen & Johannsen, 2020). Reduced GLO1and Nrf2 expression, together with the increase of KEAP1, were observed in the skeletal muscle of T2DM patients compared to healthy control subjects (Kold-Christensen & Johannsen, 2020; Nigro et al., 2019). In accordance, activators of Nrf2 induce increased Glo1 mRNA, protein, and activity and decrease levels of MGO *in vivo* and in HepG2 cells (Jyotiska Chaudhuri et al., 2016; Cheng et al., 2012; Xue et al., 2012), and a clinical trial of a coformulation of the Nrf2 activators trans-resveratrol and hesperetin in healthy overweight and obese individuals showed improved glycaemic control and vascular inflammation and function via induction of Glo1 (Xue et al., 2016). Conversely, Glo1 is negatively regulated by hypoxia inducible factor 1α (HIF-1α) binding to ARE under hypoxic conditions and, accordingly, MGO is increased during hypoxia (Hanssen et al., 2015) (Nigro et al., 2019). Other consensus sequences in the GLO1 gene promoter are for the transcription factors specificity protein 1 (SP1), NF-κB, CCAAT-enhancer-binding protein (C/EBP), and AP-1 (Schalkwijk & Stehouwer, 2020).

Implications of MGO in the pathogenesis of diabetes and diabetic complications

Indeed, the pathogenesis-associated decline in Glo1 expression and/or an increase in the formation of MGO, was estimated to lead to a 2–3-fold increase in plasma and tissue-specific increase in the concentration of MGO (Rabbani & Thornalley, 2022), which has been suggested be a major factor in low-grade inflammation and cell tissue dysfunction driving the development of chronic disease or complications of metabolic, vascular, and other diseases (Rabbani et al., 2016b). As briefly mentioned, MGO has been implicated in the progression of diabetes and its associated complications (Nigro et al., 2019; Schalkwijk & Stehouwer, 2020) and its levels are increased in diabetic patients (Dhananjayan et al., 2019; Rabbani & Thornalley, 2014b). Elevated MGO levels and its derived AGEs are considerable markers of carbonyl overload, oxidative stress, and the resultant damage in diabetes (Dhar et al., 2008; Hollenbach, 2017; Lin et al., 2012; Rabbani et al., 2016a). It was observed in diabetic patients that the degree of tissue damage and disease progression in diabetic complications, such as diabetic nephropathy , polyneuropathy cardiovascular, disease and mortality in type 1 and 2 diabetes (Kold-Christensen & Johannsen, 2020) positively correlated with plasma concentrations of MGO and with plasma concentrations of MGO-derived hydroimidazolone (Schmoch et al., 2017).

MGO may not only be reflection of increased hyperglycaemia but also itself induce insulin resistance and *β*-cell dysfunction, thereby promoting the onset and progression of T2DM (Dornadula et al., 2015; Moraru et al., 2018). Several rodent models were able to corroborate the ability of MGO to induce insulin resistance and directly damage pancreatic insulin secreting *β*-cells (Bora & Shankarrao Adole, 2021; Nigro et al., 2014; Tym, 2014; Vlassara & Uribarri, 2013). *In vivo* chronic MGO administration to rats induced systemic dysmetabolism, as indicated by higher FFA levels, insulin resistance and impaired insulin secretion and glucose intolerance, which are all characteristic of T2DM (Dhar et al., 2011). In another similar study in rats, in addition to reducing pancreatic insulin protein expression , MGO administration reduced pancreatic Nrf2 protein expression (Cheng et al., 2015), which would lead to decreased GLO1 expression.

MGO experimentally exerted major deleterious effects on INS-1E cells through the impairment of both insulin action and secretion, independently from ROS production, and the inhibition of pancreatic and duodenal homeobox 1 mRNA expression (Fiory et al., 2011), a crucial factor in pancreas organogenesis, the maturation and identity preservation of *β*-cells, and of their role in normal insulin function (Ebrahim et al., 2022). MGO contributes to MIN6 cell dysfunction *in vitro* through damage to collagen IV, impairment of *β*-cell attachment to the extracellular matrix protein, and increased protein turnover (Tym, 2014). MGO induced cytotoxicity in INS-1E cells primarily through ROS production, triggering the mitochondrial apoptotic pathway and ER stress (C. Liu et al., 2017). Similarly, in INS-1E cells exposed to AGEs, ROS-mediated cell death was observed, mainly as a result of mitochondrial dysfunction (Lin et al., 2012).

Furthermore, MGO has been shown to negatively influence systemic metabolism by inducing glucose intolerance and insulin resistance. MGO reduced the activity of plasma membrane glucose transporter-4 (GLUT-4) as well as pancreatic glucose sensor GLUT2 thereby reducing glucose uptake in diabetic animals as well as in isolated pancreatic islets (Dornadula et al., 2015; Shamsaldeen et al., 2016). MGO treated HepG2 cells showed increased serine phosphorylation of IRS1 (Afridi et al., 2016; Cheng et al., 2012), which was also observed in adipocytes (Ng et al., 2022). Similar results were obtained in mouse aortic endothelial cells treated with MGO and S-p-bromobenzylglutathione-cyclopentyl-diester (BBGC), a GLO1 inhibitor, the inhibition of the IRS1/Akt/eNOS pathway was also observed, thereby blunting NO generation. In the same study, chronic administration of MGO to C57BL/6 mice impaired whole-body insulin sensitivity and induced endothelial IR (Nigro et al., 2014). It was also

reported that acute exposure to MGO inhibits insulin-stimulated phosphorylation of Akt at Ser473 and Thr308 and extracellular-regulated Kinase 1/2 (ERK 1/2) leading to impaired insulin signalling in L6 skeletal muscle cells (Riboulet-Chavey et al., 2006).

Regarding important diabetic complications, MGO has been strongly and ubiquitously linked to their development and to has been shown to exert cytotoxicity to the respective cell types. MGO levels are considered a biomarker of both non-diabetic and diabetic chronic kidney disease - higher plasma MGO levels were shown to be associated with a decline in the estimated glomerular filtration rate (Hanssen et al., 2019). In patients with chronic renal disease, up to sixfold higher plasma levels of MGO have been reported and increase with the severity of disease (Henning et al., 2014; Lu et al., 2011; Nakayama et al., 2008; Rabbani et al., 2007). MGO-derived hydroimidazolone MG-H1 was increased 8-fold and 30-fold in stage 4 CKD in patients without and with diabetes, respectively, likely reflecting an increased MGO glycation status in diabetes. MG-H1 increased progressively in accordance with the decline in renal function (Rabbani et al., 2023). Elevated levels of MGO-derived AGEs were found to be independent risk factors for the progression of diabetic kidney disease, specifically, and may serve as biomarkers to improve the reliability of early diagnosis (Ding et al., 2024). MGO-derived AGEs cause injury and nephrotoxicity as demonstrated in human embryonic kidney cells (Gupta et al., 2022) through oxidative stress, inflammation and necrosis. MGO-induced inflammation was also observed in a human kidney epithelial cell line, as a result of activated inflammatory signalling pathways p38 mitogen-activated protein kinase (MAPK), ERK, and c-JUN N-terminal kinase (JNK) and accordingly, increased expressions of NF-κB and inflammatory cytokines TNF-α, interleukin- (IL) 1β, and IL-6. Interestingly, MGO also increased NOX4 and PKC expression while decreasing Nrf2 and GLO1 expression (D. Kim et al., 2021).

Plasma MGO levels are also associated with increased CVD prevalence in T1D patients (Hanssen et al., 2017), and increased CVD prevalence and mortality in individuals and T2DM, which is the leading cause of death in T2DM patients (Hanssen et al., 2018; Koska et al., 2018). The importance of MGO in endothelial dysfunction in diabetes has been demonstrated in experimental models, where it has been suggested to be partly through the formation of AGEs which exert damage both directly as well as via AGE-RAGE (Bora & Shankarrao Adole, 2021; Stirban et al., 2014). Amadori products and AGEs are able to inhibit NOS activity (Ren et al., 2017; Soro-Paavonen et al., 2010), increase ROS generation and activate PKC isoforms

(Cai et al., 2010), which have been linked to decreased NO production in smooth muscle cells and inhibition of insulin-stimulated expression of eNOS in endothelial cells *in vitro* (Bora & Shankarrao Adole, 2021; Giacco et al., 2010). *In vitro*, MGO decreased Nrf2 expression, increased ROS generation, mitochondrial dysfunction and triggered mitochondrial apoptotic pathways in human endothelial cells (Wang et al., 2022; Zhang et al., 2021). In healthy mice treated with a GLO1 inhibitor, MGO deposition in aortic wall was enhanced, and *in vitro*, MGO triggers human aortic endothelial cell dysfunction by activating the JNK/p38 MAPK pathway (Wang et al., 2019). *In vivo* MGO administration or the use of BBGC, to increase levels of MGO to those observed in diabetic mice, increased vascular adhesion and inflammation and enhanced atherogenesis in euglycemic apolipoprotein E knockout mice similarly to what is observed in hyperglycaemic mice with diabetes (Tikellis et al., 2014). RAGE deletion did not completely prevent inflammation or vascular damage, suggestive of alternative mechanisms mediating MGO-induced cytotoxicity. MGO was also shown to trigger ER stress and apoptosis in rat vascular smooth muscle cells, both of which may play an important role in atherosclerosis progression in vascular diseases (Kırça & Yeşilkaya, 2022). MGO could also contribute to atherosclerosis through the AGE-led oxidation and glycation of low-density lipoprotein cholesterol (LDL-C) (Bora & Shankarrao Adole, 2021).

The pro-thrombogenic effects of MGO could also play an important role in the enhanced risk for stroke in diabetic individuals, as shown by increased platelet aggregation in response to MGO *in vitro* (Hadas et al., 2013). Low platelet GSH levels, GPx-1 and SOD-1 expression were observed in diabetes, which were associated with elevated MGO levels and MGO-adduct formation in platelets (B. Wang et al., 2018). Furthermore, exacerbated stroke-induced brain injury in diabetes was correlated with a higher brain MGO/GSH ratio (B. Wang et al., 2016). Chen et al. also reported that *in vitro*, MGO induced human brain microvascular endothelial cell dysfunction and apoptosis, and impaired angiogenesis *in vivo* (Chen et al., 2022).

A role for MGO in the development of diabetic cardiomyopathy has also been suggested, through the induction of inflammation and ER stress (Nam et al., 2015; B. Vulesevic et al., 2016). Moreover, elevated MG-H1 levels are associated with retinopathy in patients with T2DM (Fosmark et al., 2007; Fosmark et al., 2006). In experimental settings, MGO treatment mimics important aspects of diabetic retinopathy such as retinal and macular damage (Y.-C. Chang et al., 2015; Fosmark, 2008; Kim et al., 2012; Y. S. Kim et al., 2014; Sekar et al., 2023),

and plays a pathogenic role in microglial activation, vascular damage, and neuroretinal dysfunction in the absence of hyperglycaemia (Akagawa et al., 2018; Schlotterer et al., 2019). It was also suggested to play an important role in age-related macular degeneration (Yoon et al., 2012).

Furthermore, MGO might play a key role in the progression of diabetes-related cognitive disorders, as it has been associated with the progression of diabetic neuropathy (Brings et al., 2017; Matafome et al., 2017; Qi et al., 2022), neurodegeneration and cognitive decline i.e. dementia and Alzheimer's (Angeloni et al., 2014; Beeri et al., 2011; Hansen et al., 2016; Jiang et al., 2014; Lissner et al., 2022; Liu et al., 2013; Srikanth et al., 2013).

Lastly, MGO treatment can negatively influence collagen metabolism and differentiation of human bone marrow-derived stromal cell-derived osteoblasts through a RAGE independent mechanism (Waqas et al., 2022), possibly explaining the increased risk of fractures and musculoskeletal disorders observed in diabetes (Saito et al., 2014). MGO was also shown to slow diabetic wound healing (Berlanga et al., 2005; H. Li et al., 2019; Wang et al., 2017) and Jiang et al. reported that MGO-derived AGEs in human diabetic wounds is increased, whereas the expression of GLO1 is decreased, and in a T2DM mice model and in-vitro studies, MGO-induced macrophage dysfunction leads to dysfunctional inflammatory response and slower wound healing (Jiang et al., 2022). Higher plasma MGO levels were also associated with more adverse outcomes in severe limb ischemia and amputations in T2DM patients (Hanssen et al., 2021).

Glycation and AGE formation

One of the recognised significant mechanisms that drive the damaging effects of MGO is through glycation and the formation of AGEs, leading to important tissue damage that is observed in diabetes (Beisswenger et al., 2003; Nigro et al., 2019), as many studies have indeed shown increased MG-derived AGEs and glycation in diabetic humans (Matafome et al., 2017). MGO that escapes metabolism is able to induce glycation leading to the intra- and extra-cellular formation of AGEs at a rate up to 20,000 times that of glucose and is believed to be the most important source of AGEs (Angeloni et al., 2014; Rabbani & Thornalley, 2014b; Rosca et al., 2005). MGO mainly reacts with lysine, arginine, and cysteine residues of proteins, nucleic acids and phospholipids to produce a variety of structurally distinctive, irreversible AGEs

(Dhar et al., 2008; Hollenbach, 2017; Li et al., 2018; Nigro et al., 2019; Rabbani & Thornalley, 2022; Rosca et al., 2005; Zheng et al., 2016).

MGO interactions with arginine most importantly form, through Maillard reactions, compounds named hydroimidazolones: N^{δ}-(5-hydro-5-methyl-4-imidazolone-2-yl) ornithine, , (MG-H1) and other related structural isomers, 2-amino-5-(2-amino-5-hydro-5-methyl-4-imidazolon-1-yl)pentanoic acid (MG-H2), and 2-amino-5-(2-amino-4-hydro-4-methyl-5-imidazolon-1-yl) pentanoic acid (MG-H3) (Angeloni et al., 2014). These compounds can undergo further modifications; yielding either Nδ-(4-carboxy-4,6-dimethyl-5,6-dihydroxy-1,4,5,6-tetrahydropyrimidine-2-yl)-L-ornithine (THP) or argpyrimidine (Nδ-(5-hydroxy-4,6-dimethylpyrimidine-2-yl)-l-ornithine) (Angeloni et al., 2014; Hollenbach, 2017). Further minor arginine-derived adducts are the MGO-mediated N!-carboxyethylarginine (CEA) (Hollenbach, 2017) which in recent studies has emerged to be more important than previously estimated(Kold-Christensen & Johannsen, 2020) . MG-H1 is the most abundant (>90% of MGO adducts) and functionally important AGE in physiological systems (Kold-Christensen & Johannsen, 2020; Nigro et al., 2019). MGO reactions with lysine residues form Nε-(1-carboxyethyl)-L-lysine (CEL) and Nε-(1-carboxymethyl)-L-lysine (CML) adducts and the lysine dimer 1,3-di(Nε-lysino)-4-methyl-imidazolium (MOLD) (Angeloni et al., 2014; Hollenbach, 2017) (Chen et al., 2017). MGO can form with one lysine and one arginine residue, 2-ammonio-6-(2-[(4-ammonio-5-oxido-5-oxopentyl) amino]-4-methyl-4,5-dihydro-1H-imidazol-5-ylidene amino) hexanoate (MODIC) (Angeloni et al., 2014).

Protein alterations by MGO usually involve nucleophilic addition at arginine, lysine and cysteine residues at the N-terminus amino group (Dhar et al., 2008). While oxidation does not mediate these alterations, does accelerate the degree of their formation (Beisswenger et al., 2003). As arginine is a widely present compound in functional sites of proteins, glycated arginine residues, result in a loss of positive charge, and electrostatic interactions involved in the side-chain guanidine group and ligand-binding interactions, resulting in protein dysfunction and possibly total loss of function of the original protein (Hollenbach, 2017; Nigro et al., 2019; Schmoch et al., 2017) .

MGO is formed intracellularly though a small proportion may leak out glycate extracellular proteins as well, such as albumin (Cerf, 2013; Khan et al., 2015). MGO has indeed been reported to move across membranes such as plasmalemma and mitochondrial membranes and

hence is able to attack different intracellular targets, including mitochondrial complexes and proteins. The MGO induced glycation of mitochondrial proteins could lead to mitochondrial protein dysfunction , enzyme inactivation, and an immune response which are all associated with an increased production of ROS which can further damage cellular structures (Hollenbach, 2017; Nigro et al., 2019).

MGO has previously been directly linked to mitochondrial dysfunction, which is strongly evidenced by the identification of and the presence of reactive dicarbonyls and MGO-derived modifications within the mitochondria in response to hyperglycaemia (Rabbani & Thornalley, 2008; Rosca et al., 2002; Rosca et al., 2005). Accordingly, the overexpression of Glo1 in C. elegans decreased dicarbonyl glycation of mitochondrial proteins, ROS generation and markers of both dicarbonyl glycation and oxidative damage (Rabbani & Thornalley, 2008). The incubation of mitochondria with MGO concentrations approaching the pathological range in diabetes results in the formation of MG-H1 on several mitochondrial proteins and increases the generation of superoxide as a result of impaired electron transport chain function (Rosca et al., 2002; Rosca et al., 2005). A two- to three-fold increase in oxidative stress has been associated with small increases in MG-H1 alterations of mitochondrial proteins, most likely due to electron leakage from the electron transport chain complex III (Schalkwijk & Stehouwer, 2020). Indeed, studies have revealed that MGO preferably damages complex III as MGO-induced imidazole epitopes are widely observed in critical protein components of this complex (Rosca et al., 2005).

The inhibition of complex III can originate in 70–80% of superoxide in the ubiquinone cycle (Rosca et al., 2005), and consistently, MGO treatment increases mitochondrial superoxide production and $ONOO^-$ production, which can be reversed by a dicarbonyl scavenger treatment, aminoguanidine (Rosca et al., 2005). This would also lead to the collapse of the mitochondrial transmembrane potential, and reduced mitochondrial respiration results in reduced ATP production, further compromising mitochondrial and cellular stability and function (Angeloni et al., 2014). Several studies also report that MGO inactivates Cu,Zn SOD by covalent crosslinking, releasing copper ions from the enzyme promoting the cytotoxic misfolding and aggregation process of SOD1 (Angeloni et al., 2014; Polykretis et al., 2020), and thus further increasing cellular oxidative stress. This is also well demonstrated in *ex vivo* studies where erythrocytes drawn from diabetic patients showed a significantly increased

glycation of SOD1 as well as a lower enzymatic activity compared to control subjects (Kawamura et al., 1992)

Furthermore, increased levels of circulating glycated insulin, which shows reduced biological activity, have been observed in diabetic animal models and humans with T2DM (Hunter et al., 2003; McKillop et al., 2001). Indeed, incubation of human insulin with MGO leads to alterations to the β-chain of insulin, specifically at the N-terminus on the arginine residue via Schiff base formation (Jia et al., 2006). This forms MGO-insulin adducts that impair the insulin mediated glucose uptake by cells, decreases insulin clearance through liver cells and hinders the autocrine control of insulin release from pancreatic β-cells ((Nigro et al., 2019; Shamsaldeen et al., 2016). Moreover, glucose-induced insulin secretion insulin-stimulated glucose uptake can be also hindered through direct MGO-glycation of IRS proteins. Exposure of INS-1E cells to non-cytotoxic MGO concentrations yields the formation of CEL– and argpyrimidine–IRS1 adducts (Fiory et al., 2011). These modifications suppress the insulin-induced activation of IRS1/ phosphatidylinositol 3 kinase (PI3K)/Akt (Nigro et al., 2019). Changes in IRS synthesis and/or degradation may have caused these results, but the authors were able to rule it out as IRS protein levels were unaltered after MGO treatment. Treatment with aminoguanidine and N-acetylcysteine, MGO scavengers, reversed the MGO inhibitory effects and the formation of AGE adducts on IRS (Fiory et al., 2011; Shamsaldeen et al., 2016). MGO seems to impair insulin expression, secretion and action which are fundamental to the pathogenesis of diabetes. This is in line with a report that pharmacological induction of GLO1 activity improves insulin sensitivity and glycaemic control in patients (Xue et al., 2016).

In data obtained from yeast, it has been shown that MGO directly modifies cysteine residues in the functional homologue of mammalian AP-1, Yap1, whose DNA-binding activity depends on the redox regulation of a conserved cysteine residue (Maeta et al., 2004). Direct MGO-modification of Yap1 is sufficient for translocation to the nucleus and activation of its target genes, thus indicating that MGO regulates this transcription factor positively. It is worth noting that the promotor region of human GLO1 contains consensus sites for NF-κB, as well as for AP-1 (Ranganathan et al., 1999), suggesting that MGO may play a role in the redox regulation of these transcription factors in this manner, in both physiological and pathophysiological processes. Furthermore, it was discovered that a novel MGO-derived AGE, termed MICA, formed between a cysteine and an arginine residue in the KEAP1 protein, leads to impaired dimerization and therefore, disrupting nuclear translocation of Nrf2, and the subsequent

transcriptional activation of GLO1 (Bollong et al., 2018). This may explain the decreased GLO1 expression levels that are observed in conditions where dicarbonyl stress is increased. Indeed, MGO glycation can cause pathological changes to a plethora of proteins including various key proteins within the cell to disrupt cellular function (Queisser et al., 2009). One possibility is this being a result of DNA damage due to MGO attack on DNA, direct attacks on proteins involved in transcription and translation by MGO, and finally glycation on proteins themselves. A proteomics analysis conducted in endothelial cells revealed that 17% of proteins had low-level MGO modification including those involved in protein synthesis, protein folding, kinase signalling, glycolysis, and gluconeogenesis. In particular, heat shock protein (HSP) 90-β was one with the highest number of modifications (Irshad et al., 2019).

HSPs are multimolecular complexes that are constitutively expressed in cells under normal growth conditions, accounting for up to 5–10% of total cellular protein (Hendrick & Hartl, 1993). They function as molecular chaperones, regulating intracellular protein transport in the cytosol, ER and mitochondria as well as protein repair and degradation, and the refolding of misfolded proteins when ER stress persists for an extended period of time (Raghunath et al., 2018). A variety of physiological, pathological, or environmental stressors, including oxidative stress and apoptotic stimuli can substantially upregulate HSPs (Dubey et al., 2015). In addition to HSP90-β, MGO also modifies HSP6, HSP27, and α-crystallin, which has HSP domains (Lai et al., 2022). This may interrupt HSP interactions with other proteins, and impair chaperone function hindering their role in ER stress relief (Raghunath et al., 2018). Nevertheless, MGO-modified proteins are presumably processed by the HSP pathway through sensing and binding of an unshielded, surface hydrophobic MG-H1 residue. These are recognised as misfolded proteins, which are transported to the proteasome for proteolysis (Hollenbach, 2017). This would activate the UPR (Irshad et al., 2019; Rabbani et al., 2021), which as previously mentioned, has been recognised as a driver of insulin resistance and low-grade inflammation linked to T2DM and the development of vascular complications of diabetes and NAFLD (Rabbani & Thornalley, 2022).

When MGO-modified proteins cannot be refolded, they are targeted for degradation through the UPS (Rabbani et al., 2021). However, these damaged, dysfunctional proteins can accumulate in the cell, increasing cell death and the risk of irreversible tissue damage. This can be due to some being resistant to proteolysis but also as a result of decreased activity/efficiency of intracellular proteases (Nigro et al., 2019). The UPS is the major proteolytic system in the

cell and thereby regulates the levels of numerous proteins that control gene expression and cell division, as well as responses to stress and inflammation. Damaged or modified intracellular proteins become targeted for proteolysis by polyubiquitin chains attaching to Lys-48, and subsequently degraded in the 26S proteasome. The 20S core proteasome, which carries out proteolytic processes, and the 19S regulatory complex are the two primary multiprotein components that constitute the 26S proteasome. Through the ubiquitin-interacting motifs of its S5a subunit, the 19S regulator complex binds to ubiquitinated proteins, unfolds them, and transports them into the 20S core proteasome where they are subsequently degraded (Goldberg, 2003; Queisser et al., 2009).

The proteasome promptly eliminates oxidised, damaged proteins as a defence mechanism against aggregated and damaged proteins. An undisturbed function of the UPS is crucial for cellular processes to occur normally (Queisser et al., 2009). Indeed, many hepatocyte functions are regulated by this system. These include cell cycle check points and activation of transcription factors such as NF-κB, and hypoxia HIF-1α (French & Bardag-Gorce, 2005). The loss of proteasomes or the inhibition of the UPS can lead to hepatocellular injury including proliferation and apoptosis, and the blunting of essential gene expression that is dependent on transcription factor activation by the proteasome, possibly impeding the inflammatory response of the liver and the response to oxidative injury (French & Bardag-Gorce, 2005). There is evidence of this to also be a consequence of MGO glycation.

In yeast, an unfocussed gene deletion analysis showed strains deleted for genes of ubiquitin-dependent protein degradation were more sensitive to glyoxal and MGO toxicity (Hoon et al., 2011). In fibroblasts, increased dicarbonyl stress caused increased Golgi-to-ER retrograde traffic of proteins (Ashour et al., 2020), further supporting the idea of increased misfolding and the need for the induction of various refolding mechanisms by the ER as a result of dicarbonyl stress (Rabbani et al., 2021). An abundance of ubiquitin-protein ligases is found in HAECS under high glucose/dicarbonyl stress (Irshad et al., 2019) and several reports have demonstrated that high glucose levels lead to an acceleration of proteasomal activity and sustained NF-κB activation (A. Bierhaus et al., 2001; Queisser et al., 2009; Schalkwijk & Stehouwer, 2020), while others have shown impaired proteasome function in diabetes (Schalkwijk & Stehouwer, 2020). Moreover, previous reports also demonstrated that AGEs influence proteolytic degradation through decreased cathepsin activity, suggesting that AGEs affect proteolysis (Sebekova et al., 1998).

In HFD mice, liver proteasome activity is reduced, which causes ER stress, UPR activation and IR (Otoda et al., 2013). In line with this notion, approximately 20% of proteins with an N-terminal signal peptide were differentially secreted from mouse steatotic hepatocytes compared to normal hepatocytes, and some of these proteins were found to induce IR and pro-inflammatory signalling (Kim & Yang, 2021; Ruth C. Meex et al., 2015). Furthermore, *in vitro* experiments by Spanos et al. revealed that, in HepG2 cells, GLO1 is hyperacetylated, ubiquitinated and degraded in response to an accumulation of intracellular lipids, leading to an increase in MGO (Spanos et al., 2018). The above-described findings indicate that a substantial fraction of the hepatic proteome is heavily disturbed during NAFLD progression, including GLO1, which further favours MGO accumulation, and altogether may significantly impair energy homeostasis and systemic metabolism.

One study carried out by Queisser et al. found that proteasome activity is decreased *in vitro* and *in vivo* under conditions of chronic hyperglycaemia. Similarly, when incubated with MGO, they found that MGO covalently modified 20S proteasome subunits. The expression level of the catalytic 20S proteasome subunit was not altered but its proteasomal chymotrypsin like activity was significantly reduced. In contrast, levels of regulatory 19S proteasomal proteins were decreased. In diabetic Ins2Akita, STZ diabetic, and nondiabetic and diabetic GLO1 knockdown mice, chymotrypsin-like activity was also reduced and MGO modification of the 20S-2 subunit was increased (Queisser et al., 2009). Hyperglycaemia induced MGO accumulation was sufficient to cause direct proteasome dysfunction and accumulation of malfunctioning ubiquitinated proteins in these models as a result. The results indicate a strong link between hyperglycaemia and impairment of crucial cell functions. Moreover, the inhibition of proteasome function in human foetal hepatocytes and hepatoma cells lead to apoptotic cell death coupled with enhanced expression of IL-8, both at the protein and the mRNA levels. This increase in IL-8 is independent of NF-κB activation and is associated with an increase in JNK and activator protein-1 (AP-1) activity (Joshi-Barve et al., 2003), which corroborates the propositions of Rabbani et al., suggesting that chronic low-grade inflammation may be a pathogenic consequence of proteome disturbances caused by MGO and the consequent aberrant activation of the UPR (Rabbani et al., 2021).

Furthermore, MGO-derived AGEs, through RAGE interaction, as mentioned previously, triggers various signal transduction pathways which involve NF-κB and the production of pro-inflammatory cytokines (Hollenbach, 2017) and increases overall ROS production. Serum

MGO-AGEs and soluble RAGE are associated with NAFLD even in nondiabetic patients (Palma-Duran et al., 2018) and elevated MGO-modified proteins were also found in patients with cirrhosis compared to controls (Ahmed et al., 2004), which correlated with severity of the disease (Šebeková et al., 2002). In patients with NAFLD, patients with abnormal ALT were found to have higher serum levels of IL-6 and statistically higher levels of serum IL-8, indicating that some degree of liver damage is correlated with increasing levels of circulating proinflammatory cytokines (Chu et al., 2007) . In adult NAFLD patients, increased serum levels of MG-H1 were significantly correlated with body mass index (Spanos et al., 2018), and plasma MGO-derived CML accumulation was found to be significantly associated with the grade of hepatic steatosis and the grade of hepatic inflammation in obese patients, reflected by increased hepatic gene expression levels of IL-8 and CRP (Gaens et al., 2012). In a diabetic mouse model, AGE supplementation aggravates inflammation and renal and heart complications in the animals (Nigro et al., 2019). This is consistent with findings of a study where the overexpression of GLO1 in mouse models reduces MGO-induced inflammation and prevents ventricular dysfunction (Nigro et al., 2019).

Interestingly, in addition to increasing the phosphorylation of ERK, and IKK, AGEs induced activation of SREBP-1-c and subsequent lipid accumulation in HepG2s, which could aggravate IR and become a source of inflammation (Mahali et al., 2014). Furthermore, liver steatosis and inflammation led to elevated levels of circulating MGO-AGEs (Bijnen et al., 2019). AGEs are thus not only a reflection debilitating hyperglycaemia but may also play a notorious role in hepatocyte inflammation in diabetes, dysfunction and apoptosis, driving liver tissue damage and the predisposition to liver disease. In a rat cirrhosis model, decreased liver GLO1 expression was observed, which led to MGO accumulation, increased production of MGO-AGEs, and RAGE activation, causing inflammation and stress (Hollenbach et al., 2017). Interestingly, the deletion of RAGE deletion does not prevent intrahepatic AGEs accumulation, inflammation, GLO-1 inhibition, and the development of liver steatosis in mice under obesogenic conditions which suggests an alternate RAGE-independent mechanism of liver dysfunction mediated by MGO (Bijnen et al., 2018; Wouters et al., 2021).

Cellular mechanisms involved in MGO-induced cytotoxicity

MGO, has the potential to increase oxidative stress in many cell types, (Hollenbach, 2017; Li et al., 2018; Lin et al., 2012; Paramita & Wisnubroto, 2018; Wang et al., 2022), including rat and human hepatocytes, *in vitro*, and *in vivo* in rodent models, (Bensellam et al., 2012; Dong

et al., 2010; Seo, Ki, et al., 2014; Seo, Seo, et al., 2014) not only through AGE-RAGE signalling, but also through other mechanisms.

MGO, similarly to glucose, can undergo autoxidation and therefore generate ROS (P. Thornalley et al., 1984; P. J. Thornalley et al., 1984; Yamaguchi & Nakagawa, 1983). ROS, RNS and free radicals are also generated during the formation of and degradation of MGO (Angeloni et al., 2014). The autooxidation of AA to form MGO is also a source of carbon centred radicals and superoxide (Dutra et al., 2001; Hiraku et al., 1999). The formation of MGO catalysed by SSAO from AA or by GalO from acetol is coupled to H_2O_2 production (Johnson et al., 1985; Kalapos, 2008; Yu et al., 2003). H_2O_2 is also a by-product of the conversion of MGO to pyruvate by glyoxal oxidase (Kersten & Kirk, 1987; Leuthner et al., 2005); Finally, the nonenzymatic reaction from acetoacetate to MGO also produces ROS, in the presence of myoglobin, haemoglobin, manganese, cytochrome c, or peroxidase (Takayama et al., 1976; Vidigal & Cilento, 1975).

Furthermore, in addition to glycating and inactivating SOD (Angeloni et al., 2014; Polykretis et al., 2020), MGO has the ability to directly impair antioxidant enzyme activity. MGO depletes GPx1 (Sultan et al., 2018), inactivates glutathione reductase (Vander Jagt et al., 1997) which might a result of MG-induced glycation of arginine residues (Jia et al., 2006). MGO also reduces GSH while increasing GSSG content in many cell types, including HepG2 cells (Seo, Ki, et al., 2014; Seo, Seo, et al., 2014) and isolated murine hepatocytes (Kalapos et al., 1991, 1992) impairing glyoxalase system activity, and further aggravating dicarbonyl and oxidative damage (Wu & Juurlink, 2002). MGO treatment in mice decreased also liver GSH content and the activities of SOD and CAT (Choudhary et al., 1997; Seo, Ki, et al., 2014; Seo, Seo, et al., 2014), which are important enzymes for counteracting mitochondrially produced ROS (Cioffi et al., 2017; Mazzoli et al., 2019). The mice demonstrated increased plasma ALT and AST levels, and LPO, plasma ALT and AST, indicating liver injury and confirming MGO's potential for hepatic oxidative damage. Choudhary et al. showed that methylglyoxal treatment in mice causes mitochondrial dysfunction and induced apoptosis in mouse hepatocytes (Choudhary et al., 1997). The depletion of SOD2 and GPX antioxidant abilities in mice exacerbates insulin resistance and the progression of NAFLD to steatohepatitis and fibrosis (Nascè et al., 2022; Saponaro et al., 2015; Tilg et al., 2017) and remarkably, steatotic livers from HFD mice also showed lower levels of reduced GSH as well as increased liver tissue levels of MG-H1 (Santini et al., 2022), further corroborating this link.

Indeed, MGO's effect on the mitochondria is also of great importance to the rise in cellular oxidative stress, as previously covered. Furthermore, MGO increased the activation of uncoupling protein 2 (UCP-2), further increasing oxidative stress, and reportedly collapses the mitochondrial membrane potential (MMP), resulting in reduced mitochondrial respiration and ATP production, further compromising mitochondrial and cellular stability and function in pancreatic β-cells and HepG2 cells (Bo et al., 2016; Dornadula et al., 2015; Li et al., 2018; Rosca et al., 2005; Seo, Ki, & Shin, 2014; Seo, Seo, et al., 2014). Bovine aortic endothelial cells incubated with high glucose and with BBGC revealed that mitochondrial MGO concentration increases approximately threefold under hyperglycaemia (Pun et al., 2014).

MGO and MGO-derived AGEs also are directly involved in the accumulation of ROS and inflammation through the activation inflammatory pathways such as NF-κB and all MAPK pathways - p38 MAPK, JNK and ERK, which has been suggested to be mediated through the production of strong oxidants (Wu & Juurlink, 2002)(Chu et al., 2016; D. Liu et al., 2022; Y. Wang et al., 2019). In HepG2 cells, MGO triggered NF-κB activation and increased mRNA expression of pro-inflammatory cytokines, TNFα and IFN-γ (Cha et al., 2019). MGO treatment also led to an increase in PKCα in HepG2 cells, coupled with increased superoxide levels (Afridi et al., 2016). As briefly mentioned, the deletion of RAGE deletion does not prevent hepatic or vascular inflammation in animal models (Bijnen et al., 2018; Tikellis et al., 2014; Wouters et al., 2021). In addition, it was reported that proinflammatory signalling mediated by SETD7 was inhibited by overexpression of GLO1 (El-Osta et al., 2008). This suggests that the XBP1/SETD7 pathway could be involved in increased low-grade inflammation in T2DM and may be a pathogenic consequence of activation of the UPR by dicarbonyl stress, and in particular, via XBP-1 (Rabbani et al., 2021).

The impact of MAPK and NF-κB on IL-8 production was investigated in human intestinal cells (Caco-2 and HT-29) after stimulation by MGO and glyoxal and both compounds induced a dose-dependent enhancement of IL-8 secretion. Furthermore, inhibitors of MAPK p38 (SB 203580 and 239063), ERK1/2 (PD 98059) and NF-κB activation (SM-7368 and SC-514) reduced IL-8 secretion, however, superoxide dependent MAPK p38 activation seems to be the most dominant pathway for IL-8 secretion in intestinal cells (Kuntz et al., 2010). Interestingly, MGO treatment (up to 30 μM) of neutrophils isolated from diabetic patients either did not alter, or decreased, the production of TNF-α, IL-8, and IL-6, but effectively increased the release and expression of these cytokines in neutrophils obtained from controls. The neutrophils from

T2DM patients were also more vulnerable to apoptosis, which was exacerbated by *in vitro* MGO treatment (Wang et al., 2007). MGO and TNF-α were also shown to synergize in promoting endothelial cell death *in vitro*, which was associated with increased angiopoietin 2 expression and reduced Bcl-2 expression (Branka Vulesevic et al., 2016). Furthermore, overexpression of p65 inhibited TNF-α induced cell death; however, in the presence of MGO, overexpression of p65 caused far greater TNF-induced cell death. These findings suggest that MGO may play an important role in modulating the expression of NF-κB-responsive genes and that this, in cells with constitutive NF-κB activation, favours cellular death in response to stressful stimuli such as inflammation (Laga et al., 2007). NF-kB activation may also downregulate GLO1 expression, as NF-κB antagonizes the transcriptional activity of Nrf2 (Angelika Bierhaus et al., 2001; Hanssen et al., 2015; Liu et al., 2008; Nigro et al., 2019; Tym, 2014), further reinforcing dicarbonyl stress and inflammation. This is corroborated by observations in humans, where systemic inflammation downregulates GLO1 mRNA expression (G.H. Driessen et al., 2021). Furthermore, as outlined in the previous section, MGO treatment *in vitro* or administration *in vivo* leads to a downregulation of Nrf2 expression (Cheng et al., 2015; D. Kim et al., 2021; Wang et al., 2022; Yamamoto et al., 2018).

Indeed, JNK, PKC, p38 MAPK, and NF-κB pathways activation play a fundamental role in MGO-induced cell apoptosis and necrosis. MGO treatment significantly induced the expression of proapoptotic proteins (Bax, Bcl-2, Caspase-3, and PARP) and the phosphorylation of MAPK signalling proteins (ERK, JNK, and p38) in murine mesangial cells (Lee et al., 2020) which attenuated by L-Cysteine and aminoguanidine, a dicarbonyl scavenger. GLO1 inhibition also triggered the mitochondrial apoptotic pathway via Hsp27 p53 and NF-κB in irradiated MCF-7 breast cancer cells (Antognelli et al., 2014), and induced the activation of the stress-activated protein kinases JNK1 and p38 MAPK, which led to caspase activation and apoptosis in glyoxalase I-overexpressing human lung cancer cells (Sakamoto et al., 2001). Noteworthy, several genes involved in apoptosis belonging to p53 signalling pathway, (i.e., insulin-like growth factor binding protein 3, sestrin 2, p21) are significantly modified and upregulated by MGO (Nigro et al., 2019).

Interestingly, in an in vitro study by Laga et al., the authors suggest that in L929 cells, up to 1000μM MGO may be responsible for tipping the balance towards TNF-induced cell death which could be via JNK activation and c-Jun phosphorylation (Laga et al., 2007). Synergy between MGO and TNF-α was also demonstrated to enhance apoptotic endothelial cell death,

which was linked to decreased Bcl-2 expression and increased angiopoietin 2 expression (Branka Vulesevic et al., 2016). In human osteoblasts MGO treatment triggers apoptosis with observable specific apoptotic biochemical changes, oxidative stress JNK and NF-κB activation, mitochondrial membrane potential changes, cytochrome C release, increased Bax/Bcl-2 protein ratios, and activation of caspases (caspase-9, caspase-3) and p21-activated protein kinase 2 (PAK2). A JNK-specific inhibitor was able to reduce MGO-induced apoptosis and activation of caspase-3 and PAK2 in this study (Chan et al., 2007).

In human retinal pigment epithelial cells, MGO induces cell death not through caspase-dependent manner, but via ROS formation, decreased MMP, intracellular calcium elevation and ER stress mediated CHOP activation. Cell death in this case was prevent by inhibiting ROS generation and ER stress (C. M. Chan et al., 2016). Indeed, MGO triggers mitochondrial apoptotic cell death in many cell types including HepG2 cells, (C. M. Chan et al., 2016; Gao et al., 2016; Seo, Ki, et al., 2014; Sheader et al., 2001; Wang et al., 2022; Zhang et al., 2021). In HepG2 cells, this was characterised by loss of MMP increased ROS, PARP cleavage, increased caspase 3 and ATP depletion (Seo, Seo, et al., 2014). MGO- induced HepG2 apoptosis was partially reversed by pre-treatment with antioxidants suggesting the importance of oxidant species in the apoptotic process (Seo, Ki, et al., 2014). In the same cell type, treatment with methylglyoxal-derived AGEs also increased ROS and apoptosis-related factors such as Bax, p53, and Caspase 3 (Kang et al., 2023).

As previously discussed, DNA single-strand breaks activate PARP (Dornadula et al., 2015). PARP reduces levels of cellular NAD+ and ATP and triggers mitochondrial apoptotic pathways. As MGO reportedly collapses the mitochondrial transmembrane potential and induces MPTP formation (Dornadula et al., 2015; Du et al., 2003; Seo, Ki, et al., 2014), it may lead to the abnormal opening of mitochondrial transition pore and thereby the rapid release of caspase activators characteristic of apoptosis (Dornadula et al., 2015; Du et al., 2003). Inhibition of PARP in endothelial cells protected against the methylglyoxal-mediated cell dysfunction and apoptosis, however, it had no effect on cell inflammation (Bates & Mabley, 2010). Furthermore, in HepG2 cells and pancreatic β-cells, resveratrol demonstrated the potential to improve mitochondrial function, prevent apoptosis, increase AMP-activated protein kinase (AMPK) and sirtuin 1 activity, suppress of proinflammatory cytokines induced NO production, and inhibit of PARP activation (Cheng et al., 2015; Ku et al., 2012). Noteworthy, while methylglyoxal causes intracellular and intranuclear damage that leads to apoptosis, it

also has the potential to glycate and hence damage the cell membrane leading to a chaotic necrotic cell death, observed in RINm5F insulinoma cells, and in rat pancreatic β-cells (Cheng et al., 2015).

Indeed, histopathological changes in the livers of patients with DM can be observed. Patients with DM present with increased hepatic lipid accumulation (Jiang et al., 2020; Kupriyanova et al., 2021)and animal models of streptozotocin (STZ)-induced and alloxan-induced DM rats revealed a reduction in rough endoplasmic reticulum and glycogen content, as well as a reduction and damage to hepatic mitochondrial and nuclear structures (Lucchesi et al., 2015; Welt et al., 2004). In progressive disease, NASH is further characterized by hepatocyte ballooning, necrosis near steatotic hepatocytes, and mild inflammation, which may explain the consequent development of fibrosis and the progression into more severe and morbid stages of the disease mentioned above (Marchesini et al., 2003; Starley et al., 2010). When associated with diabetes, NAFLD usually shows severe inflammation and fibrosis on liver biopsy and a high tendency toward a more rapid progression of fibrosis (Macêdo et al., 2014).

Although data in liver and hepatocytes is relatively scarce, the weight of the available evidence is evocative of the cytotoxic potential of MGO, through increased oxidative stress, ER stress, inflammation, and the induction of apoptotic and necrotic pathways. Thus, it may play an important role in increasing susceptibility of liver disease in T2DM patients, who are exposed to increased intracellular MGO levels, as well as tissue and serum levels.

Methods of studying dicarbonyl stress

Fasting plasma MGO concentrations are higher in T1D (Han et al., 2007) and T2D (X. Kong et al., 2014; Scheijen & Schalkwijk, 2014), and increase transiently during the postprandial period in both normoglycemic and diabetic individuals (Beisswenger et al., 2004; Maessen et al., 2015). Measurements have estimated that typical plasma concentrations of MGO in healthy individuals range between ~60–250 nM (Henning et al., 2014; Rabbani & Thornalley, 2014b; Scheijen & Schalkwijk, 2014) and in pathological conditions, from 190nM to up to 742nM (Dhananjayan et al., 2019; Rabbani & Thornalley, 2014b). Consistently, up to 90–99% of cellular MGO is reversibly bound to macromolecules such as biopolymers and small-metabolite thiols as hemithioacetals and amines as hemiaminals and the remainder is unbound (Angeloni et al., 2014; Kold-Christensen & Johannsen, 2020; Nigro et al., 2019). Additionally, its half-life is short in a biological environment and therefore (Allaman et al., 2015b; Nigro et

al., 2019), it is likely that the intracellular concentrations of MGO are considerably higher than plasma levels. Cellular MGO levels have been estimated at ~0.8–5 μM MGO (Dobler et al., 2006; Phillips et al., 1993; Xue et al., 2012).

There are various ways to increase intracellular MGO in experimental settings. Most previous studies investigating the role of MGO in cellular toxicity and dysfunction were conducted using exogenously applied MGO in concentrations that are relatively higher than what is observed in diabetes. The concentrations of MGO that have been used to assess its acute toxic effects in most previous *in vivo* and *in vitro* studies on different cells and targets (endothelial and hepatic cells, mitochondria, LDL) range from 0.1 to 10 mM (Afridi et al., 2016; S. H. Cha et al., 2019; Chang et al., 2005; de Souza Prestes et al., 2019; Prestes et al., 2022; Seo, Ki, et al., 2014). *In vivo* studies are scarcer, but exposure protocols also use non-physiological doses of MGO (ranging from 100 to 400 mg/kg body weight) (W.-C. Chang et al., 2015; Seo, Ki, et al., 2014; Yılmaz et al., 2018). Although providing valuable insight into the cellular effects of MGO, this method does not reflect accurately the natural occurrence of MGO accumulation within the cell. This may also be problematic as MGO may react with extracellular proteins and cell membranes and the extent of its penetration into the cell may differ from what is observed in diabetic conditions, where MGO is likely to be formed intracellularly. It should also be emphasized that in most of these studies, MGO was derived from commercial stock solutions, which have been contaminated with formaldehyde and other substances (Rabbani & Thornalley, 2014b).

Other mechanisms to increase intracellular MGO consist of inhibiting MGO breakdown by the glyoxalase system, through siRNA technology and genetic manipulation to abrogate GLO1 expression, or pharmacologically, with the use of S-p-bromobenzylglutathione cyclopentyl diester (BBGC), a cell-permeable prodrug inhibitor of GLO1 (Rabbani & Thornalley, 2022). Genetic manipulation to suppress GLO1 expression such as GLO1 knockdown exacerbates hyperglycaemia-induced damage in diabetes (Masania et al., 2016; Rabbani et al., 2016a; Thornalley, 1998; Vander Jagt & Hunsaker, 2003) as reflected in several studies, in both cell cultures and animal models, by the subsequent increase in free MGO as well as MG-derived AGEs and oxidative stress (Kold-Christensen & Johannsen, 2020; Shamsaldeen et al., 2016). GLO1 Knockout in flies and fish also demonstrate decreased insulin sensitivity and increased blood glucose and lipid accumulation, which are core characteristics of diabetes (Kold-Christensen & Johannsen, 2020).

Cell permeable GLO1 inhibitor prodrugs have been developed to induce an acute profound increase in MGO to cytotoxic levels as a prospective treatment for cancer—particularly for high Glo1 expressing-related multidrug-resistant tumours (Rabbani & Thornalley, 2022; Sakamoto et al., 2001). The prototype GLO1 inhibitor BBGC, is a potent, competitive inhibitor of GLO1 (Rabbani & Thornalley, 2022; Rabbani et al., 2016a). BBGC is a prodrug that delivers the active GLO1 inhibitor, S-p-bromobenzylglutathione (BBG), which is one of the most potent inhibitors with an inhibitor constant K_i of 160 nM for human GLO1 (Allen et al., 1993). BBG however displays poor membrane permeability and susceptibility to cleavage and degradation by cell surface γ-glutamyl transferase (γ-GT). Rabbani, Thornalley et al. were able to overcome this by developing BBGC through the diesterification of BBG on the carboxylate groups of the γ-glutamyl and glycyl residues to render it resistant to cleavage by γ-GT (Rabbani & Thornalley, 2022). Once BBGC enters the cell, the ester groups are hydrolysed by cellular non-specific esterases to form the active inhibitor BBG (Rabbani & Thornalley, 2022).

The inhibition of cell growth in hepatocellular carcinoma HUH7cells was achieved using 10 μM BBGC (Michel et al., 2019). They predict that selective increase in intracellular MGO levels in response to inhibition of GLO1 is favoured by a higher glycolytic rate and can reach over 12-fold for ca. 24 h of the cell growth cycle (Rabbani et al., 2018). *In vitro*, pharmacological inhibition of GLO1 by BBGC does increase in the cellular concentration of MGO and induction of apoptosis in experiments with endothelial cells and tumour cells (Dobler et al., 2006; Sakamoto et al., 2001; Thornalley et al., 1996) and in bovine aortic endothelial cells incubated with high glucose and BBGC revealed that mitochondrial MGO concentration increases approximately threefold under hyperglycaemia (Pun et al., 2014).

BBGC has become a valuable research tool to explore the effect of pharmacologically induced dicarbonyl stress in experimental studies (Rabbani & Thornalley, 2022). It is now widely used to study the effect of dicarbonyl stress in diabetes (Bierhaus et al., 2012; Dobler et al., 2006; Pun et al., 2014; Tikellis et al., 2014), and cirrhosis (Hollenbach et al., 2017) as well and age-related decline in heart function (Ruiz-Meana et al., 2019).

AIMS

We hypothesised that the pharmacological inhibition of GLO1 using BBGC in hepatocytes would result in increased intracellular MGO accumulation as a model of dicarbonyl stress, which would lead to hepatic cell dysfunction.

The first aim of this study was to determine whether this model is suitable and effective in comparison to the limited studies performed to investigate the effects of MGO in hepatocytes. Next, we aimed to expand on this to further characterise the damaging effects of dicarbonyl stress in hepatocytes, in terms of its effects on cell viability, cell survival and inflammation and to examine the cellular pathways involved in causing any dysfunction that may be observed, to determine whether these pathways could potentially contribute to the increased risk of liver disease in diabetic patients.

2. Materials and Methods

2.1 Materials

The human hepatoma HepG2 cell line was obtained from European Collection of Authenticated Cell Cultures. RPMI-1640 media, trypsin-EDTA (0.05%), heat-inactivated foetal calf serum and penicillin-streptomycin (10,000 U ml^{-1}) were purchased from Gibco Thermo Fisher Scientific (Oxford, UK). MGO, S-p-bromobenzylglutathione cyclopentyl diester, Aminoguanidine and Alagebrium were from MedChemExpress. WWL113 was obtained from Cayman Chemical (Michigan, USA)

Bradford reagent and Laemmli sample buffer were purchased from Bio-Rad Laboratories Ltd (Hertfordshire, UK). [4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), bovine serum albumin, phosphate buffered saline, 2-mercaptoethanol, tetramethylrhodamine ethyl ester perchlorate (TMRE), sodium palmitate and hydrogen peroxide solution 30% (w/w) were obtained from Sigma-Aldrich (Poole, UK). BD OptEIA™ Human IL-8 ELISA kit, Annexin V-FITC, propidium iodide and annexin V binding buffer were purchased from BD Pharmingen (Berkshire, UK) and 2′,7′-dichloro-dihydro-fluorescein-diacetate (DCFH-DA) was purchased from Millipore UK Ltd (Hertfordshire, UK). Human GDF-15 DuoSet ELISA, (Catalog #: DY957) and Human FGF-21 DuoSet ELISA (Catalog #: DY2539) were purchased from R&D Systems, Inc. P450-Glo™ CYP3A4 Assay and Screening System was obtained from Promega (Promega UK) and Carboxylesterase 1 (CES1) Specific Activity Assay Kit (ab109717) from Abcam (Cambridge, UK). The RT-qPCR primers were obtained from Eurofins Genomics (Wolverhampton, UK).

Anti-CHOP antibody (Cat#: sc-7351; RRID: AB_627411), goat anti-rabbit IgG (Cat#: sc-2004; RRID: AB_631746), mouse IgG antibody (Cat#: sc-516102; RRID: AB_2687626) and anti-GAPDH antibody (Cat#: sc-25778; RRID: AB_10167668) were obtained from Santa Cruz Biotechnology (Texas, USA). Anti-GRP78 antibody (Cat#: 3216-1; RRID: AB_2279866) was obtained from Epitomics (California, USA). ECL western blotting detection reagents were obtained from Thermo Fisher Scientific (Massachusetts, USA).

Rainbow™ Marker (Cat#: RPN800E) was purchased from Sigma-Aldrich (Poole, UK) and PageRuler Plus prestained protein ladder (Cat#: 26619) was obtained from Thermo Fisher Scientific (Oxford, UK). Goat anti-rabbit AlexaFluor™ 488 secondary antibody (Cat#: A-11008; RRID: AB_143165) and goat anti-mouse AlexaFluor™ 488 secondary antibody (Cat#: A32766; RRID: AB_2762823) were obtained from Invitrogen Thermo Fisher Scientific (Oxford, UK).

All other reagents and cell culture plastics were purchased from Thermo Fisher Scientific (Oxford, UK) and Sigma-Aldrich (Poole, UK).

Buffers and solutions

The buffers and solutions used in this study are listed below in alphabetical order.

0.5 M Tris (Western blot)

0.5 M Tris-HCl

Buffer adjusted to pH = 6.8

1.5 M Tris (Western blot)

1.5 M Tris-HCl

Buffer adjusted to pH = 8.8

Blocking buffer (Western Blot)

5% (w/v) non-fat milk powder in Tris-buffered saline with Tween 20 (TBST)

Blocking Buffer (immunocytochemistry)

1% BSA and 0.1% Triton X-100 in Phosphate buffered saline

Hank's Balanced Salt Solution (immunocytochemistry)

0.14 M NaCl

0.005 M KCl

0.001 M $CaCl_2$

0.0004 M MgSO4·7H2O

0.0005 M $MgCl_2 \cdot 6H_2O$.

0.0003 M $Na_2HPO4 \cdot 2H_2O$

0.0004 M KH_2PO_4

Buffer was adjusted to pH 7.2.

Phosphate buffered saline buffer

137 mM NaCl

2.7 mM KCl

8 mM Na_2HPO_4

2 mM KH_2PO_4

Radioimmunoprecipitation assay buffer (Western blot)

50 mM Tris-HCL, pH 8.0

150 mM NaCl

1 mM sodium orthovanadate

1 mM NaF

0.5% sodium deoxycholate

0.1% sodium dodecyl sulphate (SDS)

1% Triton X-100

Running buffer (Western blot)

25 mM Tris

190 mM glycine

0.1% SDS

Stripping buffer (Western blot)

0.2 mM glycine

1 g SDS mM NaCl

1% Tween 20

Buffer was adjusted to pH 2.2

Tris-buffered saline (TBS) buffer (Western blot)

20 mM Tris

150 mM NaCl

Buffer was adjusted to pH 7.6

Transfer buffer (Western blot)

25 mM Tris

190 mM glycine

20% methanol

0.1% SDS

2.2 Methods

2.2.1 Cell culture

All experiments in our study involved the use of the human hepatoma HepG2 cells. HepG2 cells are frequently used in a wide range of studies as in vitro alternative to primary human hepatocytes, most commonly to assess hepatotoxicity (Arzumanian et al., 2021; Donato et al., 2015). HepG2 cells are nontumorigenic cells with high proliferation rates and an epithelial-like morphology that exhibit the key characteristics of hepatocytes and perform many differentiated hepatic functions (Arzumanian et al., 2021; Donato et al., 2015).

HepG2 cells were cultured as an adherent monolayer in 75 cm^2 single-use sterile flasks (Corning, Germany). HepG2 cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), and 1% penicillin-streptomycin. The cells were incubated in a humidified incubator at constant physiochemical conditions: 37ºC, 95% air and 5% CO_2. The cells were passaged twice a week (every third or fourth day) under aseptic conditions in a class II laminar flow cabinet. Prior cell harvesting, RPMI-1640 medium and 0.05% w/v trypsin were warmed up in a water bath at 37ºC. Then, the HepG2 cells were checked under the microscope to ensure the cells were healthy and to estimate confluence. Upon confluence (> 80%), the adherent monolayer of HepG2 cells was enzymatically harvested by adding 3 mL of 0.05% w/v trypsin-EDTA (0.53 mM) before being incubated at 37ºC and 5% CO_2 for 3 min. After cell detachment, the trypsin was neutralised with 6 mL of RPMI-1640 medium (supplemented with heat-inactivated 10% FCS and 1% penicillin-streptomycin). Then, the cell suspension was centrifuged for 5 min at 300 × g. After centrifugation, the supernatant was removed, and the cell pellet was resuspended in 12 mL of RPMI-1640 medium. The cell suspension was divided into four single-use sterile 75 cm^2 flasks (2.5 or 3 mL of cell suspension in each flask) in 13 mL of RPMI-1640 medium per flask. The cells in the flasks were then incubated for three to four days at 37ºC and 5% CO_2.

In order to prepare the cells for plating, the following protocol has been adopted. Upon confluence (> 80%), HepG2 cells were harvested and centrifuged as described above. Then, the cells were resuspended in RPMI-1640 medium (supplemented with heat-inactivated 10% FCS and 1% penicillin-streptomycin) in order to obtain the appropriate seeding density depending on the application (Table 2.1). Optimal seeding densities of HepG2 cells for the different applications were pre-determined to reach the target number of cells (Table 2.1). In order to obtain the respective seeding density, the number of total cells were counted using a haemocytometer and to confirm, a Countess II FL Automated Cell Counter (Thermo Fisher Scientific). As part of routine checks, 0.4% trypan blue solution was added to the cells to rapidly assess cell viability using Countess II FL Automated Cell Counter.

After plating, HepG2 cells were incubated at 37°C in a humidified 37ºC incubator with 5% CO_2 for 24 or 48 hrs prior to conducting the treatment protocol (Table 2.1). HepG2 cells from passage 14 to passage 60 were used. The table below summarises the empirically determined cell seeding density, cell culture plate used and incubation time prior treatment protocol respective to each technique used in this study (Table 2.1).

Application	Seeding density (in cells/well)	Cell culture plate used	Incubation time prior treatment (in hours)
IL-8, FGF21 and GDF15 release measurements	$14x10^4$ cells	48-well plate	48
Cell viability assay (MTT assay)	3.5×10^4	96-well plate	24
Apoptosis and necrosis analysis using Annexin V-FITC and propidium iodide staining	7.5×10^5	6-well plate	24
Intracellular ROS generation (DCFH-DA assay)	2.0×10^5	6-well plate	48
Mitochondrial superoxide generation (MitoSOX probing)	5×10^5	6-well plate	24
Mitochondrial membrane potential changes (DiOC6 and TMRE probing)	$7.5x\ 10^5$	6-well plate	24
CYP3A4 activity assay	3.5×10^4	96-well plate	48
CES1 activity assay	$14x10^4$ cells	48-well plate	48
Cellular ATP assay	3.5×10^4	Opaque walled 96-well plate	24
RT-qPCR	0.5×10^6	6-well plate	48
Western Blot	1.0×10^6	10-cm cell culture dish	48
Immunocytochemistry (MG-H1, NF-κB)	0.5×10^5	Poly-ornithine-coated coverslips placed in 6-well plates	48

Table 2.1 HepG2 cell plating and incubation conditions for the experiments conducted in this study.

2.2.1 Cell viability (MTT assay)

Principle

Cell viability was determined by the 3-([4,5-dimethylthiazole-2-yl])-2,5-diphenyltetrazolium bromide (MTT) assay which involves the enzymatic reduction of MTT to MTT-formazan by mitochondrial dehydrogenases, a by-product of mitochondrial respiration (Mosmann, 1983). The quantity of MTT-formazan is directly proportional to the number of viable HepG2 cells.

Protocol

The following protocol was performed as first described by Mosmann (1983). A fresh MTT solution (0.5 mg of MTT/mL in 3% heat-inactivated FCS RPMI-1640 media) was prepared prior running the MTT assay. Following the appropriate experimental protocol, the culture media was removed from all the wells before and replaced by 100 µL of freshly prepared MTT solution (0.5 mg/mL) to each well. The cells were then incubated for 30 to 40 minutes in a humidified 37ºC incubator with 5% CO_2. After observing a colouring reaction, the MTT solution was in turn carefully removed from all the wells and 100 µL dimethyl sulfoxide (DMSO) was added to each well in order to solubilise the MTT-formazan purple crystals. The quantity of MTT-formazan was measured using a spectrophotometer (BioTek Synergy HT Microplate Reader, USA) at 540 nm.

Data analysis

The absorbance measurements of the treated cells were expressed as % of the absorbance observed in vehicle (DMSO)-treated cells (control).

2.2.2 Interleukin 8 secretion measurements

Protocol

Following cell growth and treatment protocol, the supernatant was collected and frozen at -20°C prior to being assayed for IL-8 release, using a human IL-8 ELISA set (BD OptEIA™ Human IL-8 ELISA Set, BD Pharmingen, Berkshire, UK), according to manufacturer's instructions.

A dilution test consisted of serially diluting a sample in order to select a dilution factor was conducted to ensure the absorbance values of each sample fit within the standard curve. This

was derived using the lyophilised standard IL-8 provided, reconstituted with 1.0 mL of deionized water to yield a stock standard, which was subjected to serial dilutions. A dilution of (1:5) in assay buffer for the collected samples was selected to conduct the studies, and each diluted sample was pipetted into its respective well on Nunclon assay plates pre-coated with the human IL-8 antibody provided. The assay was conducted according to manufacturer's instructions and following the final incubation, 50 µL of Stop Solution was added prior to immediately reading the absorbance at 450 nm and 570 nm with a spectrophotometer (BioTek Synergy HT Microplate Reader, USA).

Data analysis

Wavelength correction was first performed by subtracting absorbance 570 nm from absorbance 450 nm for every well. Then, the average absorbance value for the blank control standards was subtracted from all the absorbance values, and a standard curve was plotted. The secreted IL-8 levels were calculated and normalised against total cellular protein concentration, determined by the Bradford assay. The concentration of the IL-8 in the sample was determined by interpolating the blank control subtracted absorbance values against the standard curve. An example of the calculations to obtain the amount of IL-8 in the samples is shown below:

$$IL\text{-}8\ (pg/mL) = (((Absorbance\ at\ 570\ nm - Absorbance\ at\ 450\ nm)) - Blank\ (mean\ of\ duplicates)) \times 0.3077$$

The resulting value was multiplied by the appropriate sample dilution factor, to obtain the concentration of IL-8 in the sample. IL-8 measurements were the normalised to total protein content determined by the Bradford assay (see 2.2.3). Data was expressed pg/µg of protein.

2.2.4 Growth differentiation 15 secretion measurements

Protocol

Following cell growth and treatment protocol, the supernatant was collected and frozen at -20°C prior to being assayed for GDF-15 release, using a human GDF15 ELISA set (Human GDF-15 DuoSet ELISA, R&D Systems, Inc), according to manufacturer's instructions.

A dilution test consisted of serially diluting a sample in order to select a dilution factor was conducted to ensure the absorbance values of each sample fit within the standard curve. This was derived using the lyophilised standard GDF15 provided, reconstituted with 1.0 mL of deionized water to yield a stock standard, which was subjected to serial dilutions. A dilution of (1:400) in assay buffer for the collected samples was selected to conduct the studies, and

each diluted sample was pipetted into its respective well on Nunclon assay plates pre-coated with the human GDF15 antibody provided. The assay was conducted according to manufacturer's instructions and following the final incubation, 50 µL of Stop Solution was added prior to immediately reading the absorbance at 450 nm and 570 nm with a spectrophotometer (BioTek Synergy HT Microplate Reader, USA).

Data analysis

Wavelength correction was first performed by subtracting absorbance 570 nm from absorbance 450 nm for every well. Then, the average absorbance value for the blank control standards was subtracted from all the absorbance values, and a standard curve was plotted. The secreted GDF15 levels were calculated and normalised against total cellular protein concentration, determined by the Bradford assay (see 2.2.5). The concentration of the GDF15 in the sample was determined by interpolating the blank control subtracted absorbance values against the standard curve as described in 2.2.2. Data was expressed pg/µg of protein.

2.2.4 Fibroblast growth factor 21 secretion measurements

Protocol

Following cell growth and treatment protocol, the supernatant was collected and frozen at -20°C prior to being assayed for FGF21 release, using a human FGF21 ELISA set (Human FGF-21 DuoSet ELISA, R&D Systems, Inc), according to manufacturer's instructions.

A dilution test consisted of serially diluting a sample in order to select a dilution factor was conducted to ensure the absorbance values of each sample fit within the standard curve. This was derived using the lyophilised standard FGF21 provided, reconstituted with 1.0 mL of deionized water to yield a stock standard, which was subjected to serial dilutions. Following this, we realised sample concentration was in fact necessary. To achieve this, 500 µL of each sample was processed using Amicon® Ultra-0.5 Centrifugal Filters, 10 kDa MWCO (Sigma Aldrich, Merck KGaA, Darmstadt, Germany), spun at 14,000 × g at room temperature for 15 minutes to concentrate the sample 27-fold.

Following centrifugation, 10 µL of each sample was diluted in assay buffer and pipetted into its respective well on Nunclon assay plates pre-coated with the human FGF21 antibody provided. The assay was conducted according to manufacturer's instructions and following the final incubation, 50 µL of Stop Solution was added prior to immediately reading the absorbance

at 450 nm and 570 nm with a spectrophotometer (BioTek Synergy HT Microplate Reader, USA).

Data analysis

Wavelength correction was first performed by subtracting absorbance 570 nm from absorbance 450 nm for every well. Then, the average absorbance value for the blank control standards was subtracted from all the absorbance values, and a standard curve was plotted. The secreted FGF21 levels were calculated and normalised against total cellular protein concentration, determined by the Bradford assay (see 2.2.5). The concentration of the FGF21 in the sample was determined by interpolating the blank control subtracted absorbance values against the standard curve as described in 2.2.2. Data was expressed pg/mg of protein.

2.2.5 Bradford protein assay

Principle

The Bradford assay, developed by Marion Bradford, is a colorimetric assay used to determine the total protein concentrations in a sample (Bradford, 1976). The principle the Bradford assay is that the binding of protein molecules such as lysine, histidine and arginine, to the Coomassie dye, resulting in a change of colour from brown to blue. The protein-dye binding represents the various concentrations of proteins.

Protocol

To perform the Bradford protein assay, a standard curve was plotted by using different concentrations of BSA solution (0, 0.0625, 0.125, 0.25, 0.5 and 1 mg/mL) prepared by serially diluting a 1 mg/mL BSA stock solution (30 mg of BSA in 30 mL of distilled H_2O) in distilled H_2O. The standards and samples were assayed in duplicates. The cell lysate for each treatment was obtained by adding 0.2 mL of 0.1 M NaOH to the cells. The cell lysates constitute the samples to be assayed as they contain the proteins extracted from the cells. The standards and samples (10 µL) were pipetted into each well of a 96-well microplate before adding 200 µL of Bradford dye reagent (acidified Coomassie dye) to each well. A spectrophotometer (BioTek Synergy HT Microplate Reader, USA) was used at 595 nm to measure the absorbance of the standards.

Data analysis

The average absorbance value for the blank control standards was subtracted from all the absorbance values. Then, a standard curve was plotted. An example of a plotted standard curve is shown below (Figure 2.1). The concentration of the protein in the sample was determined by interpolating the blank control subtracted absorbance values against the standard curve.

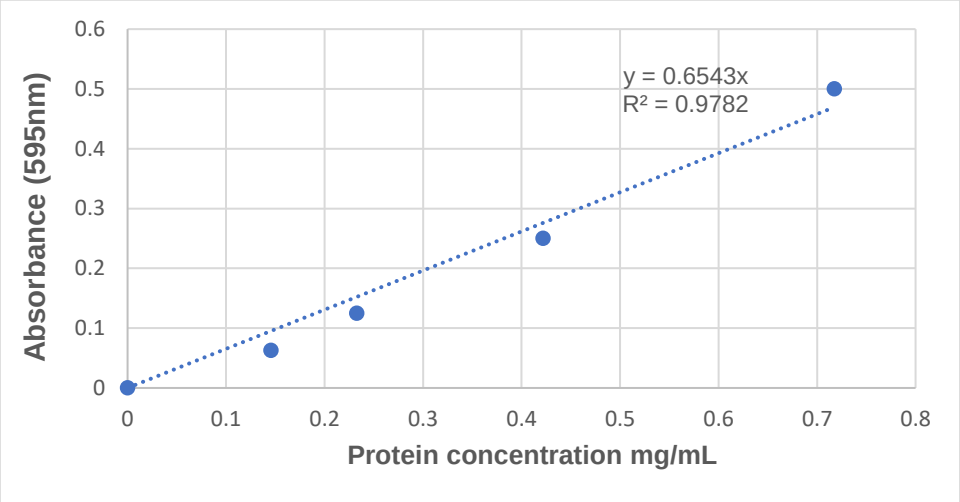


Figure 2.1. An example of a representative protein standard curve. A standard curve was plotted using the average (of duplicates) blank control subtracted absorbance value for each standard concentration against the target protein concentration (0 – 1 mg/mL) of the standard. Blank-subtracted data values (mean of duplicates) are graphed.

2.2.6 Determination of cellular ATP levels

The following protocol was established as per manufacturer's guidelines (Promega, Wisconsin, US) as well as method optimisation. Then, cells were exposed to BBGC (10 or 20 µM) for 24 hrs at 37$_{o}$C and 5% CO_2. Control wells containing medium without cells were also prepared to determine background luminescence. Following treatment protocol, the plate and its contents were equilibrated to room temperature for approximately 30 mins. Then, a volume (100 µL) of CellTiter-Glo® 2.0 Reagent (containing the firefly luciferase enzyme and substrate) equal to the volume (100 µL) of cell culture medium was added in each well. The contents were then mixed for 2 mins on an orbital shaker at 300 rpm to induce cell lysis. The plate was then incubated at room temperature for 10 mins to stabilise the luminescent signal before recording luminescence with a luminometer with an integration time of 1 second per well. Data were expressed as the % luminescence relative to vehicle treated control samples.

2.2.6 Analysis of cell death by flow cytometry

It is important to detect cell death and distinguish between apoptosis and necrosis as an indicator of cellular stress. Whereas apoptosis is a regulated type of cell death that is generally triggered by normal healthy processes, necrosis is the premature death of cells, triggered by external factors or disease. Hepatocyte death, via apoptosis or necrosis, was detected using flow cytometric analysis.

Apoptosis and necrosis levels were detected by Annexin V and propidium iodide (PI) staining followed by flow cytometric analysis. Annexin V is commonly used to probe apoptotic cells, as it recognises phosphatidylserine (PS) externalisation during early apoptosis. It can also stain the PS that remains on the inner face of the membrane of cells undergoing late apoptosis or primary necrosis. PI detects the loss of plasma and nuclear membrane integrity, a classic characterisation of late apoptotic and necrotic cells (Brauchle et al., 2014).

Protocol

This protocol was optimised by testing several parameters (*i.e.*, concentration of PI and harvesting method) after exposure to a positive control (50µM Camptothecin for 24 hours). Camptothecin induces apoptosis by inhibiting the activity of the enzyme DNA topoisomerase-I (Morris & Geller, 1996).

Briefly, HepG2 cells were treated with BBGC 5, 10, and 20 µM for 24 hours prior to treatment. Following treatment protocol, the cells were enzymatically harvested with trypsin/EDTA (0.05%). Then, the cells were centrifuged for 5 min at 300 x g and washed twice by gently resuspending cells with phosphate buffered saline (PBS) (3 mL) with intermittent centrifugation steps. Cells were then resuspended in Annexin V binding buffer (1 mL) (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) and 100 µL of cell suspension from each sample (~10^5 cells/mL) was transferred for staining with Annexin V-FITC (5 µL) (BD Pharmingen, Cat#: 556419) and PI (2 µg/mL) (BD Pharmingen, Cat#: 556463) according to the manufacturer's instructions. After a 15-minute staining period in the dark and at room temperature (25°C)., the cells were centrifuged for 5 min at 300 × g and resuspended in 400 µL of Annexin V binding buffer. The cells were then immediately subject to flow cytometric analysis (BD C6 Flow Cytometer; BD Biosciences; Becton-Dickinson Co., Franklin Lakes, NJ, USA). The flow cytometer used was subject to routine maintenance and was calibrated

prior each experiment using beads purchased from the manufacturer to ensure accuracy of the data (BD C6 Flow Cytometer; BD Biosciences; Becton-Dickinson Co., Franklin Lakes, NJ, USA).

Additionally, compensation controls were performed for each experiment. These include:

- Unstained vehicle control (DMSO only)
- Vehicle control stained with Annexin V FITC only
- Vehicle control stained with PI only

These controls were used to analyse the data as described below.

Data analysis

For each flow cytometric analysis, 10,000 events were recorded with the BD C Sampler Plus software (BD Biosciences, New Jersey, USA). The recorded events were analysed using the BD Biosciences software and the FlowJo software (Treestar Inc, Ashland, US). A gating strategy was put in place to eliminate cell debris and doublets/clumps of cells using the unstained vehicle-treated (control) cells. To eliminate debris and dead, an FSC-A vs SSC-A density plot was drawn up. FSC-A vs SSC-A plots were used for cell population gating and to identify any changes in the scatter properties of the cells. Cell debris and cell clumps tend to have lower forward scatter (FSC) levels and are found at the bottom left corner of an FSC vs SSC density plot, therefore a gate was set up to exclude cell debris.

Then, to obtain singlets (excluding doublets), an FSC-A vs FSC-H plot was created and the HepG2 cells along the densest diagonal were gated. The same gate was used for the analysis of all the samples to ensure reproducibility. Ultimately, a total of approximately 95% of the events were analysed. Then, to set up the quadrants, the unstained control was used. The quadrants were set as to ensure that all the unstained cells were included in the lower left quadrant. An example of the gates and the quadrant set-up is shown below (Figure 2.2).

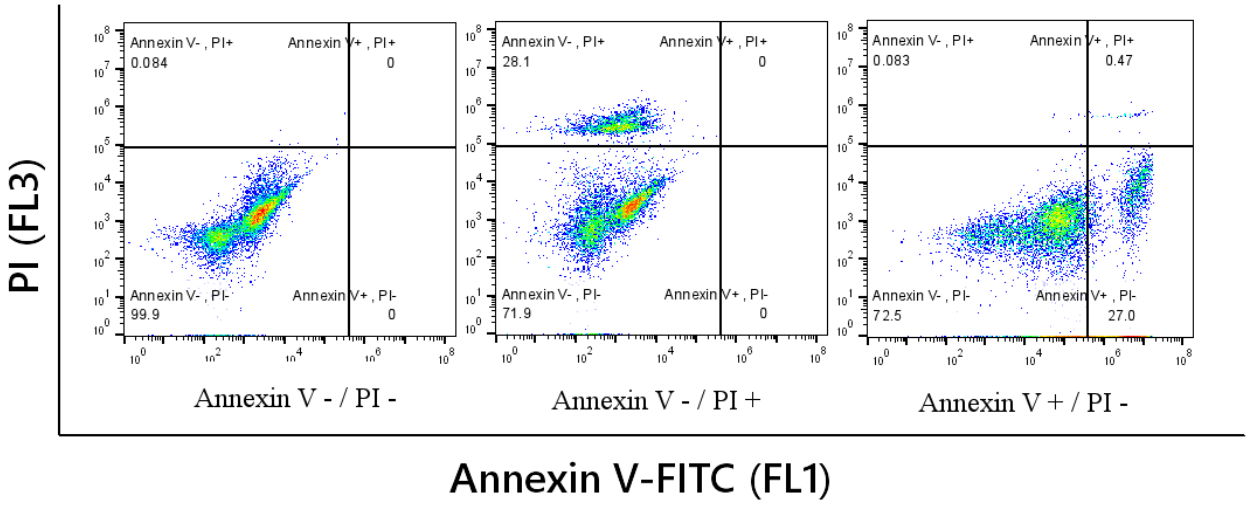


Figure 2.2. Gating strategy implemented in our study. HepG2 cell population and singlets of a control sample were discriminated based on scatter parameters (FSC-A vs. FSC-H). The gating strategy was based on unstained and compensation controls on a FL1 vs. FL3 plot. Each of the four quadrants represent the live cells (Annexin V-, PI- - lower left quadrant), cells undergoing early apoptosis (Annexin V+, PI- - lower right quadrant) or late apoptosis/early necrosis (Annexin V+, PI+ - upper right quadrant) and dead cells (Annexin V-, PI+ - Q1). These plots were created using the FlowJo software.

The compensation controls were used to reduce the spillover fluorescence in the two channels, using the BD C6 Accuri Software. To reduce the spillover fluorescence in the FL1 channel (Annexin V-FITC), the control stained with PI only was used as shown in Figure 2.2. Similarly, to reduce the spillover fluorescence in the FL3 channel (PI), control stained with Annexin V-FITC only was used.

Then, a plot of the compensated FL1 channel vs. FL3 channel was drawn using the Flow Jo software (Tree Star Inc., USA) to visualise the percentage of viable, early apoptotic, late apoptotic/early necrotic, and dead cells for each sample. Viable cells remained unstained (Annexin V-FITC−/PI−). Early apoptotic cells showed Annexin V-FITC+/PI− staining patterns due to PS externalisation while late apoptotic and primary necrotic cells exhibited Annexin V-FITC+/PI+ staining patterns due to a loss of plasma and nuclear membrane integrity (Brauchle et al., 2014). Cells that stain positive for PI and negative for Annexin V-FITC are considered as dead.

2.2.7 Messenger RNA extraction and quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Principle

Quantitative reverse transcription polymerase chain reaction (PCR) is used to analyse the expression of genes of interest. RT-qPCR consists of transcribing the starting material, RNA, into complementary DNA (cDNA) by reverse transcription. The cDNA serves as the template for the qPCR reaction. Here, we analyse the mRNA expression of key markers of ER stress (*i.e.*, *CHOP* and *GRP78*), key pro-inflammatory cytokines (*IL8, Tnfα, IL6*), hepatokines (*FGF21* and *GDF15*), drug metabolising enzymes (*CYP3A4*, *CES1*) and SARS-CoV2 receptors (*ACE2* and *TMPRSS2*).

Protocol

Sample preparation

Following cell growth and treatment protocol, HepG2 cells were enzymatically harvested with 1 mL of trypsin/EDTA (0.05%). After trypsinisation for 1 to 2 minutes, 2 mL of 1640 RPMI media supplemented with 10% heat-inactivated FCS was added to neutralise the trypsin. Then, 1 mL of cell suspension from each sample was transferred to RNAse-free microfuge tubes (Thermo Fisher Scientific). Cells were then centrifuged at 300 × g for 5 minutes. Following centrifugation, the supernatant was completely removed, and the cell pellets were immediately stored at -80^{o}C.

Total RNA extraction

The cell pellets were thawed at room temperature (~25^{o}C). Total RNA was isolated from HepG2 cells using the RNeasy Mini Kit (Qiagen, Cat#: 74104, Hilden, Germany) according to the manufacturer's instructions with several alterations as per method optimisation. In summary, HepG2 cells were disrupted in 350 µL buffer RLT and homogenised. Then, 350 µL of 70% ethanol was added to the lysate, thus creating conditions that promote selective binding of RNA to the RNeasy membrane supplied. Then, 700 µL of the lysate was applied to the RNeasy Mini spin column which was centrifuged at ≥ 8,000 × g for 1 minute. Contaminants were washed away, and high-quality RNA was eluted in 40 µL RNAse-free water. Elution was done in two steps: the RNA was firstly eluted in 20 µL of RNAse-free water and centrifuged

before adding another 20 µL of RNAse-free water and centrifuged again. RNA was then transcribed into cDNA. The concentration of RNA was determined using a Nanodrop® Lite spectrophotometer (Thermo Fisher, USA), with nuclease-free water as zero absorbance reference. A Nanodrop® Lite spectrophotometer also provides a A260/A280 ratio that allows the assessment of purity. Values close to 2 are generally accepted as 'pure' RNA. Once the purity was deemed acceptable, and the amount of RNA was determined, then reverse transcription was carried out.

Reverse Transcription

Reverse transcription of RNA to cDNA was performed using QuantiTect Reverse Transcription Kit (Qiagen, Cat#: 205311, Hilden, Germany) as per manufacturer's instructions. The genomic DNA (gDNA) elimination reaction was performed by adding the components in Table 2.2 then incubating the gDNA elimination reaction mix for 2 mins at 42ºC. The volume of template RNA was calculated depending on the amount of RNA assessed earlier.

Component	Volume (in µL) /reaction
gDNA Wipeout Buffer, 7 x	2
Template RNA, up to 1 µg	3.9
RNase-free water	8.1
Total reaction volume	**14**

Table 2.2. Genomic DNA elimination reaction components

Then, the reverse transcription (RT) master mix was prepared by mixing all the components in Table 2.3, except the entire gDNA elimination reaction, in a single 0.2 mL PCR tube. This would reduce the risk of mixing up samples and contaminating one sample with another sample. We also made sure to obtain enough RT mix to add to the entire gDNA elimination reaction. For instance, to prepare five samples, we assumed we had a total of six samples to reduce the risk of pipetting errors. Then, 6 µL of this mixture was added to the entire gDNA elimination reaction (14 µL) obtained earlier. Then, the reverse transcription reaction was

incubation for 15 minutes at 42°C to initiate the reverse transcription process, followed by 3 minutes at 95°C to inactivate Quantiscript Reverse Transcriptase.

Component	Volume (in µL) /reaction
Quantiscript Reverse Transcriptase	1
Quantiscript RT Buffer, 5×	4
RT Primer Mix	1
Entire gDNA elimination reaction	14
Total reaction volume	**20**

Table 2.3. Reverse transcription reaction components

SYBR green

SYBR qPCR amplifications were performed in 25 µL reactions of Rotor-Gene SYBR Green (Qiagen, Cat# 204074, Hilden, Germany), as per manufacturer's protocol. The reactions contained 12.5 2x Rotor-Gene SYBR Green PCR Master Mix, 1 µM of forward and reverse primers, and variable volumes of RNAse free water and template DNA, depending on the amount of template DNA present in each sample as each reaction requires ≤ 100 ng/reaction of template cDNA.

RT-qPCR analyses were performed on the cDNA preparations to detect the expression of the genes *CHOP*, *GRP78*, *IL8, Tnfα, IL6*, *FGF21*, *GDF15*, *ACE2*, *TMPRSS, CYP3A4*, *CES1, PXR, FXR,* and *PPARα* using SYBR green fluorescence. The previously designed primers for each gene mentioned, and *GAPDH* are outlined in Table 2.4

Gene	Sequence	Length (bp)	Reference
GAPDH	Forward 5'- ATCACCATCTTCCAGGAGCG -3', Reverse 5'- CAAATGAGCCCCAGCCTTC -3'	20 19	(Qi et al., 2019)
CHOP	Forward 5'- TTCACTACTCTTGACCCTGCGTC -3', Reverse 5'- CACTGACCACTCTGTTTCCGTTTC -3'	23 24	(Liao et al., 2016)
GRP78	Forward 5'- CGGGCA AAGATGTCAGGAAAG -3', Reverse 5'- TTCTGGACGGGCTTCATAGTAGAC -3'	21 24	(Gu et al., 2010)
ACE2	Forward 5'- GGGATCAGAGATCGGAAGAAGAAA -3', Reverse 5- AGGAGGTCTGAACATCATCAGTG -3'	24 23	(Ma et al., 2020)
TMPRSS	Forward 5'- AATCGGTGTGTTCGCCTCTAC -3', Reverse 5- CGTAGTTCTCGTTCCAGTCGT -3'	21 21	(Ma et al., 2020)
IL8	Forward 5'- CATACTCCAAACCTTTCCACCCC -3' Reverse 5'- TCAGCCCTCTTCAAAAACTTCTCCA -3'	23 25	(Yu et al., 2011)
TNFA	Forward 5'- CGCTCCCCAAGAAGACAG -3', Reverse 5'- AGAGGCTGAGGAACAAGCAC -3'	18 20	(Reza et al., 2021)
IL6	Forward 5'- TACCCCCAGGAGAAGATTCC -3', Reverse 5'- TTTTCTGCCAGTGCCTCTTT -3'	20 20	(Reza et al., 2021)
FGF21	Forward 5'- ATGGATCGCTCCACTTTGACC -3', Reverse 5'- GGGCTTCGGACTGGTAAACAT -3'	21 21	(Zhang et al., 2018)
GDF15	Forward 5'- CCGCCAGCTACAATCCCAT -3', Reverse 5'- TGGCTAACAAGTCATCATAGGTC -3'	19 23	(Gao et al., 2016)
CYP3A4	Forward 5'- TACACAAAAGCACCGAGTGG -3', Reverse 5'- TGCAGTTTCTGCTGGACATC -3'	20 20	(Berger et al., 2016)
CES1	Forward 5'- CCAGAGAGAGTCAACCCCTTCT -3', Reverse 5'- TCCTGCTTGTTAATTCCGACC -3'	22 21	(Luo et al., 2017)
PXR	Forward 5'- AGCTGGAACCATGCTGACTT -3', Reverse 5'-AGGGGCGTAGCAAAGGGGTG-3'	20 20	(Hanioka et al., 2012)
FXR	Forward 5'- ACCAGCCTGAAAATCCTCAACAC -3', Reverse: 5'- CTCTCCATGACATCAGCATCTCAG -3'	23 24	(Attia et al., 2017)
PPARα	Forward 5'- ATGCCAGTACTGCCGTTTTC -3', Reverse 5'- GGCCTTGACCTTGTTCATGT -3'	20 20	(Xi et al., 2021)

Table 2.4. Primer sequences for RT-qPCR

The following parameters were checked as part of optimal primer design guidelines:

- Sufficiently long (18-30 nt)
- G and C content between 40-60%
- G or C at the end (GC clamp stabilises primer binding)
- Long sequences of same nt should was avoided (e.g., 4 As or Ts)
- Annealing temperature (T_m) - 5^{o}C between forward and reverse primers for one gene

The primers were diluted in EDTA to produce a final concentration of 100 µM. An example reaction setup is shown in Table 2.5.

We empirically determined an appropriate volume of template cDNA (3 µL) which was sufficient to amplify (data not shown). We added all the components of SYBR green master mix shown in Table 2.5, except the template cDNA in a PCR tube. The reaction was made to 25 mL total volume with nuclease-free water. We also made sure to obtain enough SYBR green mix for all samples. For instance, to prepare five samples, we assumed we had a total of six samples to reduce the risk of pipetting errors. Then, 22 µL of the SYBR green master mix (without the template cDNA) was added in individual 0.1 mL PCR strip tubes. Template cDNA was then added to the rest of the SYBR green master mix. The SYBR green reaction mix was then immediately subject to RT-qPCR.

Component	Volume (in µL) /reaction	Final concentration
2× Rotor-Gene SYBR Green PCR Master Mix	12.5	1x
Primer A (forward)	0.25	1 µM
Primer B (reverse)	0.25	1 µM
RNase-free water	9	N/A
Template cDNA	3	≤ 100 ng/reaction
Total reaction volume	**25 µL**	

Table 2.5. Example of reaction setup for RT-qPCR using Rotor-Gene SYBR Green.

All samples were subjected to RT-PCR in duplicates or triplicates with the following cycling conditions, as per manufacturer's instructions (Qiagen, Cat# 204074, Hilden, Germany). The cycling conditions used in this study are detailed in Figure 2.3.

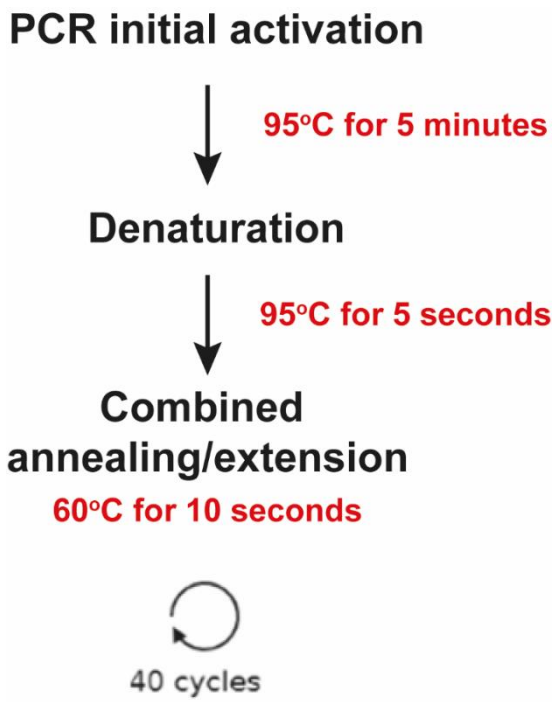


Figure 2.3. Cycling conditions for RT-qPCR. The programme was set to heat the samples to 95°C for 5 mins for the initial denaturation. The second stage was the thermo-cycling phase, which consisted of a further denaturation of 5 sec at 95°C, annealing and extension at 60°C for 10 sec as all primer sets had an annealing temperature (Tm) below 60°C. This was repeated for 40 cycles. Figure created in BioRender.

The resulting amplification curves, representing SYBR green fluorescence vs. number of cycles was used to determine a cycle threshold (Ct) value (Figure 2.4). For accurate quantitation the threshold must be set so that all amplification curves cross this threshold while in the exponential phase. Ct values will be used for data analysis and changes in gene expression were expressed as $2^{-\Delta\Delta Ct}$, the expression fold change, derived using the following formulae:

ΔCt Value control = Ct value gene of interest of the control - Ct value housekeeping gene of the control

ΔCt Value treatment = Ct value gene of interest of treated sample - Ct value housekeeping gene for the control

ΔΔCt = ΔCt Value treatment - ΔCt Value control

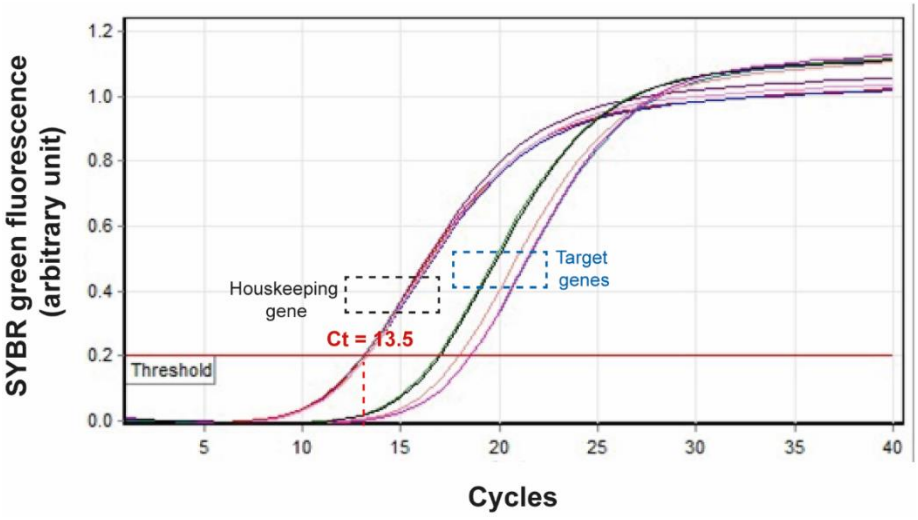


Figure 2.4. Representative amplification plot for RT-qPCR using SYBR green. Amplification curves for housekeeping and target genes from HepG2 cells. A threshold was set to obtain Ct values for housekeeping and target genes.

Melt curve

Melt curves were performed for each RT-qPCR run to confirm that a single amplicon has been generated by qPCR, confirmed by the presence of a single peak, hence indicating specific amplification. Other analysis tools, such as agarose gels can be used in conjunction with melt curves to determine the purity of an amplicon.

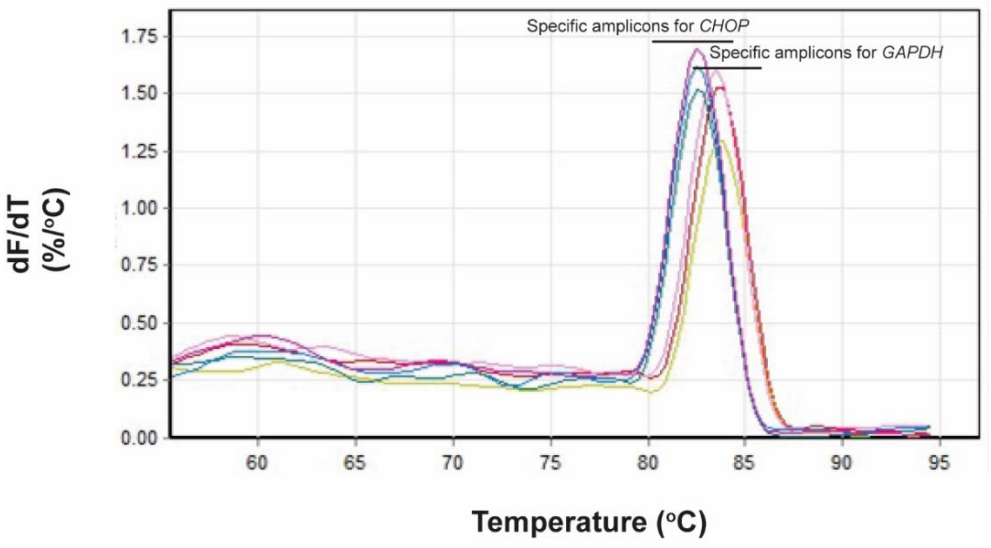


Figure 2.5. Example melt curves from RT-qPCR for *GAPDH* and *CHOP* genes. The single peak is observed for an amplicon from *GAPDH* (red, yellow and pink) or from *CHOP* (blue, green and purple), which is typically interpreted as a pure, single amplicon.

2.2.8 Measurement of protein levels by Western blot

Principle

Western blot was performed to detect and characterise specific proteins. It is based on the principle of immunochromatography, as proteins are separated into polyacrylamide gel according to their molecular weight. The proteins are then transferred onto nitrocellulose membrane and eventually detected following incubation with a specific primary and secondary enzyme (HRP) – labelled antibody. Here, we investigate the expression of the proteins CHOP, CYP3A4 and CES1, using GAPDH as a loading control.

Protocol

Lysate preparation

Following cell growth and treatment protocol, HepG2 cells were washed twice with cold PBS (10 mL) and scrapped in 10 mL of ice-cold PBS before being transferred to labelled centrifuge tubes placed on ice. The cells were then centrifuged into a pellet for 5 mins at 300 x g. Then, cells were lysed by resuspending the pellet in 200 µL of radioimmunoprecipitation assay (RIPA) buffer with a cocktail of protease and phosphatase inhibitors (0.1%). The samples were then kept on ice for 30 mins prior centrifugation at 20,000 × g for 30 mins at 4°C. The supernatants were then collected and stored at -80°C. To prepare the samples for protein electrophoresis, protein lysates were thawed on ice and a 1:1 protein lysate and 2x Laemmli buffer supplemented with 2-mercaptoethanol (5%) mixture (50 µL the lysate and 50 µL of 2x Laemmli buffer with 2-mercaptoethanol) was prepared in labelled Eppendorfs for each sample. Then, the protein lysates in Laemmli buffer were heated on a heat block. This procedure would allow the denaturation of proteins, facilitating protein electrophoresis strictly by molecular weight. The temperature for denaturation of proteins was determined empirically. A standard 95^{o}C for 5 min was initially performed, however, CHOP protein was not detected unless the proteins were heated at 70^{o}C for 10 min. Therefore, protein lysates in Laemmli were heated for 70^{o}C for CHOP expression and 95^{o}C for 5 min for CYP3A4 and CES1 expression to completely denature the proteins. GAPDH was visible with both conditions.

Protein electrophoresis

Proteins were separated onto polyacrylamide gel by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE).

To do so, a 5% polyacrylamide gel and 10% polyacrylamide gel have been prepared by adding the components in Table 2.6, in descending order.

	Polyacrylamide gel (10%)	Polyacrylamide gel (5%)
Distilled H_2O	4.3 mL	2.8 mL
1.5M Tris	2.5 mL	0
0.5M Tris	0	1.3 mL
10% w/v SDS	100 µL	50 µL
10% w/v APS	100 µL	50 µL
TEMED	14 µL	7 µL
Acrylamide 30% v/v	3.3 mL	830 µL

Table 2.6. Recipes for 10% and 5% polyacrylamide gels for Western blot. The components were added in descending order. APS, ammonium persulfate; SDS, sodium dodecyl sulfate; TEMED, tetramethylethylenediamine. n.b. 10% w/v SDS was made by adding SDS (MW = 288.38 g/mol) to 1 mL of distilled H_2O. 10% w/v APS was made by adding APS (MW = 228.18 g/mol) to 1 mL of distilled H_2O.

After preparing the 10% polyacrylamide gel, the gel was poured in a gel cassette, leaving space for the 5% polyacrylamide. The 5% polyacrylamide gel was then poured on top of the 10% or 12.5% polyacrylamide gel after it has polymerised. Immediately after pouring the 5% polyacrylamide gel, a gel comb (10- or 15-well gel comb depending on the amount of protein to be loaded) was embedded in the 5% polyacrylamide, on top of the gel cassette. After allowing the 5% polyacrylamide gel to polymerise, the comb was gently removed in order to obtain wells for sample loading.

For each protein of interest, the amount of protein to be loaded was empirically determined by loading different amounts of protein. Similarly, concentrations of primary and secondary antibodies were empirically determined by starting with the suggested manufacturer's concentration and either increasing or decreasing the concentration depending on the results obtained.

The proteins, quantified using the Bradford assay as described previously, were equally loaded into the wells of 5% polyacrylamide gel. Page Ruler™ Plus (5 µL) (Thermo Fisher, USA) or Amersham™ ECL™ Rainbow™ Marker - Full range (Sigma Aldrich, Germany) molecular weight ladder was added to the first well to ensure the identification of proteins. The denatured proteins alongside the molecular weight ladder were separated by SDS-PAGE for approximately 1 hr at 100 V at room temperature (25^{o}C).

Membrane transfer

In this step, the separated proteins were transferred out of the gel and onto a nitrocellulose membrane. The nitrocellulose membrane is popular for its high protein-binding affinity, its ability to immobilise proteins and its compatibility with a variety of detection methods including chemiluminescence. The wet transfer method was adopted and prepared according to Figure 2.7. The 5% polyacrylamide was gently cut off the rest of the gel and the remaining gel with the proteins was submerged in transfer buffer for 10 min before assembling the wet transfer sandwich (Figure 2.6). The nitrocellulose membrane, filter papers and sponges were also submerged in transfer buffer to ensure a wet transfer. Following the assembly of the wet transfer sandwich, the gel holder cassette was placed in the buffer tank filled with transfer buffer and the proteins were left to transfer for 75 min at 100 V at room temperature.

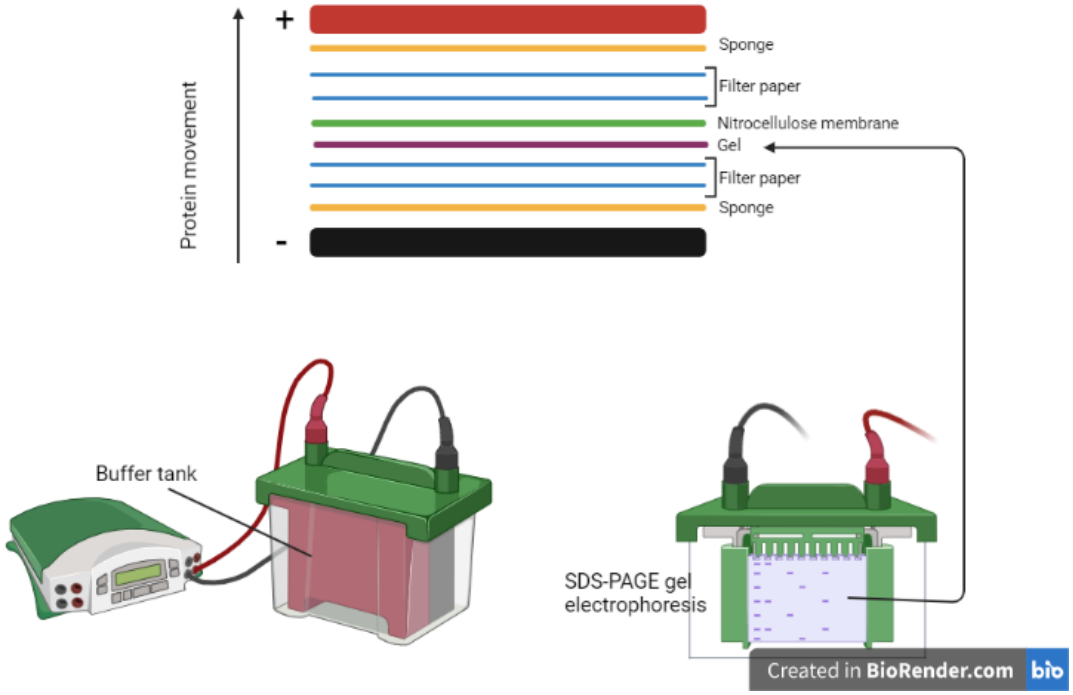


Figure 2.6. Wet transfer sandwich. The gel was removed from its cassette and the top portion containing the wells was cut off; then, the gel, nitrocellulose membrane, filter papers and sponges were equilibrated in transfer buffer; finally, the transfer sandwich was then placed in a gel holder cassette with the order illustrated above (created in Biorender).

Immunoblotting

After the wet transfer of proteins onto the nitrocellulose membrane, the nitrocellulose membrane containing the proteins was blocked with Tris-buffered saline (TBS) containing 0.05% v/v Tween 20 (TBS-T) and 5% non-fat dried milk for 1 hr at room temperature. Then, the protein was immunoblotted with the antibody of interest (dilution in 5% non-fat dried milk in 0.05% TBS-T), the mouse monoclonal anti-CHOP, anti-CYP3A4 or anti-CES1 IgG_1 κ antibody, overnight at 4°C. The excess primary antibody was washed off by washing the membrane three times with TBS-T for 10 min at 60 rpm and three times for 5 min at 60 rpm. After washing, the membrane was incubated with mouse IgGκ binding protein (BP) conjugated to Horseradish Peroxidase (HRP) for 1 hr at room temperature. Table 2.7 and 2.8 detail the primary and secondary antibodies used in this study.

Primary antibody	Company	Catalogue number	RRID	Species
Mouse monoclonal anti-GADD 153/CHOP IgG_1 κ	Santa Cruz Biotechnology, Inc.	sc-7351	AB_627411	Mouse
Mouse monoclonal anti-CES1 IgG_1 κ	Santa Cruz Biotechnology, Inc.	sc-365248	AB_10844328	Mouse
Mouse monoclonal anti-CYP3A4 IgG_1 κ	Santa Cruz Biotechnology, Inc.	sc-53850	AB_782375	Mouse
Rabbit polyclonal anti-GAPDH IgG	Santa Cruz Biotechnology, Inc.	sc-25778	AB_10167668	Rabbit

Table 2.7 List of primary antibodies used in this study.

Secondary antibody	Company	Catalogue number	RRID
HRP-conjugated mouse IgGκ BP antibody	Santa Cruz Biotechnology, Inc.	sc-516102	AB_2687626
HRP -conjugated mouse anti-rabbit IgG	Santa Cruz Biotechnology, Inc.	sc-2357	AB_628497

Table 2.8 List of secondary antibodies used in this study.

Detection

After washing, the bands were visualised with enhanced chemiluminescence (ECL) western blotting detection reagents (Thermo Fisher Scientific, USA; Cat#: 34580) and digital images were acquired using C-DiGit (LI-COR, USA) or iBright™ CL750 Imaging System (Thermo Fisher Scientific, USA).

Stripping and reprobing

Following ECL detection, blots were then stripped and reprobed with the house-keeping protein, GAPDH. After stripping the blot twice with stripping buffer for 10 min on a plate shaker (80 rpm), the blot was washed twice with PBS for 10 min. Then, the stripped blot was washed twice with 0.1% TBS-T for 5 min. The blot was then blocked with 5% non-fat milk in 0.1% TBS-T for 1 hr. Following blocking, the blot was incubated with rabbit polyclonal anti-GAPDH IgG antibody (1:1000) for 1 hour at room temperature. Then, the blots were washed 0.1% TBS-T for 45 min with 10-minute intervals (three times) and 5-minute intervals (three times). Then, the blots were incubated with mouse anti-rabbit antibody (1:5000) for 1 hr at room temperature. The bands were visualised with ECL.

Data analysis

Using the Image Studio 5.0 software (LI-COR, USA), the background was subtracted from the bands of interest before measuring densities of proteins of interest. Background-subtracted densities are proportional to protein levels. The results were then normalised to GAPDH protein levels.

2.2.9 Statistical analysis

Data are expressed as the mean ± mean standard deviation (SEM). The level of statistical significance was set at $P < 0.05$ and was assessed by one-way ANOVA followed by Bonferroni test or a student's t-test for parametric analysis. In case of non-parametric analysis, a Kruskal-Wallis test followed by Dunn's multiple comparison was performed. We used "fold matched control" and "% of control" to normalise the data obtained for comparison purposes. In some instances, technical replicates were used to ensure the reliability of single values. The average of technical replicates per treatment was calculated to obtain an independent value. Statistical analysis was performed using the Statistical Package for Social Sciences software (SPSS; IBM Corp., New York, USA). Graphs were drawn on GraphPad Prism (GraphPad Software, San Diego, USA).

3. Characterisation of S-p-bromobenzylglutathione cyclopentyl diester in Human hepatoma HepG2 cell line

Introduction

As outlined in the general introduction, dicarbonyl stress induced by the highly reactive glucose metabolite MGO may be responsible for many cellular damaging effects leading to the development of diabetic complications (Kold-Christensen & Johannsen, 2020; Nigro et al., 2019). Indeed, the pathogenesis-associated decline in Glo1 expression and/or an increase in the formation of MGO, was estimated to lead to a 2–3-fold increase in plasma and tissue-specific increase in the concentration of MGO (Rabbani & Thornalley, 2022), which has been suggested be a major factor in low-grade inflammation and cell tissue dysfunction driving the development of chronic disease or complications associated with diabetes (Rabbani et al., 2016b).

There are various ways to increase intracellular MGO in experimental settings. Most previous studies investigating the role of MGO in cellular toxicity and dysfunction were conducted using exogenously applied MGO in concentrations that are relatively higher than what is observed in diabetes, using commercial stock solutions that may be contaminated (Rabbani & Thornalley, 2014b; Schalkwijk & Stehouwer, 2020). The concentrations of MGO that have been used to assess its acute toxic effects in most previous in vivo and in vitro studies on different cells and targets (endothelial and hepatic cells, mitochondria, LDL) range from 0.1 to 10 mM (Chang et al., 2005; de Souza Prestes et al., 2019; Prestes et al., 2022), which is higher than the physiological serum levels observed in non-pathological (up to 250 nM) and pathological conditions (up to 742 nM), as well as estimates of intracellular MGO levels (~1–5 µM MGO) (Dhananjayan et al., 2019; Dobler et al., 2006; Nemet et al., 2005; Phillips et al., 1993; Rabbani & Thornalley, 2014b). In vivo studies are scarcer, with some long-term exposure protocols using high doses (ranging from 100 to 400 mg/kg body weight) (W.-C. Chang et al., 2015; Seo, Ki, et al., 2014; Yılmaz et al., 2018).

Although providing valuable insight into the cellular effects of MGO, this method does not reflect accurately the natural occurrence of MGO accumulation within the cell. To overcome

this, other mechanisms to increase intracellular MGO to more physiologically relevant concentrations may be used, through siRNA technology and genetic manipulation to abrogate GLO1 expression (Masania et al., 2016; Rabbani et al., 2016a; Thornalley, 1998; Vander Jagt & Hunsaker, 2003), or pharmacologically, with the use of S-p-Bromobenzylglutathione cyclopentyl diester (BBGC), a cell-permeable prodrug inhibitor of GLO1 (Rabbani & Thornalley, 2022). This approach may be more appropriate and reliable as studies have proven that it not only increases dicarbonyl stress to levels that matched what is observed in diabetes, but also induces a state of pathology with similar features and magnitude to diabetes *in vivo* (Kold-Christensen & Johannsen, 2020; Masania et al., 2016; Shamsaldeen et al., 2016; Tikellis et al., 2014). Indeed, BBGC has become a valuable research tool to explore the effect of pharmacologically induced dicarbonyl stress in experimental studies (Rabbani & Thornalley, 2022). *In vitro*, pharmacological inhibition of GLO1 by BBGC does increase in the cellular concentration of MGO and the induction of apoptosis in experiments with endothelial cells and tumour cells (Dobler et al., 2006; Thornalley et al., 1996). Similar results were also observed *in vivo*, where along with increased MGO and markers of MGO-induced damage, increased inflammation can be observed (Tikellis et al., 2014).

Dicarbonyl stress may be responsible for increasing the incidence of liver disease in diabetic patients. Previous studies investigating the effects of dicarbonyl stress in hepatocytes have used exogenously applied MGO or MGO-derived AGEs (Kang et al., 2023). Older studies in isolated murine hepatocytes have used concentrations ranging from 1mM to 20mM of MGO to report impaired cellular function, such as protein synthesis, and depleted GSH levels (Kalapos & de Groot, 1992; Kalapos et al., 1991, 1992). Over time, the protocols used seem to improve. In HepG2 cells treated with 0.5mM MGO for 24 hours, Afridi et al. report increased ROS generation, and an inhibition of glucose uptake and IRS1 phosphorylation (Afridi et al., 2016). Seo et al. have demonstrated that HepG2 cells treated with 3mM MGO for 36 hours leads to a loss in cell viability, mitochondrial dysfunction, increased ROS generation coupled with cellular GSH and ATP depletion, and increased apoptosis with an observable upregulation in PARP cleavage and caspase 3 levels (Seo, Ki, et al., 2014). In 2019, Cha et al. showed that treatment with 0.25mM MGO for 0.5, 2, 4, and 6 hours was able to trigger NF-κB activation and increase mRNA expression of pro-inflammatory cytokines, TNFα and IFN-γ in HepG2 cells (Cha et al., 2019). However, these studies have the same limitations as those outlined above and in chapter 1, and as of yet, no studies have been carried out to investigate alternative and more physiologically relevant methods of increasing dicarbonyl stress in hepatocytes. In

order mimic endogenous MGO accumulation, the use of cell permeable GLO1 inhibitor prodrug BBGC was used in this series of experiments to examine the effects of MGO in hepatocytes, which, to date, has not been thoroughly investigated.

Experimental protocol and methods

In a first series of experiments, HepG2 cells were exposed to MGO (0.03 mM to 1 mM) in RPMI-1640 medium supplemented with 0% FCS and 1% penicillin-streptomycin and incubated for 24 hours before measuring cell viability and IL-8 secretion (described in 2.21 and 2.2.2, respectively). Similarly, HepG2 cells were exposed to Glyoxalase I inhibitor S-pbromobenzylglutathione cyclopentyl diester (BBGC) (3 µM to 50 µM) in in RPMI-1640 medium supplemented with 3% heat-inactivated foetal calf serum (FCS), and 1% penicillin-streptomycin and incubated for 24 hours prior to measuring cell viability and IL-8 secretion. Then, HepG2 cell viability was measured after the cells were treated for 24 hours with BBGC (5 µM to 15 µM) in combination with dicarbonyl scavengers Aminoguanidine (100 µM to 500 µM) or Alagebrium (10 µM to 100 µM). The combination studies were carried out in two different ways – the cells were either treated simultaneously with BBGC and Aminoguanidine/Alagebrium or pre-treated for 4 hours with Aminoguanidine/Alagebrium prior to exposing them to the simultaneous treatment described herein. Finally, MG-H1 formation was measured in HepG2 cells by immunostaining following a 24, 48 ad 72 hours treatment with 20 µM BBGC.

In a second series of experiments, HepG2 cells were treated with BBGC (3 µM to 20 µM) for 24 hours, before carrying out flow cytometric analysis of cell death (described in 2.2.6) and measuring ROS formation using the $DCFH_2$-DA assay.

Detection of MG-H1 by immunocytochemistry (ICC)

HepG2 cells were plated on 13mm diameter glass coverslips in 6-well plates at a seeding density of 1.0×10^5 for 72 hours prior to treatment. The HepG2 cells were then treated for 4, 24 and 48 hours with BBGC 20 µM to measure intracellular MG-H1 accumulation.

Buffers and solutions

- Wash buffer: 3% BSA in PBS.

- 3.7% formaldehyde fixative solution: 1mL of 37% formaldehyde in 9 mL of PBS.

- Permeabilization solution 0.5% Triton® X-100 in PBS.

- Blocking buffer: 1% BSA + 0.1% Triton® X-100 in PBS.

After treatment is complete, the cells were washed twice with PBS then fixed to the coverslip using formaldehyde fixative solution for 10 minutes at room temperature (~25°C). This was removed and the cells were washed twice with wash buffer. The cells then underwent permeabilization using 0.5% Triton® X-100 in PBS for 20 minutes at room temperature (~25°C). After the cells were permeabilised, they were washed twice with wash buffer, and this was followed by a one-hour incubation with blocking buffer at 4°C to block the cells. It was then incubated overnight with the mouse anti-MG-H1 antibody (Abcam), diluted 1:1000-1:2000 in blocking buffer at 4°C.

The next day, the coverslips were washed 10 times in 3 changes of wash buffer before being incubated with the secondary anti-mouse antibody Alexa Fluor® 488, diluted 1:2000 in blocking buffer for 2 hours at room temperature. This was followed another 10 washes in 3 changes of wash buffer one wash with PBS. The coverslips were mounted using a DAPI-containing anti-fade mounting medium and visualised using a confocal microscope and filter sets appropriate for the label used.

The slides were scanned using confocal microscopy at 20x resolution, with the results being analysed using LAS AF to determine the intensity of the MG-H1 stain.

Detection of intracellular ROS; The dichloro-dihydro-fluorescein diacetate ($DCFH_2$-DA) assay

2',7' dichlorodihydrofluorescein diacetate ($DCFH_2$-DA) is a widely used probe for the detection of intracellular ROS. Initially colourless and cell permeable, $DCFH_2$-DA diffuses across the cell membrane where intracellular esterases deacetylate it to make $DCFH_2$, a cell impermeable molecule. The actions of multiple types of ROS (hydrogen peroxide, peroxynitrite, hydroxyl radicals, nitric oxide, and peroxy radicals) on DCFH2 oxidize it into DCF which is fluorescent (reported Ex/Em: 485–500 nm/515–530 nm) and can be detected using a flow cytometer equipped with a standard filter set for fluorescein (FL1 channel) or

visualised using confocal microscopy set to the appropriate settings (Parihar and Brewer 2007; Gomes et al., 2005; Eruslanov et al., 2010; Soh et al., 2006).

Confocal microscopy:

HepG2 cells were plated on a 6-well plate at a seeding density of 5.0×10^5 cells for 24 hours prior to treatment. After treatment protocol, cells were washed twice with 3 mL of PBS per well (37ºC) and stained with 1 mL of a 10 µM H_2DCFDA working solution which was prepared by diluting the H_2DCFDA stock solution (in DMSO) in Hank's Balanced Salt Solution (HBSS) (37ºC) for 30 minutes at 37ºC. Cells treated with H_2O_2 100µM (in HBSS) for 1 hour prior to the imaging served as positive control and an unstained control (HBSS only) was also included for each experiment as well. When the incubation was complete, the cells were washed twice with PBS and the staining solution was replaced with HBSS for the imaging process.

Cellular ROS generation was observed and photographed using an inverted confocal laser scanning microscope (Leica TCS SP5 Confocal Laser Scanning Microscope, Germany) and the accompanying Confocal LAS AF software. The Argon laser at power 0.1-10% was set and the DCF was excited using the 488 Argon (Blue) laser line (FITC filter settings) and the emission was collected at ~500-580 nm. 4 to 6 random regions of the well were imaged for each sample. The images were analysed using the ImageJ software (Maryland, USA) in order to obtain mean fluorescence intensity to quantify ROS generation. Fluorescence compensation (to avoid autofluorescence measurements) was performed by subtracting the MFI of the DCFH-DA-stained cells by the MFI of unlabelled cells.

RESULTS

The effects of Methylglyoxal on HepG2 inflammation viability

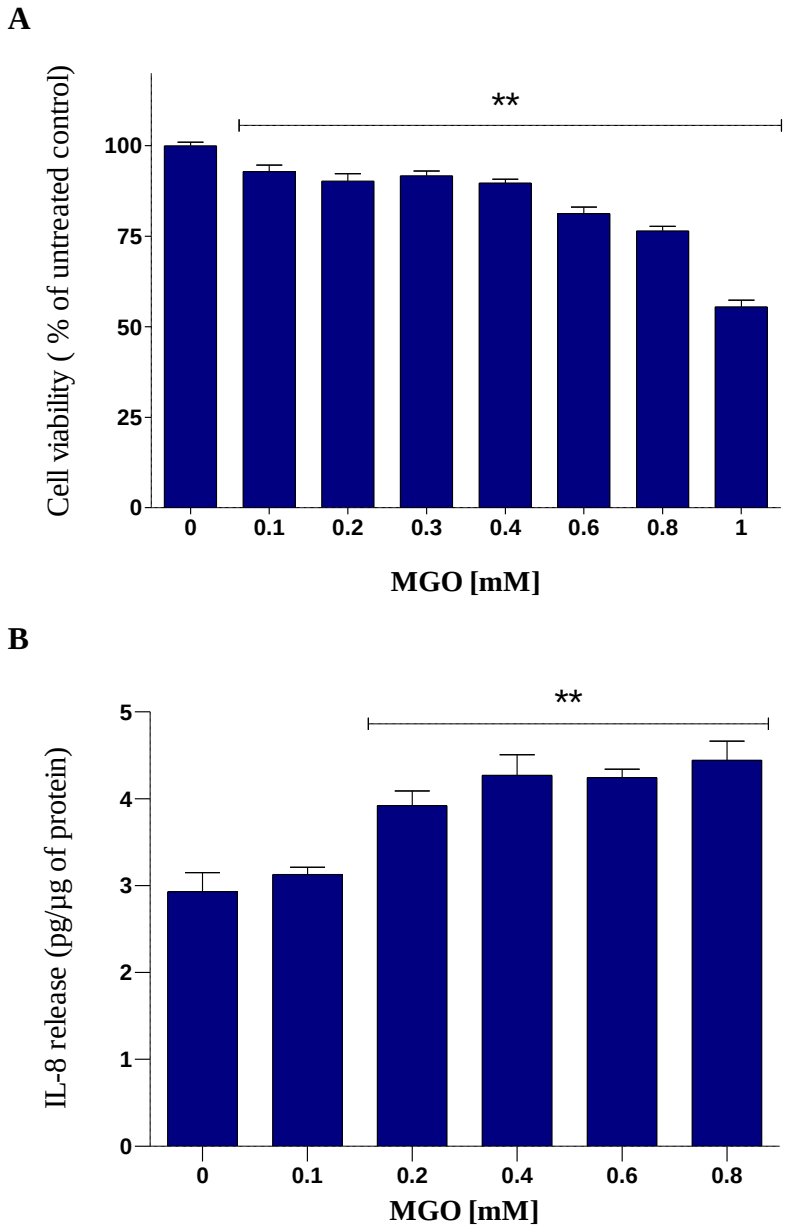


Figure 3.1. The effects of methylglyoxal treatment on HepG2 cell viability (A) and inflammation (B). 24-hour methylglyoxal treatment causes a dose-dependent decrease in HepG2 cell viability compared to untreated control, and a dose dependent increase in IL-8 secretion. Data are expressed as the mean ± SEM from n = 8 experiments for cell viability (6 replicates per experiment) and n=6 (3 replicates per experiment) for IL-secretion. Statistical analysis was carried out using with Kruskal-Wallis with Dunn's post hoc test (A) and one-way ANOVA followed by Bonferroni test where p-value $p < 0.01$ was considered significant (B); **$p < 0.01$ vs. untreated cells.

As expected, MGO treatment caused a dose-dependent reduction in cell viability (Figure 3.1.A), where significant effects start to be seen at 0.1mM MGO. 1mM MGO significantly

caused a little over 20% percent reduction after 24 hours. Direct MGO exposure also leads to a dose dependent increase in IL-8 secretion from HepG2 cells, with significance reached at concentrations ≥0.2 mM (Figure 3.1.B).

The effects of GLO-1 inhibition on HepG2 inflammation and viability

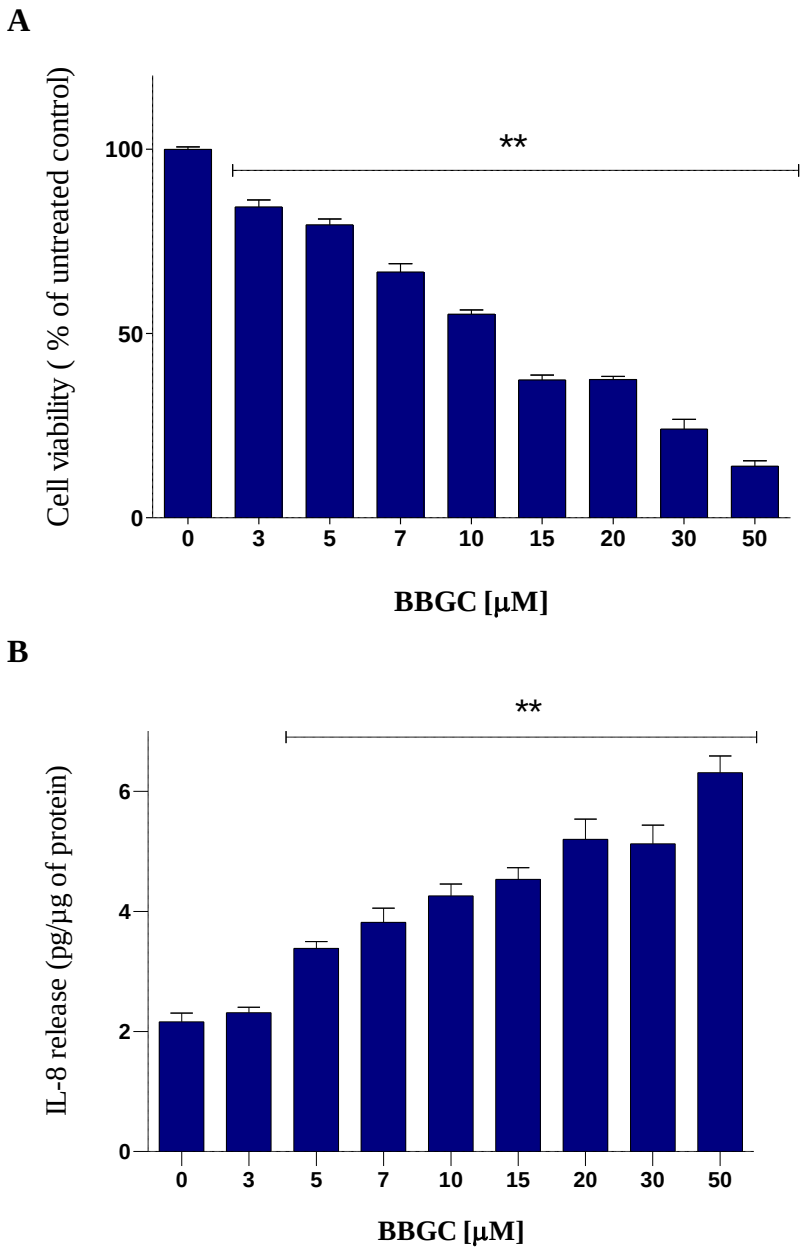


Figure 3.2. The effects of Glyoxalase 1 inhibition on HepG2 cell viability (A) and inflammation (B). Inhibition of Glyoxalase 1 following BBGC treatment caused a dose-dependent decrease in HepG2 cell viability compared to vehicle treated control (DMSO). This was coupled with a dose-dependent increase in IL-8 release. Data are expressed as the mean ± SEM from n = 4 – 8 experiments (3 to 6 replicates per experiment). Statistical analysis was carried out using with Kruskal-Wallis with Dunn's post hoc test (A) and one-way ANOVA followed by Bonferroni test where p-value $p < 0.01$ was considered significant (B); **$p < 0.01$ vs. untreated cells.

Similar to direct MGO exposure, treatment of HepG2 cells with the GLO1 inhibitor BBGC, caused a dose-dependent reduction in cell viability (Figure 3.2.A). Consistently, the inhibition of GLO-1 caused a dose-dependent increase in IL-8 release, which is known to induce monocyte attraction. (Figure 3.2.B). 3 µM BBGC failed to produce statistically significant results for IL-8 secretion.

Measurement of HepG2 intracellular oxidative stress following GLO-1 inhibition

The reduction in cell viability and IL-8 secretion as a result of GLO1 inhibition in HepG2 cells (Figure 3.2) was coupled with an increase in ROS generation, as indicated by the increased DCF fluorescence (Figure 3.3.A), indicating oxidative stress. BBGC 10 µM and 20 µM result in a 2.3 ($p < 0.05$) and 2.6 ($p < 0.01$) -fold increase in ROS, respectively (Figure 3.3.B).

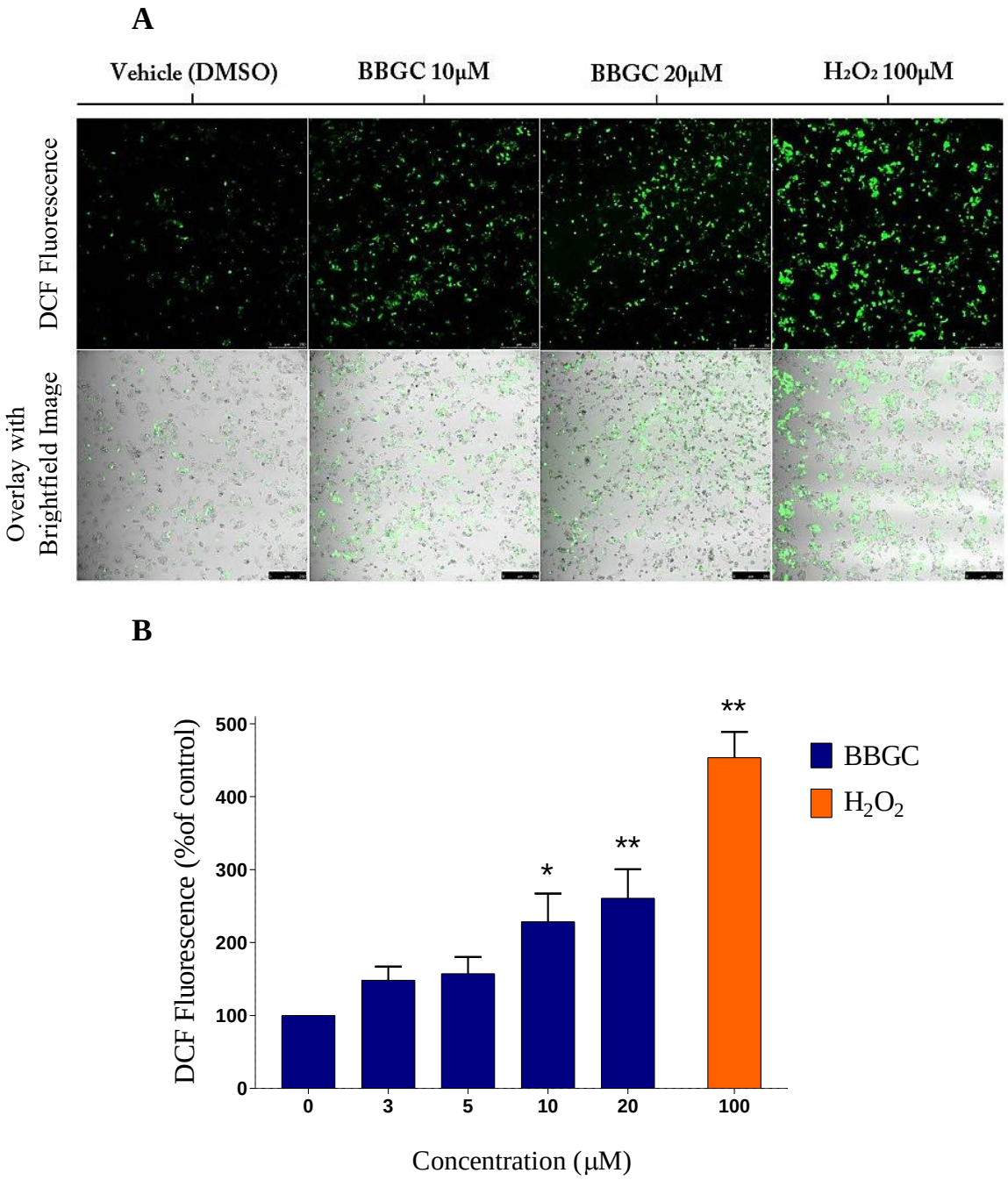


Figure 3.3. ROS formation in HepG2 cells exposed to BBGC for 24 hours. (A) Confocal images of intracellular ROS levels following BBGC treatment. The green fluorescence represents DCF which was oxidised from DCFH by ROS. A higher fluorescence signifies higher levels of ROS. (B) Quantification graphs of intracellular ROS levels in HepG2 cells following BBGC treatment determined by DCF mean fluorescence intensity using the image processing software Image J. HepG2 cells were incubated with 100 µM hydrogen peroxide (H_2O_2) for 1 hour as a positive control. Data are expressed as mean ± SEM from n = 5 independent with 1 replicate per experiment. Statistically significant differences were determined with Kruskal-Wallis with Dunn's post hoc test. * $P < 0.05$, and **$P < 0.01$ significantly different from vehicle-treated control groups.

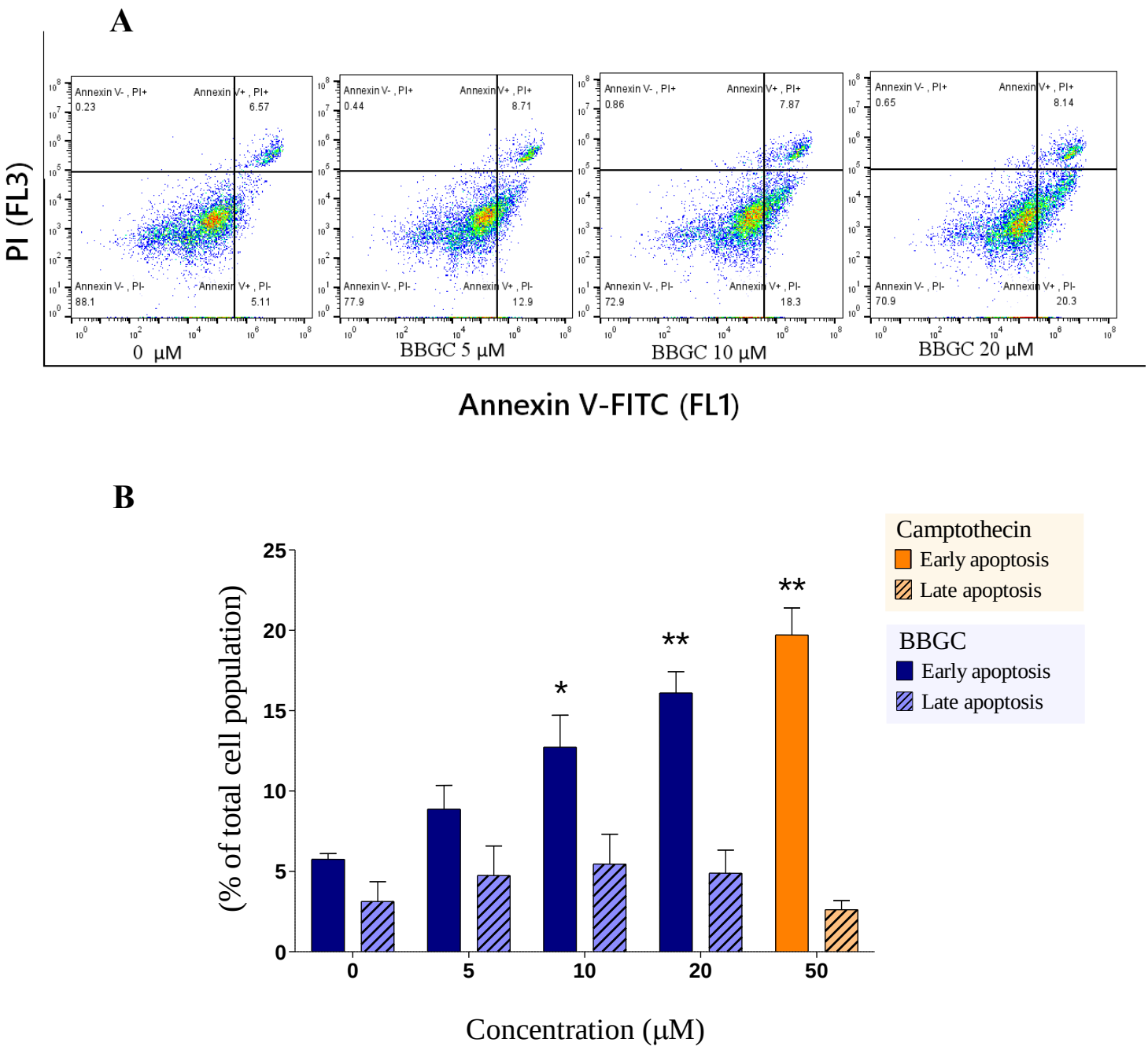


Figure 3.4. Effects of a 24-hour exposure to BBGC on HepG2 cell early apoptosis and necrosis. (A) Representative density plots for HepG2 cells treated with BBGC visualised using FlowJo (B) Quantitative representation of early apoptotic and late apoptotic cells in HepG2 cells treated with BBGC extrapolated from flow cytometry analysis where apoptosis is proportional to Annexin-V fluorescence intensity and late apoptosis is proportional to Annexin-V and PI fluorescence intensity. Data are expressed as mean ± SEM from $n = 4$ independent experiments. 50 µM camptothecin was used as a positive control. Statistically significant differences were determined using one-way ANOVA with Bonferroni post hoc test, where * $P < 0.05$ and ** $P < 0.01$ vs vehicle-treated control group.

In addition to increasing cellular ROS and IL-8 secretion, BBGC mediated dicarbonyl stress significantly triggers apoptotic cell death in HepG2 cells after 24 hours, while having little effect of necrosis. This suggests that GLO1 inhibition, through increased oxidative stress, leads to cell injury and promotes the activation of apoptotic signalling in hepatocytes.

Assessment of MGO formation following GLO-1 inhibition by immunocytochemistry (ICC)

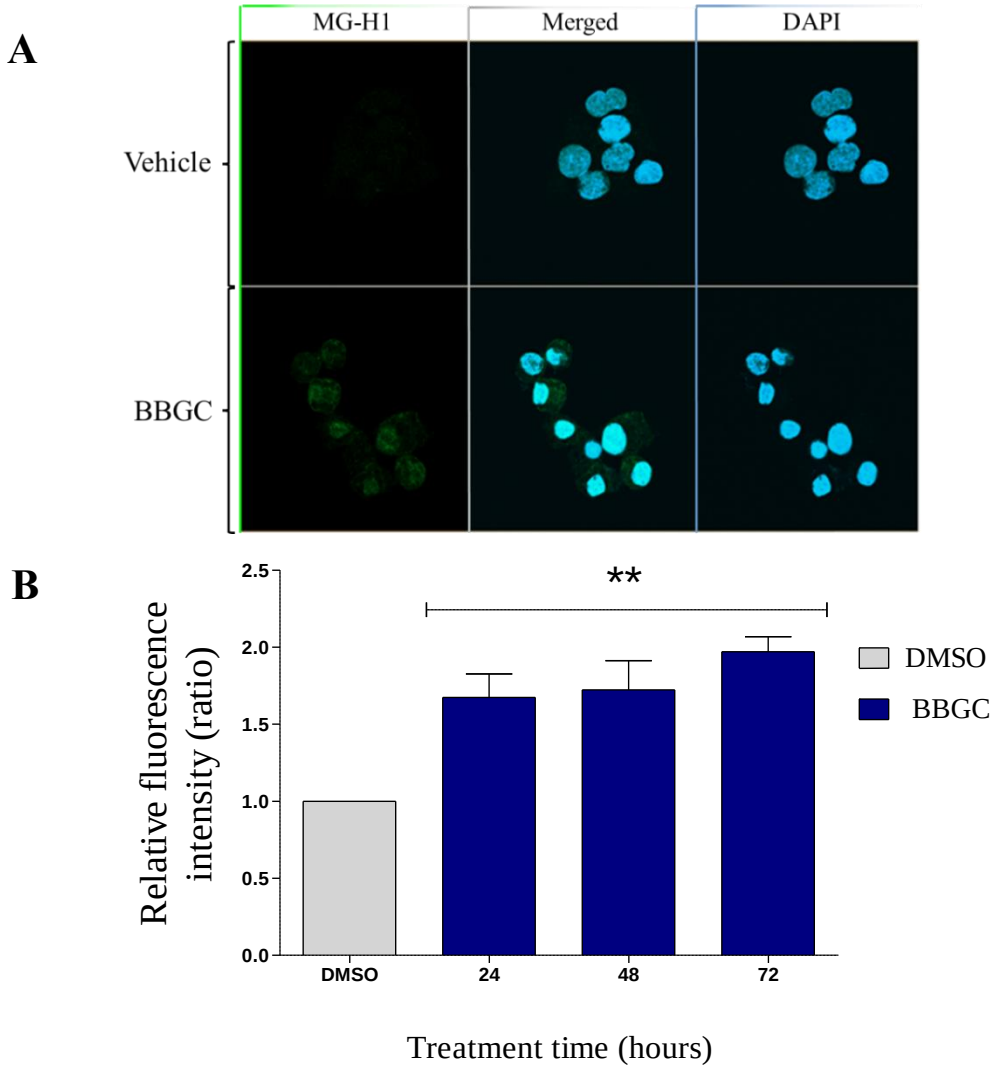


Figure 3.5. The effects of BBGC treatment on MG-H1 formation in HepG2 cells. (A) Representative images of increased fluorescent stained MG-H1 adducts, suggesting an increased formation of MGO intracellularly in HepG2 cells following GLO1 inhibition. (B) The inhibition of GLO1 leads to a significant increase in MG-H1 formation after, 48 and 72 hours. Data is represented as the ratio of the fluorescence intensity of the vehicle treated control vs. 20 µM BBGC at each respective time point. This was obtained by immunocytochemical staining of HepG2 cells followed by confocal microscope imaging. Data are expressed as the mean ± SEM from n = 4 experiments (3 replicates per experiment). Statistical analysis was carried out using one-way ANOVA where p-value $p < 0.01$ was considered significant; **$p < 0.01$ vs. untreated cells.

To verify that BBGC treatment indeed increased intracellular MGO concentrations in HepG2 cells, it was important to assess intracellular MG-H1 formation, which is the most predominant adduct derived from MGO glycation. We have identified through immunolabelling

significantly increased MG-H1 accumulation after 24, 48 and 72 hours (Figure 3.5.A). It does not appear to significantly increase with time, but we are nonetheless able to confirm that dicarbonyl stress is effectively induced after treatment, and the cells are not recovering from it as time progresses (Figure 3.5.B).

Effect of the Methylglyoxal scavengers, Aminoguanidine and Alagebrium, on the loss in cell viability induced by inhibition of Glyoxalase 1

Simultaneous treatment with the scavengers

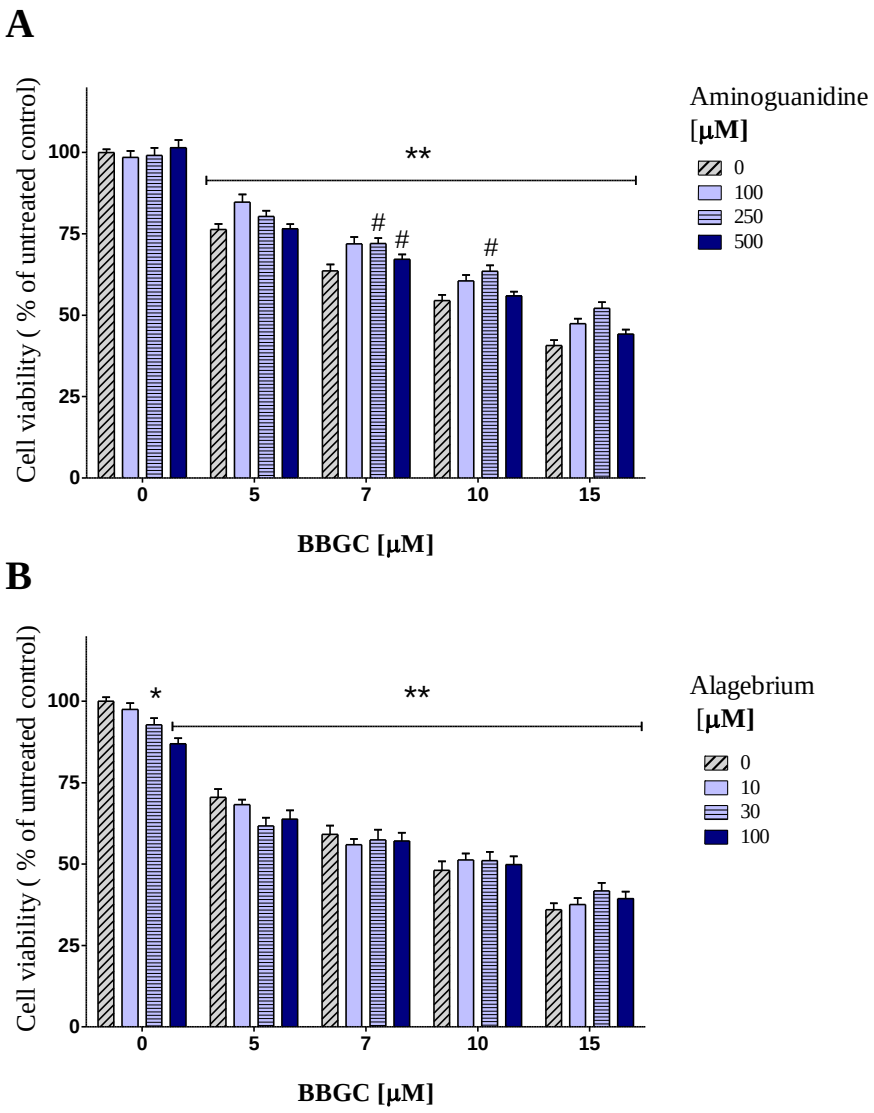


Figure 3.6. The effects of simultaneous treatment with methylglyoxal scavengers Aminoguanidine (A) and Alagebrium (B) on inhibition of GLO1 mediated loss in HepG2 cell viability. Neither Aminoguanidine nor Alagebrium when combined with BBGC were able to significantly protect against the loss in cell viability, Alagebrium to a lesser extent than Aminoguanidine. Data is expressed as the mean ± SEM from n = 2 and 5 experiments respectively, (6 replicates per experiment). Statistical analysis was carried out using with Kruskal-Wallis with Dunn's post hoc test. p-value $p < 0.05$ was considered significant; *$p < 0.05$ vs. untreated cells and # $p < 0.05$ vs. BBGC alone.

Pre-treatment with the scavengers prior to GLO-1 inhibition

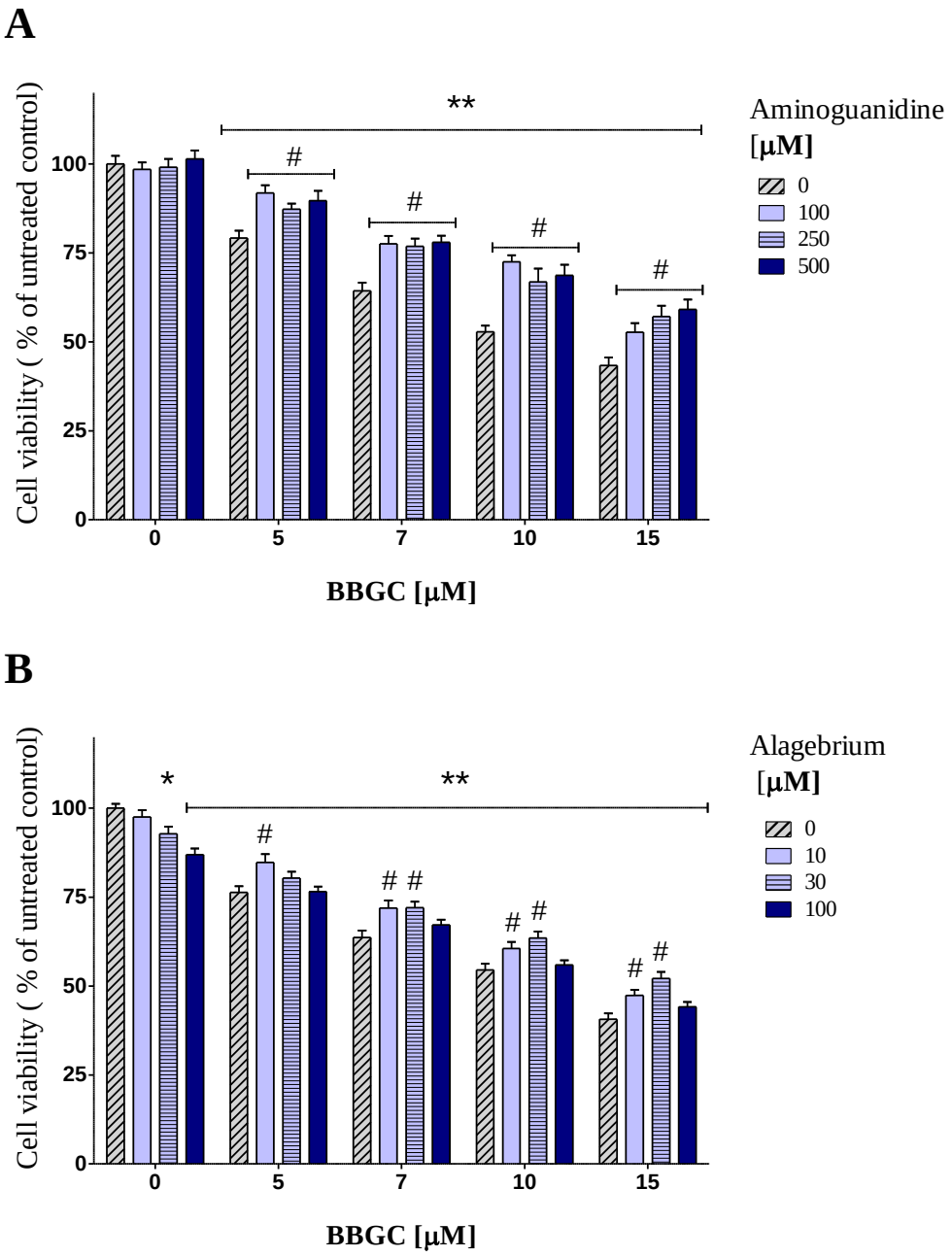


Figure 3.7. The effects of a pre-treatment with methylglyoxal scavengers Aminoguanidine (A) and Alagebrium (B) on inhibition of GLO1 mediated loss in HepG2 cell viability. The 4-hour pre-treatment has improved the ability of both Aminoguanidine and Alagebrium to protect against the loss in cell viability caused by BBGC treatment. Consistently, Aminoguanidine was more effective in producing statistically significant protection than Alagebrium. Data is expressed as the mean ± SEM from n = 4 independent experiments. Statistical analysis was performed using Kruskal-Wallis with Dunn's post hoc test. ** $p < 0.01$,*$p < 0.05$ vs. untreated cells and # $p < 0.05$ vs. BBGC alone.

When the methylglyoxal scavengers are combined with the BBGC treatment simultaneously, the scavengers fail to produce a statistically significant protection against GLO-1 inhibition mediated loss of cell viability (Figure 3.6). Alagebrium seems to produce little to no effect

using this method (Figure 3.6.B). On the other hand, the pre-treatment of the cells with the scavengers for 4 hours prior to exposing them a BBGC and scavenger combination improves the outcome (Figure 3.7). Aminoguanidine was able to attenuate the BBGC mediated loss of cell viability in a consistent, significant manner (Figure 3.7.A). Alagebrium was also able to attenuate the loss in cell viability (Figure 3.7.B), but with less significant and less consistent results. Alagebrium on its own seems to affect cell viability, which may explain why we were unable to achieve effective protection using higher concentrations (100 µM).

Discussion

The results suggest that BBGC treatment produced deleterious effects on cell viability, increased IL-8 secretion and increased apoptosis rates after 24 hours in HepG2 cells. These observations were coupled with an increase in ROS generation, emphasizing that the loss of GLO1 function plays a pivotal role in inducing inflammation and impaired cellular respiration in hepatocytes. These effects are highly likely to be mediated by increased intracellular MGO accumulation as a result of GLO1 inhibition, as indicated by the increased intracellular formation of MG-H1 adducts and by the protective effect of Aminoguanidine, a dicarbonyl scavenger,

Both MGO treatment and BBGC lead to decreased cell viability measurements, indicating lower cellular metabolic activity. This can be due to a number of stressful stimuli, including inflammatory responses and oxidative stress. Previous studies have established that MGO has the potential to increase oxidative stress in many cell types, not only through RAGE signalling, but also through other mechanisms. Similar to our findings, in HepG2 cells, the direct treatment of cells with MGO (3-10mM) also lead to a significant decrease in cell viability, increased ROS generation and an induction of apoptotic cell death. Pre-treatment with trolox (a vitamin E analogue), PEG-catalase (a hydrogen peroxide scavenger), or Mn-TBAP (SOD mimetic) attenuated the MGO-induced loss in cell viability, suggesting oxidative stress (ROS generation) plays an important role in the cytotoxic effects observed therein (Seo, Ki, et al., 2014). In line with this, treatment with MGO in many cell types, lead to an observable link between oxidative stress, genotoxicity and apoptosis (C. M. Chan et al., 2016; Hollenbach, 2017; Li et al., 2018; Lin et al., 2012; Schalkwijk & Stehouwer, 2020; Wang et al., 2022). A study with another dicarbonyl, glyoxal, in hepatocytes isolated from rats, showed that 10 µM glyoxal was sufficient to increase susceptibility to noncytotoxic concentrations of H_2O_2 , which was preceded by ROS and H_2O_2 formation, and a reduction in mitochondrial membrane

potential (Shangari et al., 2006). Furthermore, in the same model, the cytotoxicities -of fructose and glyoxal, were increased about 100-fold and 200-fold, respectively, by noncytotoxic doses of H_2O_2, which suggests that the cytotoxic mechanism of MGO likely involves the induction of oxidative stress, strongly implicating mitochondrial dysfunction in this process (Lee et al., 2009).

Oxidative stress induced by has been implicated in the development of NAFLD (Santos et al., 2013) and the development of insulin resistance and diabetes (Hollenbach, 2017). As discussed previously, excessive ROS generation can lead to many deleterious effects in the cell, including irreversible oxidative modification of lipids, proteins, and carbohydrates, leading to cellular dysfunction. Importantly, it can induce apoptosis in hepatocytes and the release of inflammatory cytokines through pathways such as NF-κB and AP-1, thereby increasing the expression of adhesion molecules and the infiltration of leukocytes, exacerbating inflammation in the liver and the progression of liver disease, which altogether can cause significant tissue damage and remodelling (Mohamed et al., 2016). Seo et al. suggest that the increase in oxidative stress in MGO-treated HepG2 cells is a result of mitochondrial dysfunction, which is highly likely and corroborated by many studies demonstrating the extent of MGO-induced mitochondrial toxicity that we have discussed in chapter1. This effect on the mitochondria may be of great importance to the dysfunction of hepatocytes and the rise in cellular oxidative stress, although glycation is not limited to mitochondrial proteins. Regardless of hyperglycaemia levels, AGEs themselves enhance the generation of ROS and impede antioxidant mechanisms (Mengstie et al., 2022). This is partly due to their interaction with their receptor RAGE, the activation of NADPH oxidase to increase the production of ROS (Li et al., 2005; Coker and Wagenknecht, 2011; Ali et al., 2015), and their ability to cause mitochondrial dysfunction (Hipkiss, 2014; Moraru et al., 2018), creating a vicious cycle that promotes AGE synthesis (Chilelli et al., 2013; Mengstie et al., 2022). AGE binding with RAGE also leads to impaired extracellular matrix structure and elicits, through ligand activation, altered intracellular signalling and gene expression (Gjorgjieva et al., 2019; Sena et al., 2013) which includes activation of nuclear factor NF-κB, which stimulates the transcription of genes encoding cytokines and growth factors (i.e. tumour necrosis factor α (TNFα)), inducing the release of pro-inflammatory cytokines such as IL-8 and increased expression of enzymatic ROS production, notably superoxide (Gjorgjieva et al., 2019; Jiang & Török, 2014; Mohamed et al., 2016; Rasmussen et al., 2008; Sena et al., 2013). AGE and RAGE levels, as well as IL-8 expression, were upregulated in steatotic HepG2 cells (Gaens et al., 2012), and Hyogo et al.

found that serum levels of AGEs were significantly elevated in NASH patients (Hyogo et al., 2007), which were found to correlate positively with the severity of liver damage. In line with this, another group also report increased α-dicarbonyls and RAGE expression in the liver of HFD mice (Petriv et al., 2021). As the liver plays a major role in the clearance of plasma AGEs, liver cells would be heavily exposed to the elevated levels of AGEs. Moreover, exposed hepatic stellate cells and myofibroblasts, responsible for fibrogenesis in chronic liver disease, were found to express the RAGE (Mohamed et al., 2016), which contributes to creating an environment where oxidative stress becomes excessive. However, due to the method used in our experiments and the time frame of the studies, this effect is unlikely mediated by AGE-RAGE interactions, but rather an oxidative stress mediated inflammatory response. It is important to remember that the genetic deletion of RAGE expression does not completely prevent inflammation in animal studies (Bijnen et al., 2018; Tikellis et al., 2014; Wouters et al., 2021), and stress-activated signalling pathways such as MAPK and NF-κB are activated by various intracellular stressors, including ROS, which are present in our study.

The activation of NF-κB by oxidative stress described herein may explain why GLO1 inhibition, leading to MGO accumulation in our studies, leads to oxidative stress generation that is coupled with increased pro-inflammatory IL-8 secretion. In HepG2 cells, 250μM MGO also induced inflammation, as evidenced by increased TNF-α and IFN-γ mRNA expression and secretion, via what the authors suggest to be NF-κB activation (S.-H. Cha et al., 2019). This corroborates a study where MGO (2mM-4mM) also increased IL-8 secretion and expression in human intestinal cells (Caco-2 and HT-29) (Kuntz et al., 2010), which was suggested by the authors to be mainly through superoxide generation. Furthermore, inhibitors of MAPK p38, ERK1/2 and NF-κB activation reduced IL-8 secretion. MGO-induced cytokine production can be observed in many studies, and in many cell types. Furthermore, hyperglycaemia was found to increase circulating inflammatory markers in wild-type but not in GLO1-overexpressing mice. Macrophages obtained from these GLO1-overexpressing mice showed no increase in TNF-α secretion in response to MGO exposure, while macrophages obtained from their wild-type counterparts did (Branka Vulesevic et al., 2016). In addition to NF-κB activation and the subsequent pro-inflammatory cytokine production, as mentioned previously, ROS has the ability to induce other transcription factors, such as HIF-1α, and AP-1, that lead to increased cytokine expression and production. Pro-inflammatory cytokines also further increase ROS generation, establishing a vicious cycle (Chatterjee, 2016). In addition to causing a significant increase in mRNA and protein expression of pro-inflammatory cytokine

IL-1β, MGO causes an extensive, oxidative stress-mediated cell death as consequence of a strong inhibition of catalase in rat hippocampal neural cells, (Di Loreto et al., 2004).

As previously discussed, damaging effects induced by oxidative stress to various cellular structures may compromise cell stability and hence favour pro-apoptotic pathways. However, apoptosis can also be directly triggered by oxidative stress. The induction of apoptosis is characterized by bio-chemical and morphological changes in the cell and is vital process for normal development and maintenance of tissue homeostasis, but abnormally increased rates of apoptosis can be a sign of tissue pathology, as detected in many chronic inflammatory and autoimmune diseases (Daniel et al., 2013). In DM, increased oxidative stress and inflammation have been established to be key mediators of cellular damage leading to various complications associated to the disease, and the role of these stressors in the induction of apoptosis in cells is a major mechanism underlying the damaging effects of hyperglycaemia. Apoptotic cell death is critical to hepatic cell damage and lesion formation in liver diseases (Daniel et al., 2013) and plays a key role in the progression of NAFLD (Afonso et al., 2019; Alkhouri et al., 2011). This is evidenced in NASH patients where significant levels of apoptosis and caspase 3 activation can be observed (Feldstein et al., 2003; Ferreira et al., 2011). Caspase 2 expression is also markedly upregulated in NAFL and NASH patients and animal models of NASH, and its deficiency reduces lipid-induced hepatocyte apoptosis and liver fibrosis (Machado et al., 2015), which emphasises the importance of these factors in the progression of the disease. In our study, we observe that in addition to reduced cell viability and ROS generation, the inhibition of GLO1 causes an increase in apoptosis in HepG2 cells. MGO was previously found to play an important role in the process of apoptosis and cell death, through several proposed mechanisms. These include ROS and GSH depletion, increased expression of NF-kB, JNK and p38 MAPK activation, inhibition of cellular respiration, DNA modification and DNA-protein cross-link resulting in DNA instability, ER stress, negative regulation of anti-apoptotic proteins Bcl-2 as well as positive regulation of pro-apoptotic proteins, and impaired mitochondrial permeability via the modification of mitochondrial proteins and release of cytochrome c (Schalkwijk & Stehouwer, 2020).

Hyperglycaemia, inflammation, oxidative stress, obesity and ageing were associated with repressed GLO1 expression and activity, which may explain why these conditions are associated with increased MGO levels (Beeri et al., 2011; He et al., 2020; Herpich et al., 2021; Rabbani & Thornalley, 2011; Sanchez et al., 2002; Schalkwijk & Stehouwer, 2020) as the loss of GLO1 function synergises with increased MGO formation to increase cellular and

extracellular MGO concentration (Rabbani et al., 2016a). In addition, GLO1 can directly activate NF-κB and AP-1, to elicit the PI3K/ Akt pathway, promoting cell survival and proliferation (Antognelli et al., 2013; Hosoda et al., 2015), rather than promoting apoptotic pathways, which is what is observed as a result of dicarbonyl stress. Hence, understanding the molecular mechanisms regulating the production and detoxification of MGO may provide important insights to delay the development of life-threatening complications of T2DM, and which have been shown to further exacerbate insulin resistance and NAFLD (Tsilingiris et al., 2021). In endothelial cells, GLO1 overexpression completely prevents high-glucose-induced MGO accumulation and AGE formation (Shinohara et al., 1998). Despite of the essential role of GLO1, data about GLO1 in fibrosis, cirrhosis or NAFLD/NASH are limited and the molecular pathways of this pathogenic progression remain elusive. The development of NAFLD and T2DM is closely linked to obesity. Through proteomic profiling, a decreased expression of hepatic GLO1 in an HFD NAFLD murine model and in tissue biopsies from paediatric NAFLD patients was observed (Spanos et al., 2018), and several studies suggest a functional role of GLO1 and dicarbonyl stress in obesity (Rabbani et al., 2016a). Hollenbach et al. studied GLO1 in a CCl4-model of cirrhosis and determined that GLO1 expression and protein was reduced in early and advanced cirrhosis in both, whole liver and primary liver cell cultures. This was indeed accompanied with significantly elevated levels of MGO and RAGE expression (Hollenbach, 2017). These results suggest that that a loss of GLO1 combined with increased MGO formation may indeed play an important role in T2DM and obesity mediated liver complications.

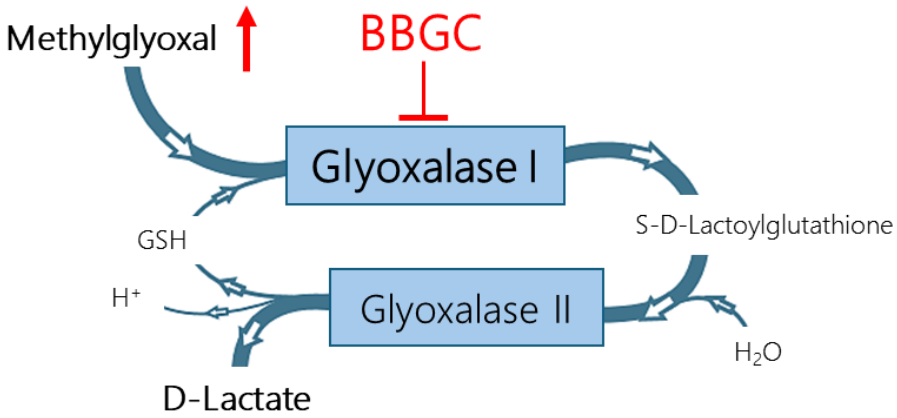


Figure 3.8. Schematic representation of S-p-bromobenzylglutathione (BBG) mode of action. Once BBGC enters the cell, the ester groups are hydrolysed by cellular non-specific esterases to form the active GLO1 inhibitor BBG. GLO1 inhibition by BBGC hinders the detoxification of MGO, resulting in increased intracellular MGO accumulation.

Rabbani et al predict that selective increase in intracellular MGO levels in response to inhibition of GLO1 is favoured by a higher glycolytic rate and can reach over 12-fold for ca. 24 h of the cell growth cycle (Rabbani et al., 2018) which as previously discussed, has been proven in studies using BBGC (Dobler et al., 2006; Thornalley et al., 1996; Tikellis et al., 2014). AGEs derived from MGO include hydroimidazolone (MG-H1), carboxyethyl lysine (CEL), and methylglyoxal lysine dimer (MOLD) (Allaman et al., 2015a; J. Chaudhuri et al., 2016; J. H. Chen et al., 2018; Vistoli et al., 2013). MG-H1 is the predominant adduct and is commonly used to assess dicarbonyl stress accumulation (Lai et al., 2022). Through pharmacological inhibition of GLO1 by BBGC in HepG2 cells, MGO accumulation is observed after 24 hours and further increases with time, at 48 hours and 72 hours respectively, as demonstrated by the visible accumulation of MG-H1 adduct formation. The increase in MGO formation is dependent on the metabolic activity of the hepatocyte and specifically its glycolytic rate, so it is possible to increase MGO accumulation in our experiments by incubating the cells in high glucose condition, which could potentially increase the damage observed. Bovine aortic endothelial cells incubated with high glucose and with BBGC revealed that mitochondrial MGO concentration increases approximately threefold under hyperglycaemia (Pun et al., 2014). In mouse aortic endothelial cells, treatment with BBGC mimicked MGO treatment in the inhibition of the IRS1/Akt/eNOS pathway (Nigro et al., 2014). *In vivo*, the use of BBGC, increased vascular adhesion and inflammation to a similar extent as MGO or high glucose treatments, in the absence of hyperglycaemia (Tikellis et al., 2014). *In vitro*, pharmacological inhibition of GLO1 by BBGC increases intracellular concentrations of MGO and induces apoptosis in endothelial cells and tumour cells (Sakamoto et al., 2001; Thornalley et al., 1996).

In order to further verify that the effects on cell viability were mediated by MGO, we aimed to reverse them by using MGO scavengers, which appears to be most successful when the cells are pre-treated with Aminoguanidine. The pre-treatment with either scavenger ensures its infiltration into the cell in sufficient amounts to be able to effectively lower readily reactive intracellular MGO. This could be explained by the mechanism of action of BBGC, as it inhibits GLO1, MGO starts accumulating in the cell as soon as BBGC starts acting upon its target, which is mainly located in the cytoplasm. The scavengers thus may enter the cell at a slower rate than BBGC, and MGO accumulation may reach toxic levels at an earlier time point than the onset of action of the scavengers. This may be one reason why improved effectiveness is obtained when the cells are preloaded with the scavengers prior to adding BBGC. Although

both compounds meet the criteria set by Lipinski's rule of five, suggesting suitable permeability, their believed mechanisms show some discrepancies.

Aminoguanidine is considered a potent scavenger of MGO owing to its dicarbonyl-directing guanidine group (Brings et al., 2017; Brownlee et al., 1986; Lo et al., 1994). Aminoguanidine was first introduced as a nucleophilic hydrazine compound and is able to directly trap reactive carbonyls formed during the Maillard reaction, especially Amadori carbonyl groups of glycated proteins, thus limiting AGE formation. Aminoguanidine also reacts with dicarbonyl compounds such as MGO, glyoxal, and 3-deoxyglucosone (Yamagishi, 2013). In animal models of diabetes, aminoguanidine effectively reduces the formation of AGEs and prevents various diabetic complications such as nephropathy (Soulis-Liparota et al., 1991; Soulis et al., 1996), retinopathy (Hammes et al., 1991), and neuropathy (Kihara et al., 1991). Studies in humans however have been terminated due to an unfavourable perceived risk-benefit ratio. Two large clinical trials in individuals with T1DM and T2DM showed a lack of efficacy and safety concerns as it led to various serious side effects (Schalkwijk & Stehouwer, 2020). Alagebrium on the other hand was developed as an AGE cross-link breaker thiazolium compound (Vasan et al., 2003; Vasan et al., 1996). Studies support that alagebrium is effective against the effects of MGO, as it reduces α-dicarbonyl compounds (Kim & Spiegel, 2013). Is has been considered to breakdown covalent bonds formed in cross-linked proteins through chemical cleavage of α-dicarbonyl carbon–carbon bonds, thereby restoring protein structure and function, but its mechanism of action remains controversial (Toprak & Yigitaslan, 2019). Although alagebrium has been developed as an AGE cross-link breaker, to date, no evidence of its ability to cleave advanced Maillard reaction cross-links has been uncovered (Schalkwijk & Stehouwer, 2020). Rather, it has been suggested that alagebrium inhibits the formation of AGEs and its likely underlying mechanisms include antioxidant activity, an ability to inhibit autooxidation of ascorbate and glucose, and ability to promote the metabolism of triosephosphates, thereby minimising the generation of MGO (Sell & Monnier, 2012; Toprak & Yigitaslan, 2019). This may explain why Alagebrium was less effective in protecting the cells against MGO, as the method used to increase its levels involved reduced MGO detoxification rather than increased generation, and aminoguanidine's ability to directly "trap" reactive carbonyls (MGO) rather than being a cross-link breaker may also explain its increased efficacy.

Altogether, these pieces of evidence help support that BBGC increases intracellular MGO levels in this line of work. We were thereby able to show for the first time that the inhibition

of GLO1 increases dicarbonyl stress in hepatocytes in a more physiological manner, which enables the further investigation of the mechanisms underlying MGO-induced hepatocyte toxicity. This would ultimately help understand the pathways that mediate the progression of liver disease in T2DM patients and explain the increased incidence and severity of liver complications in this population.

4. Mechanisms underlying Methylglyoxal-induced cellular dysfunction in HepG2 cells

Introduction

MGO has been identified as a key mediator in various diabetic complications. Previous studies investigating the effects of direct treatment with MGO have shown that MGO has the ability to increase oxidative stress, cause mitochondrial dysfunction, and ER stress, activate stress-activated signalling pathways, and induce inflammation via NF- κB activation in many cell types including HepG2 cells (S. H. Cha et al., 2019; C. M. Chan et al., 2016; Seo, Ki, et al., 2014).

MGO is mainly degraded by the glyoxalase system; in which GLO1 is the rate-limiting enzyme and in the previous chapter, we have confirmed that inhibiting GLO1 with BBGC treatment increases endogenous MGO production in hepatocytes in a more physiological manner. Several studies have confirmed its suitability as a model of pharmacologically induced dicarbonyl stress (Rabbani & Thornalley, 2022) and that it mimics the effects of MGO *in vitro* (Dobler et al., 2006; Nigro et al., 2014; Pun et al., 2014; Sakamoto et al., 2001; Thornalley et al., 1996) and *in vivo*, using rodent models (Tikellis et al., 2014).

BBGC treatment reduced cell viability, increased ROS generation, IL-8 secretion and increased apoptosis in HepG2 cells, mimicking the results seen with direct MGO exposure. Therefore, as this is a sign of MGO toxicity, the aims of this chapter are to expand on this model and identify the mechanisms underlying the damaging effects of dicarbonyl stress in hepatocytes.

Experimental protocol and methods

Experimental protocol

In a first series of experiments, HepG2 cells were exposed to GLO1 inhibitor BBGC (1 µM to 30 µM) in RPMI-1640 medium supplemented with 3% heat-inactivated FCS, and 1% penicillin-streptomycin, and incubated for 24 hours prior to assessing mitochondrial function via measuring mitochondrial membrane potential, mitochondrial superoxide generation and finally, cellular ATP levels.

In a second series of experiments, HepG2 cells were treated with BBGC (20 µM) for 24 hours, before measuring endoplasmic reticulum stress markers CHOP and Bip/GRP78 mRNA expression and CHOP protein levels.

Lastly, HepG2 cells were treated with BBGC (20 µM) for 24 hours, to measure any changes in angiotensin converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) mRNA expression in response to dicarbonyl stress.

Assessment of mitochondrial membrane potential by flow cytometric analysis using tetramethylrhodamine ethyl ester perchlorate (TMRE) probe

HepG2 cells were exposed to BBGC (1 µM to 30 µM) for 24 hours. After treatment protocol, A 50 nM TMRE working solution was freshly by diluting TMRE stock solution in prewarmed PBS (37°C). After treatment protocol, cells were washed twice with 3 mL of prewarmed (37°C) PBS. Then the cells were harvested in using prewarmed (37°C) trypsin-EDTA (0.05%). Cells were then divided into two to account for the unstained vs TMRE stained cells for each treatment.

Cells were then centrifuged for 5 minutes at 300 × g. After centrifugation, the supernatant was removed then cells were stained in 0.5 mL of 50 nM TMRE working solution. Cells were then incubated for 20 minutes at 37°C in the incubator, in the dark. For a positive control, the cells were treated with 150 µM carbonyl cyanide m-chlorophenyl hydrazine (CCCP) during the staining period. CCCP is a mitochondrial oxidative phosphorylation uncoupler and has been used in many studies as a reliable way of evaluating the loss in MMP (Cottet-Rouselle et al., 2011).

Then, the supernatant was removed and both stained and unstained cells were resuspended in 0.5 mL of prewarmed (37°C) PBS. Cell concentration was always adjusted by dilution to $2.0x10^5$ cells per 0.5 mL for all samples. The HepG2 cells were then immediately analysed using a flow cytometer BD C6 Flow Cytometer (BD Biosciences; Becton-Dickinson Co., Franklin Lakes, NJ, USA) and 10,000 events were collected for each sample.

Assessment of mitochondrial superoxide generation by immunocytochemistry analysis using MitoSOX probe

HepG2 cells were exposed to BBGC (3 µM to 20 µM) for 24 hours. A 5 mM stock solution of MitoSOX reagent (MW = 759 g/mol) was prepared by adding 13 µL of DMSO to 50 µg of MitoSOX Red reagent. The stock solution was stored at -20ºC. Recommendations for experimental protocols provided by the manufacturer were used as a starting point, and optimal labelling conditions were determined empirically.

Following cell growth and treatment protocol, the cells were washed twice with 3 mL of PBS per well (37ºC) and stained with 1 mL of MitoSOX working solution which was prepared by diluting the MitoSOX stock solution (in DMSO) in PBS (37ºC) for 30 minutes at 37ºC. An unstained control (PBS only) was also included for each experiment as well. When the incubation was complete, the cells were washed twice with PBS and the staining solution was replaced with PBS for the imaging process.

Superoxide generation was observed and photographed using an inverted confocal laser scanning microscope (Leica TCS SP5 Confocal Laser Scanning Microscope, Germany) and the accompanying Confocal LAS AF software. 4 to 6 random regions of the well were imaged for each sample. The images were analysed using the ImageJ software (Maryland, USA) in order to obtain mean fluorescence intensity to quantify superoxide generation. Fluorescence compensation (to avoid autofluorescence measurements) was performed by subtracting the MFI of the MitoSOX-stained cells by the MFI of unlabelled cells.

RESULTS

Measurement of the mitochondrial membrane potential changes following GLO-1 inhibition

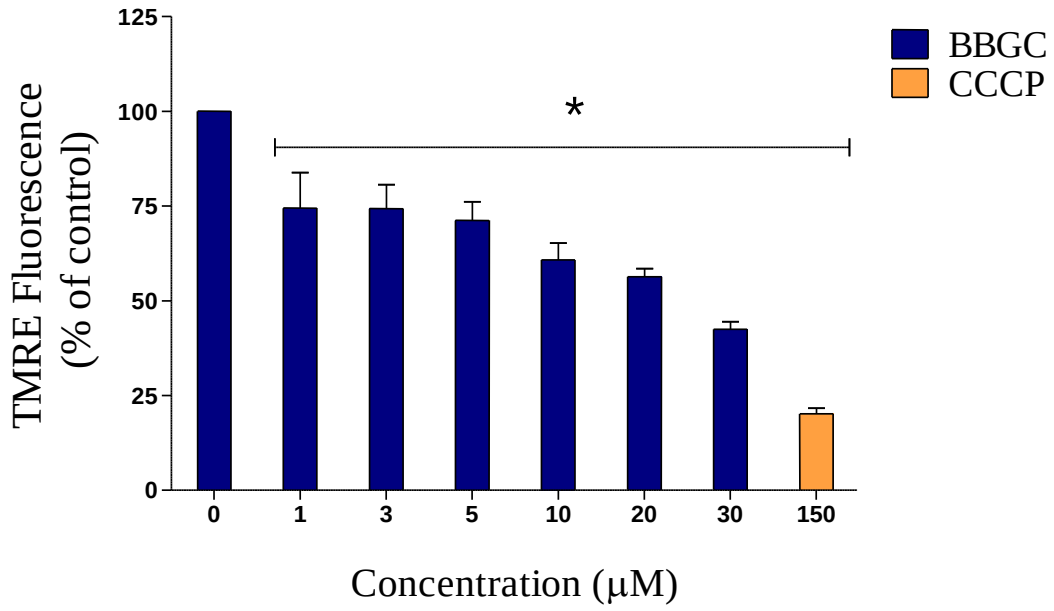


Figure 4.1. The effects of a 24-hour exposure to BBGC on mitochondrial membrane potential in HepG2 cells. TMRE fluorescence intensity is proportional to MMP. A positive control of HepG2 cells treated with 150 μM carbonyl cyanide m-chlorophenyl hydrazine (CCCP) was used to dissipate MMP. The 24-hour treatment has significantly reduced MMP. Data is expressed as the mean ± SEM from n = 6 experiments, (1 replicate per experiment). Statistically significant differences were determined using Kruskal-Wallis with Dunn's post hoc test. * $P < 0.05$, significantly different from vehicle-treated control groups.

In order to identify the potential sources of increased ROS generation that are observed (Figure 3.3), it is imperative to assess mitochondrial function. We found that BBGC mediated dicarbonyl stress significantly reduces MMP, in a dose dependent manner in HepG2 cells. Reduced MMP has long been recognized as an important mechanism instigating endogenous oxidative stress and reflects reduced mitochondrial respiration and hence mitochondrial dysfunction.

Measurement of mitochondrial superoxide generation following GLO-1 inhibition

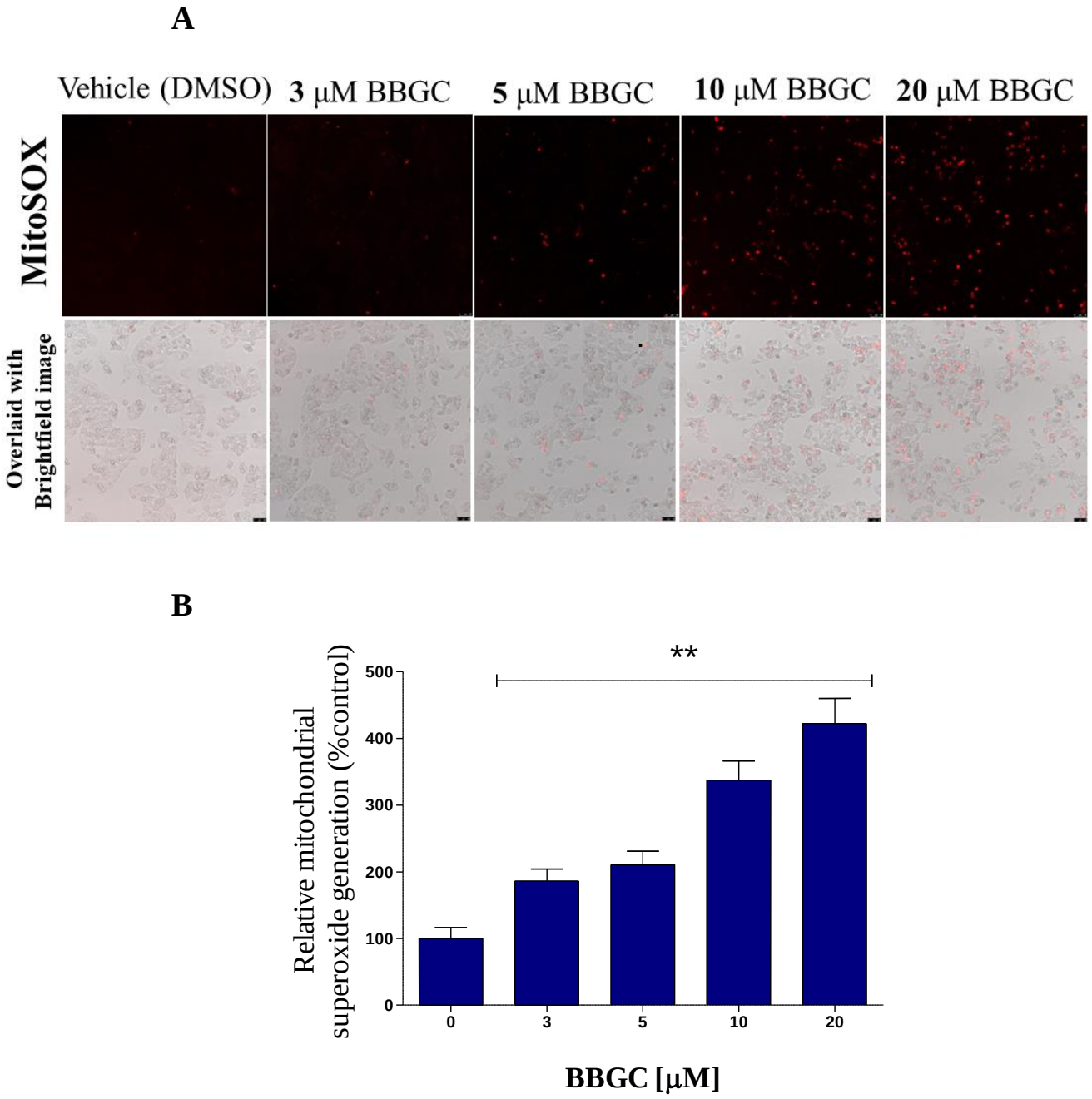


Figure 4.2. Mitochondrial superoxide formation in HepG2 cells exposed to BBGC for 24 hours. (A) Confocal images of mitochondrial superoxide levels following BBGC treatment. The pink fluorescence represents MitoSox. A higher fluorescence signifies higher levels of superoxide. (B) Quantification graphs of mitochondrial superoxide levels in HepG2 cells following BBGC treatment determined by MitoSox mean fluorescence intensity using the image processing software. Data are expressed as mean ± SEM from n = 5 independent with 1 replicate per experiment. Statistically significant differences were determined using Kruskal-Wallis with Dunn's post hoc test. ** $P < 0.01$, significantly different from vehicle-treated control groups.

As BBGC treatment leads to mitochondrial dysfunction, we wanted to confirm whether mitochondrial ROS is increased, specifically, as a result of the treatment. Indeed, GLO1

inhibition leading to intracellular MGO accumulation leads to mitochondrial dysfunction coupled with significantly increased mitochondrial superoxide generation, in a dose dependent manner (Figure 4.2). After 24 hours, 20 µM BBGC causes approximately a 4-fold increase in mitochondrial ROS.

Measurement of cellular ATP levels in HepG2 cells as a result of increased dicarbonyl stress

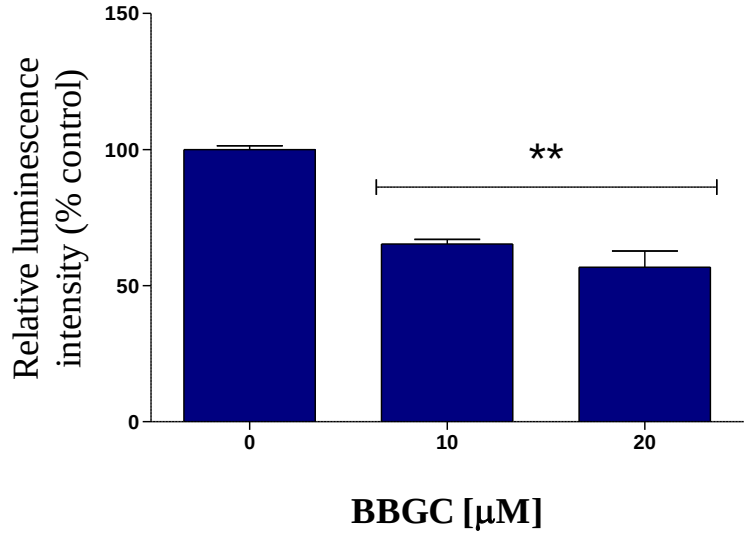


Figure 4.3. BBGC treatment decreases cellular ATP levels in HepG2 cells. HepG2 cells were exposed to BBGC for 24 hours before quantifying cellular ATP levels using the luciferase reaction followed by luminometry. Data are expressed as mean ± SEM from $n = 4$ independent experiments. Statistically significant differences were determined using Kruskal-Wallis with Dunn's post hoc test where $p < 0.01$ was considered as significant. ** $p < 0.01$, significantly different from vehicle-treated control cells.

In addition to oxidative stress, impairment to the mitochondria inevitably leads to decreased energy production and cellular dysfunction. Therefore, we measured cellular ATP levels following GLO1 inhibition after 24 hours. Accordingly, BBGC mediated dicarbonyl stress significantly reduces cellular ATP levels which would exacerbate hepatocyte dysfunction and favour cell demise (Figure 4.3).

Changes in endoplasmic reticulum stress markers CHOP and Bip/GRP78 mRNA expression and CHOP protein levels following GLO-1 inhibition

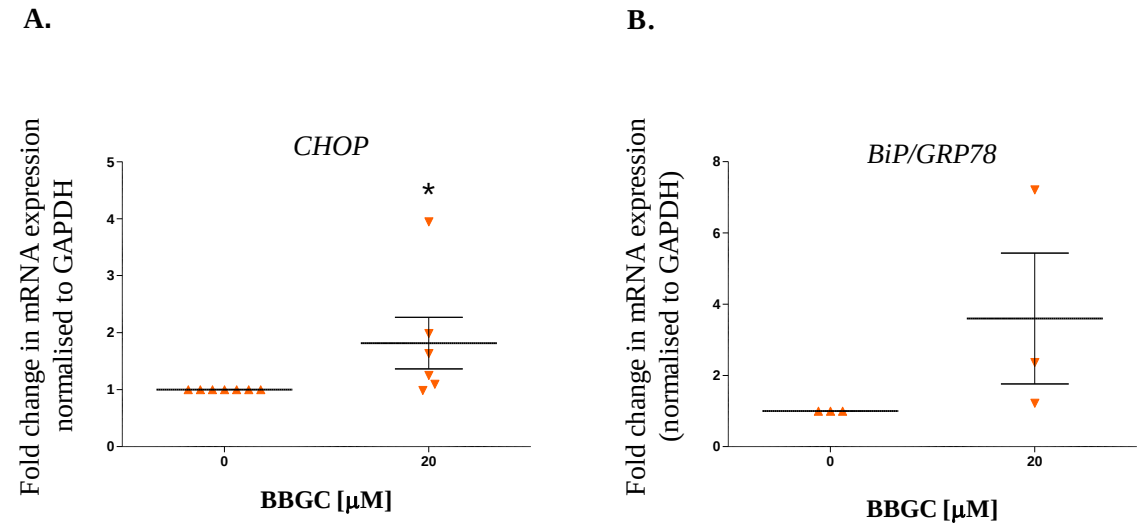


Figure 4.4. Changes in CHOP and Bip/GRP78 mRNA expression in HepG2 cells following 24-hour exposure to BBGC. mRNA expression of CHOP and Bip/GRP78 was assessed by RT-qPCR and normalised to GAPDH following a 24-hour treatment with BBGC 20 µM Data are expressed as $2^{(-\Delta\Delta Ct)}$ ± SEM *n* = 6 for CHOP, and *n*= 3 for Bip/GRP78, independent experiments. Statistically significant differences were determined using Kruskal-Wallis with Dunn's post hoc test. * P < 0.05 as compared to vehicle-treated control group.

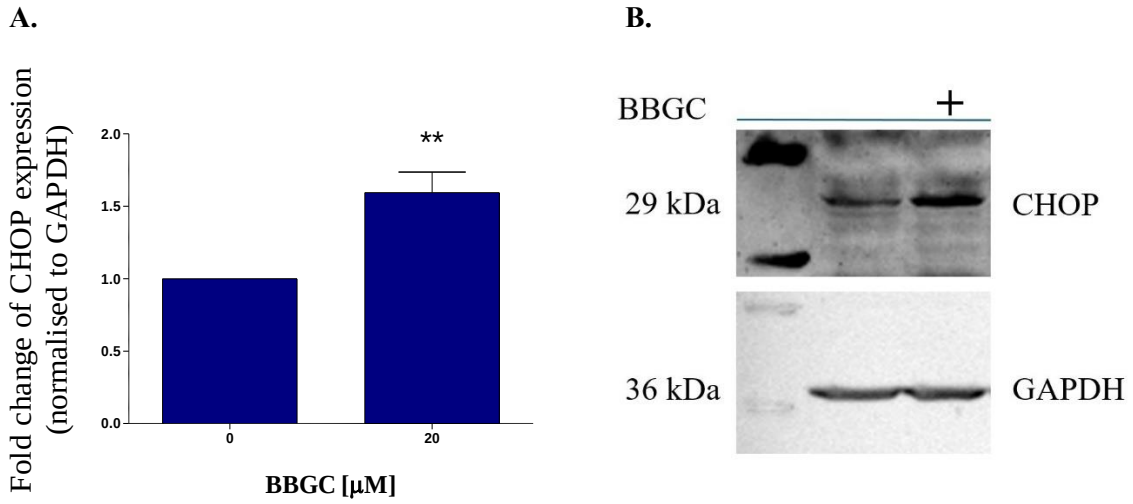


Figure 4.5 Measurement of CHOP protein expression in HepG2 cells following 24-hour exposure to BBGC. B. Representative western blot of CHOP protein expression from one independent experiment. Cell lysates were prepared following a 24-hour treatment with BBGC 20 µM. 80µg of lysates was used to conduct a Western blot analysis using antibodies against CHOP and GAPDH. A. The relative protein level was expressed as CHOP/GAPDH arbitrary units. Data are expressed as mean ± SEM *n* = 3 independent experiments differences were determined using Kruskal-Wallis with Dunn's post hoc test. ** P < 0.05 as compared to vehicle-treated control group.

ER stress is an indicator of cellular dysfunction in response to both glycative and oxidative stress. As MGO accumulation was shown to cause protein glycation (Figure 3.5), increased

ROS (Figure 3.3), mitochondrial dysfunction (Figure 4.1) and apoptosis (Figure 3.4), it is important to measure the expression key ER stress markers CHOP and chaperone protein Bip/GRP78. BBGC mediated dicarbonyl stress causes a modest but significant increase in CHOP mRNA (Figure 4.4.A) and protein expression (Figure 4.5), indicating the activation of ER stress pathways and apoptotic signalling in HepG2 cells. Although it has not reached significance, there is a trend in the increase of Bip/GRP78 mRNA expression, suggesting a possible increase in the recruitment of ER chaperone proteins in response to cell damage (Figure 4.4.B).

Measurement of angiotensin converting enzyme 2 (ACE2) and transmembrane protease serine 2 (TMPRSS2) mRNA expression in response to dicarbonyl stress

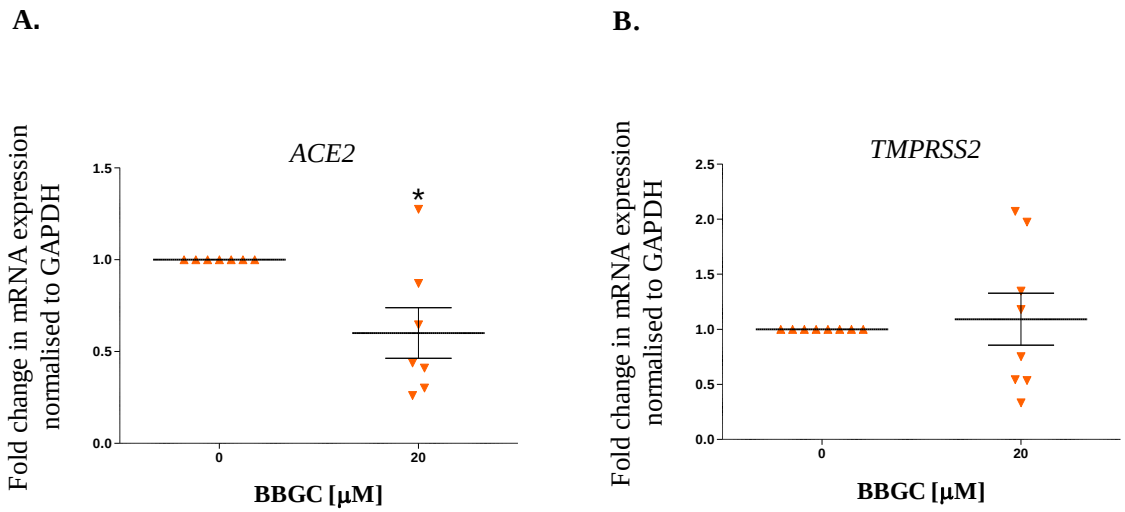


Figure 4.6. 24-hour exposure to BBGC decreases ACE2 mRNA expression in HepG2 cells. mRNA expression of ACE2 (A) and TMPRSS2 (B) was assessed by RT-qPCR and normalised to GAPDH following a 24-hour treatment with BBGC 20 μM. Data are expressed as $2^{(-\Delta\Delta Ct)}$ ± SEM n = 7 for ACE2, and n= 7 for TMPRSS2, independent experiments. Statistically significant differences were determined using Kruskal-Wallis with Dunn's post hoc test. * $P < 0.05$ as compared to vehicle-treated control group.

During the COVID-19 pandemic, we thought it would be interesting to examine whether dicarbonyl stress increases the expression of key SARS-CoV-2 host cell factors for viral entry and pathogenesis in HepG2 cells. BBGC mediated dicarbonyl stress had variable but overall, no effect on TMPRSS2 mRNA expression (Figure 4.6.B), and a significant decrease in ACE2 mRNA expression (Figure 4.6.A).

Discussion

Our result show that BBGC mediated dicarbonyl stress leads significant damaging effects of the mitochondria through the loss mitochondrial membrane potential, increased mitochondrial superoxide generation, and a decrease in cellular ATP levels. The results also indicate that MGO accumulation as a result of GLO1 inhibition leads to ER stress, reflected by the increased CHOP and Bip/GRP78 expression. MGO has previously been linked to mitochondrial dysfunction and ER stress (C. M. Chan et al., 2016; Seo, Ki, et al., 2014), and this may explain the increase in oxidative stress and apoptosis observed in the previous chapter.

Mitochondria are believed to play an important role in pathologies that are linked to glycative stress, such as DM (Brownlee, 2001). As mitochondria play a pivotal role in liver cell function and maintenance, their damage inevitably leads to liver dysfunction (Wang et al., 2020). Numerous NAFLD models also show increases in mitochondrial ROS generation, which has been suggested to be a major factor in the onset and progression of the illness (Morris et al., 2011). Mitochondrial dysfunction in hepatocytes results in low fatty acid oxidation, decreased hepatic ATP levels, and increased hepatic oxidative stress (Muriel et al., 2021; Smith & Adams, 2011b; Softic et al., 2019; Softic et al., 2020). Even under normal physiological conditions, the mitochondria is an important source of cellular ROS, which are mainly generated by the leaking of electrons from complex I and III of the electron transport chain, resulting in the reduction of molecular oxygen to superoxide (Nohl et al., 2005). Here we observe an MGO-induced decline in mitochondrial respiration, paired to increased mitochondrial superoxide generation in HepG2 cells, as well as increased intracellular ROS levels. Many studies suggest that this phenomenon is a major source of oxidative stress in diabetes (Schalkwijk & Stehouwer, 2020) and this effect on the mitochondria may be of great importance to the dysfunction of hepatocytes and the rise in cellular oxidative stress.

Endothelial cells treated with MGO leads to an induction of oxidative stress by stimulating superoxide production, (Chan & Wu, 2008; Miyazawa et al., 2010; Nagaraj et al., 2005)**,** similar to our findings. Nevertheless, substantial evidence exists to implicate MGO in mitochondrial toxicity (Rabbani & Thornalley, 2008; Rosca et al., 2002; Rosca et al., 2005). A considerable number of studies suggest that this may be a result of that direct intracellular protein glycation by MGO (Rosca et al., 2002; Rosca et al., 2005). A two- to three-fold increase in oxidative stress has been associated with small increases in MG-H1 alterations of mitochondrial proteins,

most likely due to electron leakage from the electron transport chain complex III (Brouwers et al., 2011; Schalkwijk & Stehouwer, 2020). More recently, isolated rat liver mitochondria incubated with 0.1–10 mM MGO found that MGO decreased MMP and mitochondrial viability and hindered complex II activity (5 mM and 10 mM). In this model, MGO also inhibited complex I oxidative phosphorylation, LEAK respiration, and the electron transport chain (Prestes et al., 2022). Our findings further corroborate a previous report where MGO causes increased ROS generation in HepG2 cells, disruption of mitochondrial function and increased MPTP formation (Seo, Ki, et al., 2014), suggesting it plays a crucial role in methylglyoxal induced apoptosis (He et al., 2020). The prevention of MGO-induced apoptosis by cyclosporin A further support the hypothesis that the toxic effects of MGO might be a direct result of mitochondrial impairment.

In a recent study by Sekar et al., the effects of metformin and the AMPK activator A769662 on MGO-induced diabetic retinopathy in mice was compared, as well as evaluated cytotoxicity and mitochondrial function in MGO-treated ARPE-19 cells. Both metformin and A769662 were able to reverse MGO-induced mitochondrial superoxide generation, but not the MMP loss, in ARPE-19 cells in an AMPK-dependent manner as evidenced by the loss of this effect after AMPK inhibition or silencing (Sekar et al., 2023). Although these effects of MGO can be reversed by both, the authors suggest that the reduction of mitochondrial ROS production rather than restoration of cytosolic ROS level contributes to the protective effects of metformin and A769662.

Moreover, thehe activation of AMPK also exerts long-term effects at the level of both gene expression and protein synthesis and activates mitochondrial biogenesis (Reznick & Shulman, 2006). They were able to confirm that MGO inhibits AMPK activity and leads to mitochondrial fission, inhibition of mitochondrial biogenesis and autophagy. In addition, they reported an AMPK-dependent upregulation of GLO1 expression via AMPK-Nrf2-ARE axis, consistent with previous findings (Matzinger et al., 2020; Xue et al., 2012). This may have been corroborated in human studies, where they observed T2DM patients receiving metformin treatment showed decreased plasma MGO levels and increased GLO1 activity in peripheral mononuclear cells, red blood cells and atherosclerotic lesions (Peters et al., 2018). Previous studies describe metformin is a MGO scavenger (Kinsky et al., 2016), but Sekar et al. suggest the results of their study show that the beneficial action of metformin against MGO toxicity is mainly through AMPK activation, especially given that metformin applied up to 4 h after-MGO

treatment still exerts similar protection as during co-treatment (Sekar et al., 2023). Moreover, in human neuroblastoma cells, emodin, a cytoprotective agent, was able to protect against MGO-induced mitochondrial dysfunction, and this protection was abolished after inhibiting AMPK or the silencing of the Nrf2 or heme-oxygenase-1 (HO-1) (de Oliveira et al., 2021). Therefore, these studies further emphasised the importance of AMPK/Nrf2/ signalling and suggests the inhibition of AMPK by MGO may constitute a substantial mechanism in its cytotoxic effects.

The collapse of the mitochondrial transmembrane potential and reduced mitochondrial respiration results in reduced ATP production, further compromising mitochondrial and cellular stability and function (Dornadula et al., 2015; Li et al., 2018; Rosca et al., 2005; Shamsaldeen et al., 2016). Indeed, mitochondrial integrity is essential for cell survival, and decreased MMP is an early indicator of apoptosis (He et al., 2020). Increased oxidative stress also predisposes mitochondrial DNA to attacks by free radicals (Muriel et al., 2021) which can also negatively impact mitochondrial biogenesis (Cioffi et al., 2017). Alterations in mitochondrial integrity may also explain the decline in cell viability that is observed in the previous chapter. We also observe in our study decreased ATP production in HepG2 cells in response to MGO, which is clinically relevant as patients with T2DM also exhibit reductions in hepatocellular ATP concentrations and ATP synthase flux (Kumar et al., 2021; Schmid et al., 2011; Szendroedi et al., 2009; Tilg et al., 2017), which was associated with both peripheral and hepatic IR (Schmid et al., 2011).

Moreover, although we did not measure this, MGO reportedly inhibits cellular antioxidant systems, and depletes GSH content in many cell types, including HepG2 cells (Angeloni et al., 2014; Seo et al., 2014) and in the liver of MGO-treated murine models (Choudhary et al., 1997). Importantly, the initial essential step of MGO detoxification is the GLO1 mediated formation of hemithioacetal, which is formed by a spontaneous reaction of MGO and reduced GSH, which makes GLO1 activity highly dependent on intracellular GSH availability/content (He et al., 2020). Alterations of the activities of the enzymes of the GSH synthesis pathways are linked to impaired hepatic lipid metabolism and the development of steatosis and insulin resistance (Nascè et al., 2022) and glutathione depletion is thought to play an important role in NAFLD development (Santacroce et al., 2023) and therefore, the oxidative or non-oxidative depletion of reduced GSH and subsequent inadequate functioning of the glyoxalase system

may further aggravate dicarbonyl and oxidative damage, as evidenced in a study where GSH depletion in rats induced the accumulation of MGO in the liver (Masterjohn et al., 2013).

These detrimental effects altogether promote the onset of hepatic steatosis and aggravate IR, (Kumar et al., 2021; Matthew Morris et al., 2013; Mohamed et al., 2016; Smith & Adams, 2011b), increase hepatic oxidative stress , chronic inflammation, ATP depletion and cellular death via the activation of caspase-mediated apoptotic pathways (Muriel et al., 2021; Nascè et al., 2022; Softic et al., 2019; Softic et al., 2020). This may possibly be exacerbated by increased DNA single strand breaks damage and PARP activation (Dornadula et al., 2015) as a result of oxidative stress or MGO glycation. PARP reduces levels of cellular NAD+ and ATP and triggers mitochondrial apoptotic pathways. Inhibition of PARP in endothelial cells protected against the methylglyoxal-mediated cell dysfunction and apoptosis (Bates & Mabley, 2010).

Indeed, several studies have identified ROS mediated damage as a major factor in apoptosis induction, an observation that was confirmed by antioxidant treatment (Daniel et al., 2013). More importantly, Seo et al. have shown that antioxidant treatment prevents MGO-induced apoptosis in HepG2 cells, as well as cyclosporin A, an inhibitor of MPTP formation (Seo, Ki, et al., 2014). Excessive cellular ROS can lead to damage of the mitochondrial inner membrane compounds, leading to mitochondrial fission, and increased apoptosis rates in several cell types (Qian et al., 2019; Wu et al., 2011; Richard J Youle & Alexander M Van Der Bliek, 2012). In hepatocytes, mitochondrial damage, including mitochondrial respiratory chain injury and abnormal mitochondrial fusion, due to altered structure and function, is observed in a variety of chronic liver diseases, including hepatic steatosis, non-alcoholic fatty liver disease, and viral hepatitis (Fletcher et al., 2019; Ogrodnik et al., 2017). Studies also report marked apoptosis in the diabetic liver, and the hydroxyl radical is believed to play a part in the activation of the Bax-caspase pathway which sheds light on the mechanisms underlying hyperglycaemia-induced liver cell apoptosis (Daniel et al., 2013). As MGO reportedly collapses the mitochondrial transmembrane potential and induces MPTP formation (Dornadula et al., 2015; Du et al., 2003; Seo, Ki, & Shin, 2014), it may lead to the abnormal opening of mitochondrial transition pore and thereby the rapid release of cytochrome c from the mitochondrial membrane into the cytoplasm, which induces apoptosis by forming complexes with Apaf-1, activating procaspase 9, and subsequently, downstream effector caspases 3, 6 and 7 (Dornadula et al., 2015; Du et al., 2003)(Daniel et al., 2013), which constitutes the mitochondrial apoptotic

pathway. Therefore, the damaging effects of MGO on the mitochondria of HepG2 cells may play an important role in the increased apoptosis that is observed.

In addition to damaging mitochondrial proteins and causing mitochondrial dysfunction, MGO can also lead to oxidative stress by damaging other proteins and cellular structures, which as discussed in chapter 1, can elicit the activation stress-signalling pathways and cause ER stress, both of which are associated with an increased production of ROS, which through further oxidative-damage cellular structures, initiate a vicious cycle (Hollenbach, 2017; Nigro et al., 2019). Chan et al. have proven that inhibiting ROS generation or ER-stress can attenuate MGO-induced ROS production and cell death in human retinal pigment epithelial cells (C. M. Chan et al., 2016). Notably, we now know that at least in liver, dysfunction of the ER and mitochondria are linked phenomena (Wellen & Hotamisligil, 2005). As discussed in chapter 1, protein damage and the accumulation of damaged and misfolded proteins leads to ER stress, which when remains unresolved and persistent, triggers apoptosis. One of the key components of metabolic health and apoptotic pathways involves organelle homeostasis, and the role of the ER in MGO-induced cytotoxicity cannot be overlooked. To further understand the increased rates of apoptosis rates in HepG2 cells in response to MGO, we decided to look at important ER stress markers CHOP and BiP/GRP78.

The inhibition of GLO1 in HepG2 cells in our study increased BiP/GRP78 and CHOP mRNA expression, and we were able to measure increased CHOP protein levels, indicating UPR activation and ER stress. BiP/GRP78 is increased in human aortal endothelial cells (HAECs) under high glucose conditions in vitro, which was coupled with increased intracellular MGO and MGO-modified proteins. Elements of the UPR ATF6, PERK, and IRE1α were also activated, and the silencing of GLO1 in these cells, similarly increased intracellular MGO and activated the UPR. An increased abundance of HSPs and increased heat shock factor 1 expression was also observed likely reflecting MGO-induced proteotoxic stress (Irshad et al., 2019). As previously mentioned, CHOP plays an important role in ER stress-induced apoptosis through a variety of pathways, including the mitochondrial apoptotic pathway.

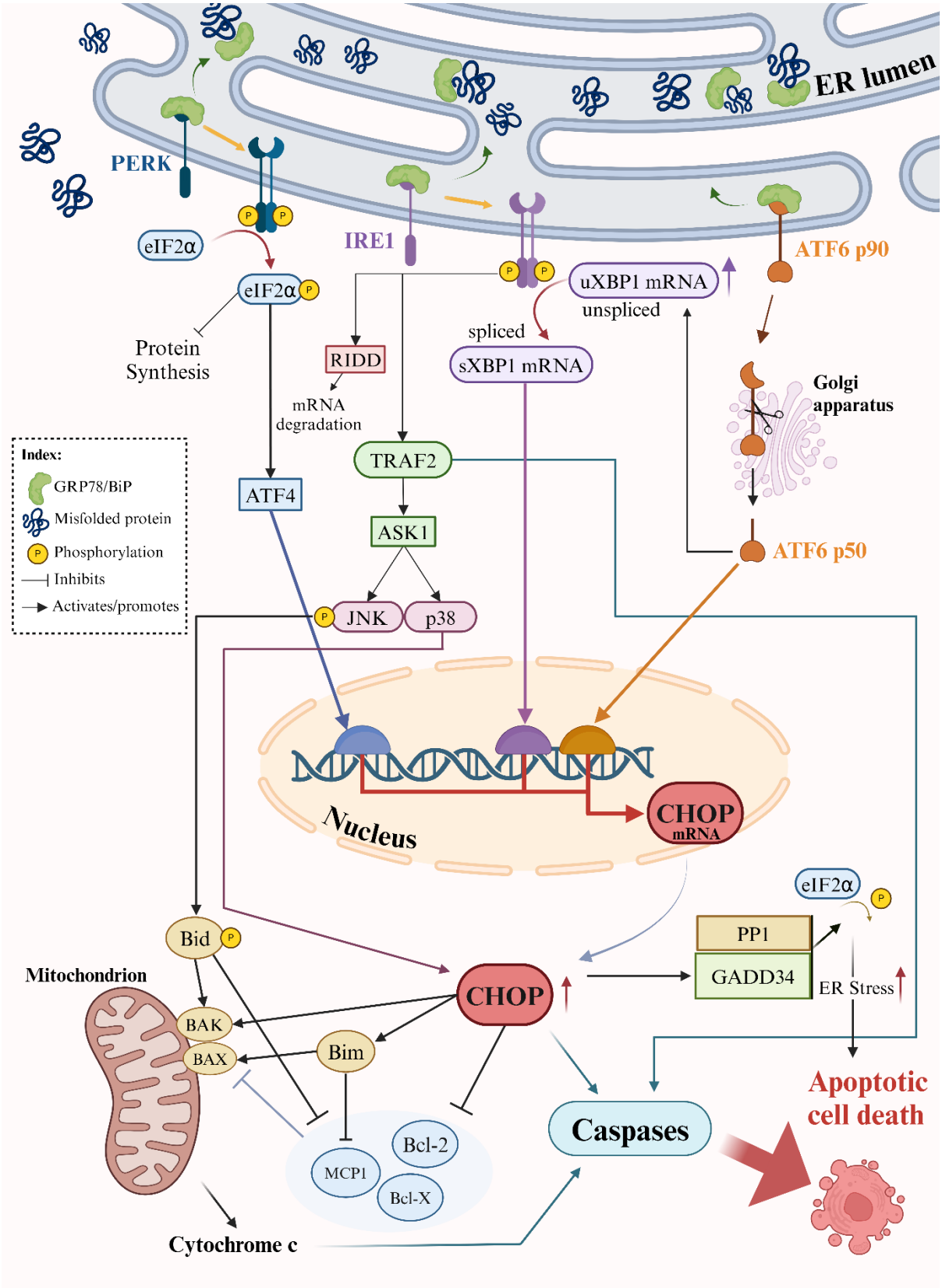


Figure 4.7. Cellular pathways engaged in CHOP-induced apoptosis. BiP/GRP78, an ER chaperone, inhibits the three sensors of the UPR, IRE1, PERK, and ATF6 under normal physiological conditions. Upon ER stress or misfolded protein accumulation, GRP78 dissociates from all UPR transducers, subsequently activating their respective downstream signalling. The activation of PERK increases the phosphorylation of the alpha subunit of

the translation protein eIF2, which attenuates protein translation to reduce ER protein overload, while upregulating ATF4 mRNA, which targets the activation of proapoptotic CHOP and other UPR target genes. IRE1 undergoes homodimerization and autophosphorylation, and its activation mediates the activation of its RNase domain to produce spliced XBP1 mRNA, which is the active isoform of XBP1 that is translocated to the nucleus to increase CHOP transcription. The RNase domain of IRE1 also regulates the RIDD (regulated IRE1-dependent decay) pathway, where IRE1 degrades ER membrane-localized mRNAs through its RNase activity. Similarly, during ER stress, the cytosolic domain of IRE1 interacts with TNF Receptor Associated Factor 2 (TRAF2) and activates the downstream kinase ASK1, enhancing apoptotic JNK and p38 MAPK signalling. ATF6, is translocated to the Golgi apparatus, where it undergoes cleavage by site-1 and site-2 proteases to yield ATF6 p50. The cleaved ATF6 p50 is then translocated to the nucleus and activate the transcription of target genes of ATF6, including CHOP and XBP1. In case of severe and sustained ER stress, proapoptotic signalling becomes dominant and leads to apoptotic cell death. CHOP triggers the intrinsic apoptotic pathway through the inhibition of BCL-2, BCL-XL, MCL-1, and the upregulation of BIM, which regulates BAX-BAK-mediated mitochondrial outer membrane permeabilization. This leads to cytochrome c release and the caspase cascade Thus, CHOP-mediated death signal is transmitted to the mitochondria, which functions as an integrator and amplifier of the death pathway. CHOP also stimulates expression of cell surface death receptor DR4 and DR5, which sensitizes cells to pro-apoptotic stimuli, presumably via the extrinsic apoptotic pathway involving caspase 12 and caspase 8. Association of TRAF2 with activated IRE1 also leads to activation of caspase 12. CHOP can directly activate GADD34 (DNA damage protein), which, combined with phosphatase 1 protein (PP1), dephosphorylates eIF2α, and results in protein translation recovery, increased ER stress, and cell apoptosis (Hu et al., 2018; Oyadomari & Mori, 2004; Sano & Reed, 2013; Turpin et al., 2021). Created with BioRender.com.

Several studies were conducted to establish the link between MGO induced ER stress and cell death. In human retinal pigment epithelial cells (ARPE-19), exogenously applied MGO was reported to induce ARPE-19 cell death through a ROS-dependent manner, involving MMP loss, intracellular calcium increases and ER stress (C.-M. Chan et al., 2016), activating the initiators of typical UPR signal transduction pathways: PERK-eIF2aATF4, IRE1-XBP1 and ATF6 and consistently, the protein expressions of BiP/GRP78 and CHOP were upregulated. Another study by Choi et al. investigating MGO-induced apoptosis in endothelial cells further corroborated that MGO-induced apoptosis is dependent on ER stress with both exogenously applied MGO and the use of a GLO-1 inhibitor (Choi et al., 2016). This is similar to what we have observed in HepG2 cells. The importance of ER stress in MGO-mediated cell dysfunction has been demonstrated by the use of chemical inhibitors of ER stress which significantly decrease the MGO-induced ROS generation as well as cell death (C.-M. Chan et al., 2016).

MGO increased activation of the PERK-eIF2α-ATF4-CHOP pathway and JNK pathway in endothelial cells in a time-dependent manner, however the use of a specific inhibitor of JNK was not able to reverse MGO-induced apoptosis, nor the MGO-mediated reduction of cell viability. Thus, to further confirm whether MGO-induced apoptosis was regulated via CHOP-dependent degenerative pathway both *in vitro* and *in vivo*, cells transfected with CHOP siRNA and a CHOP deficiency mouse model showed a significantly diminished MGO-induced endothelial apoptosis. It was also found that CHOP deficiency prevented MGO–induced aortic endothelial dysfunction (Choi et al., 2016). Collectively, these results indicate

that MGO leads to cell apoptosis in an ER stress and specifically CHOP-dependent manner and leads to the upregulation of key ER stress response elements. These findings may shed light on the mechanisms behind the increased ROS, ER stress and apoptosis rates observed in the hepatocyte toxicity following GLO1 inhibition in our experiments, suggesting a key role of ER stress. In INS-1E cells, MGO treatment and GLO1 knockdown also declined MMP, and this was associated with increased oxidative stress, increased expression of ER stress mediators BiP/Grp78 and p-PERK and activated the mitochondrial apoptotic pathway, which was reversed with the use of Aminoguanidine and ER stress inhibitor 4-phenylbutyrate (4-PBA) (C. Liu et al., 2017). However, in this study, MGO treatment down-regulated Ire1α, and apoptosis was effectively reduced by the JNK inhibitor SP600125.

ER stress has been implicated in other cellular processes in addition to apoptotic cell death. We have shown that both CHOP and BIP increases in HepG2 cells in response to dicarbonyl stress and this may affect hepatocyte function. The IRE1 and XBP1 components of the ER stress pathway play key roles in hepatocyte function. Liu et al. has demonstrated that Ire1α down-regulation by Ire1α siRNAs mimics MGO-induced cytotoxicity through the mitochondrial apoptotic pathway (C. Liu et al., 2017). In bone marrow-derived progenitor cells, MGO has been shown to cause severe dysfunction by directly binding to IRE1 in vitro, diminishing its activity (Hainan Li et al., 2019) which may impair its ability to cleave XBP1 mRNA. In general, proper activation of XBP1 is important for metabolic homeostasis in the liver. However, it was also demonstrated that increased IRE1α/XBP1 axis activation leads to the acetylation of IRS1/2, aggravating IR in the liver, and decreases the synthesis of Bax inhibitor 1 (Cao et al., 2017; Cao et al., 2023; Lebeaupin, Vallée, Rousseau, et al., 2018; Lisbona et al., 2009), which favours ER stress, calcium imbalance, ROS accumulation, and metabolic dysregulation (Lebeaupin et al., 2020). Thus, the aberrant activation of the IRE1α/XBP1 could prove detrimental to hepatocyte survival under metabolic stress. While IRE1α could improve hepatic lipid homeostasis, XBP1 can stimulate the transcription of numerous adipogenesis-related genes, including PPARγ and C/EBPα (Xia et al., 2020), as demonstrated in mice (So, Hur, Tarrio, Ruda, Frank-Kamenetsky, Fitzgerald, Koteliansky, Lichtman, Iwawaki, & Glimcher, 2012), where an improvement in hepatic steatosis was achieved by activating IRE1α and suppressing the production of XBP1. It is important to consider that hepatic insulin action and glucose homeostasis are preserved by insulin induced IRE1α activation, which is believed to be mediated by GDF15 transcription activation, which

encourages hepatic autophagy, relieves ER stress, and/or reduces forkhead box O (FoxO)1 expression (Cao et al., 2023).

While the activation of all signalling pathways involved in the amino-acid regulation of CHOP is required to achieve maximal induction, studies suggest that the PERK/eIF2*α*/AT4 signalling pathway plays an essential role in the induction of CHOP in ER stress, and is dominant over that of the ATF6 and Ire1/XBP-1 signalling pathways (Oyadomari & Mori, 2004), which may explain why we are able to observe an induction of CHOP, despite propositions that MGO may diminish IRE1α activity. Higher eIF2a phosphorylation has also been linked to the pathology of NAFLD (Xia et al., 2020). In addition to its pro-apoptotic role, aberrant activation of the PERK pathway is also suggested to contribute to IR in the liver. IR and glucose intolerance may be improved in diet-induced obese mice by liver-specific inhibition of the PERK/eIF2α/ATF4 pathway or by depleting ATF4 (Oyadomari et al., 2008; Seo et al., 2009). The underlying mechanisms may be linked to the transcriptional regulation of many targets, including glutamic pyruvate transaminase 2 (GPT2), a promotor of gluconeogenesis, endogenous inhibitor of AKT tribbles homolog 3 (TRB3) and PPARγ (Cao et al., 2023). Nevertheless, it was also demonstrated that the eIF2α/ATF4 pathway, could promote the expression of FGF21, a hepatokine with beneficial effects on metabolism as it reduces glucose intolerance in DIO mice and mediates metformin's effects on appetite and weight loss (Cao et al., 2023).

Furthermore, Rabbani et al. suggested that chronic low-grade inflammation may be a pathogenic consequence of proteome disturbances caused by MGO glycation, and the consequent aberrant activation of the UPR, mainly through Bip/GRP78 mediated XBP-1 activation, and that this involved upregulation of NF-kB signalling, and subsequent transcription of RAGE, IL-8 and GRP78 in particular (Rabbani et al., 2021). Although all three pathways of the UPR have been linked to triggering inflammation, XBP-1 is believed to play major role in dicarbonyl stress mediated inflammation (Rabbani et al., 2021). A remarkable finding confirmed this link as the overexpression of GLO1 was able to inhibit SETD7 proinflammatory cascade - NF-κB activation, increases IL-6, IL-8, RAGE, and GRP78, in models of transient and persistent hyperglycaemia (El-Osta et al., 2008). Moreover, the inhibition of proteasome function in human foetal hepatocytes and hepatoma cells lead to apoptotic cell death coupled with enhanced expression of IL-8, both at the protein and the mRNA levels. This increase in IL-8 is independent of NF-κB activation and is associated with

an increase in JNK and activator protein-1 (AP-1) activity (Joshi-Barve et al., 2003). Thus, we can hypothesise that the enhancement in IL-8 secretion that is observed following GLO1 inhibition in our experiments is potentially due to the impairment of proteasome activity, and that IL-8 secretion represent the response to a profound disturbance in hepatocyte function.

Interestingly, the induction of CHOP was reported to perturb the cellular redox state by depletion of cellular GSH (McCullough et al., 2001) and as discussed in chapter 1, ER stress leads to increased ROS generation (Nascè et al., 2022). ER stress is predicted to enhance both a loss of reduced GSH and amino-acid insufficiency, and thereby impair protein synthesis, and we know that diminished GSH levels only favours oxidative stress and MGO accumulation, rendering hepatocytes even more vulnerable to toxicity. This was also observed as a result of protein aggregation in lipotoxic conditions and dysfunctional hepatic lipid metabolism, where increases in ER phospholipid composition were found to disturb protein synthesis and induce ER stress (Gjorgjieva et al., 2019).

Another interesting finding in our studies was the downregulation of angiotensin converting enzyme (ACE)2 mRNA following GLO1 inhibition in HepG2 cells. During the course of this PhD project, the COVID-19 pandemic was ongoing, and several significant observations were made in diabetic patients. Patients with diabetes have an increased risk of COVID complications and in human studies dysfunctions of ACE2/Ang1-7 was proposed to be one of the important mechanisms underlying the inflammation and poor disease outcomes following COVID-19 infection in these patients (Lu et al., 2022). COVID-19 receptor entry points ACE2 and transmembrane serine protease 2 (TMPRSS2) are upregulated in patients with diabetes (D'Onofrio et al., 2021; Liao et al., 2020; Lu et al., 2022) and NAFLD (Meijnikman et al., 2021), including in in liver samples, although not specific to hepatocytes. High glucose or d-galactose treatment *in vitro* increases ACE2 and TMPRSS2 expression in several cell types, including macrophages, lung epithelial cells and embryonic mouse hypothalamic neurons (Baristaite & Gurwitz, 2022; Nandula et al., 2021; Srivastava & Mussa, 2022). In cultured endothelial cells, high glucose exacerbated the SARS-CoV-2 Spike protein-mediated reduction of ACE2 but had no effect on TMPRSS2 (Zhang et al., 2023). Human and humanised-ACE2 expressing mouse primary hepatocytes, where steatosis and inflammation were induced by methionine and choline deprivation, were also more vulnerable to SARS-CoV-2 infection (Mercado-Gómez et al., 2022). Thus, we hypothesised that ACE2 and/or TMPRSS2 would be upregulated by dicarbonyl stress. This was not found to be the case in hepatocytes, but to the

contrary, dicarbonyl stress led to a downregulation of ACE2 mRNA and had no effect on TMPRSS2. Therefore, the increases that were observed in clinical and experimental setting may either not occur in hepatocytes or be mediated by hyperglycaemia or glucose per se rather than dicarbonyl stress.

Nevertheless, the renin angiotensin system (RAS) has increasingly been recognized to play an important role in the metabolic system and the development of diabetes, and more specifically in lipid and glucose homeostasis and cellular metabolism (Macêdo et al., 2014). The RAS a single cascade system where the enzyme renin converts cleaves its substrate angiotensinogen to form angiotensin I (Ang I), which is then transformed into angiotensin II (Ang II) by ACE (Macêdo et al., 2014; Santos et al., 2018). The ultimate effector of the RAS on systemic blood pressure, AngII, has emerged as an important contributor to NAFLD development and progression (Matthew Morris et al., 2013). Ang II mediates its biological responses through two G-protein-coupled receptors, mainly the Ang II receptor type 1 (AT1) and Ang II receptor type 2 (AT2) (Macêdo et al., 2014). This forms the ACE/Ang II/AT_1 axis. The RAS also has a counter-regulatory axis where ACE2 cleaves the terminal leucine from ANG-I to generate the intermediate peptide Ang-(1-9) peptide which in turn is converted to the vasodilator peptide Ang-(1-7) by ACE or other peptidases such as neutral endopeptidase (NEP) (Macêdo et al., 2014; Santos et al., 2018; Simões e Silva et al., 2013). ACE2 can also directly generate ANG-(1–7) by cleaving the terminal phenylalanine residue from Ang-II (Simões e Silva et al., 2013). Therefore, Ang II is the most important substrate for Ang-(1-7) synthesis by ACE2 and this peptide mediates its activity through the G protein-coupled receptor Mas (Santos et al., 2018; Simões e Silva et al., 2013). This forms the ACE2/Ang-(1-7)/MAS axis. Several studies support the idea that ACE2 plays a beneficial role in the RAS, and highlight the importance of seeking therapies that increase its activity in the prevention and treatment of diabetes and its complications (Macêdo et al., 2014). In several disease states, the activation of the ACE/Ang II/AT_1 receptor axis, causes deleterious effects (Simões e Silva et al., 2013).

Ang II levels are elevated in diabetes and in NAFLD (Bindom & Lazartigues, 2009; Giacchetti et al., 2005; K. M. Kim et al., 2021; Marcus et al., 2013; Ribeiro-Oliveira Jr et al., 2008; Sansoè et al., 2020), due to ACE overactivation and potentially insufficient compensation by ACE2. In the liver, Ang II levels in NAFLD patients were significantly higher than in patients without, which correlated with inflammatory markers (IL-1β). Thus, Ang II levels were deemed an independent risk factor of NAFLD development (Y. Li et al., 2019). In the context of the

metabolic dysfunction and disease states like obesity and diabetes, high levels of ANG II together with hyperglycaemia can form a self-reinforcing positive feedback loop that aggravates vascular and tissue damage thus contributing to the development of the debilitating complications of diabetes (Santos et al., 2018). Furthermore, Ang II blocking drugs, such as ACE inhibitors (ACEis) and angiotensin receptor blockers (ARBs), have been also found to reduce the development of NAFLD in large amounts of literature (Hirose et al., 2007; Kudo et al., 2009; Matthew Morris et al., 2013; Paschos & Tziomalos, 2012; Rong et al., 2010; Yokohama et al., 2004). Importantly, MGO administration in rats increased circulating levels of Ang II (Dhar et al., 2014; Schupp et al., 2005).

The disruption of this RAS axis is implicated in high fructose-induced NAFLD, as evidenced by the increased ACE activity and Ang II protein expression in a study of rats fed high fructose as well as the reported dramatic decrease in the protein levels of ACE2, Ang (1–7), and Mas receptor in hepatic tissue (Attia et al., 2022). Given the substantial correlation between serum and tissue AGEs and fructose, this finding is significant. When treated with chrysin, which upregulates the ACE2/Ang (1-7)/Mas axis, they were able to protect the rats from developing liver steatosis. The authors suggest this indicates that an overactive ACE and a repressed ACE2 may play an important role in the pathogenesis of high fructose induced NAFLD by promoting the oxidative stress, inflammation, hyperglycaemia, and hypertriglyceridemia induced by fructose. Furthermore, according to Yang et al., the lack of ACE2 further exacerbated these effects in mice with a high fructose intake (M. Yang et al., 2020). Cao et al. also showed that in ACE2 knockout mice, hepatic steatosis, oxidative stress, and inflammation were enhanced. In vitro, treatment with ACE2 and Ang (1–7), ameliorated inflammation, oxidative stress, and hepatic steatosis in FFA-induced HepG2 cells. Moreover, overexpression of ACE2 reduced hyperglycaemia and steatosis in db/db mice (Cao et al., 2016). Increased levels of Ang II can contribute to NAFLD pathogenesis through multiple mechanisms, including the induction of mitochondrial dysfunction, ROS generation, depletion of cellular and mitochondrial glutathione (Doughan et al., 2008; N. Yang et al., 2020), proinflammatory cytokine production, IR, and de novo lipogenesis, thus resulting in FFA flux to the liver and promoting the increase of TG (de Kloet et al., 2010). These mechanisms have been thoroughly discussed in a review by Morris et al. (Matthew Morris et al., 2013).

Therefore, the dicarbonyl-stress mediated decrease in the expression of ACE2 and the potential to subsequently increase hepatic Ang II levels may contribute to the increased risk of NAFLD

in diabetes. This decrease may be mediated by MGO-induced inhibition of AMPK which is a regulator of ACE2 expression. (Clarke et al., 2014; Gugliucci, 2009). Dysfunction of hepatic AMPK induced by hyperglycaemia represents a key mechanism for hepatic lipid accumulation, inflammation and hyperlipidaemia associated with diabetes (Entezari et al., 2022).

In conclusion, GLO1 inhibition by BBGC and the resultant dicarbonyl stress not only mimics the cytotoxicity of direct MGO exposure but also seems to mimic the underlying cellular pathways mediating these damaging effects. This model has allowed us to demonstrate for the first time that intracellularly formed MGO causes mitochondrial dysfunction, increased mitochondrial superoxide generation and increased the ER stress markers CHOP and Bip/Grp78 expression in HepG2 cells. These pathways are all critical to hepatocyte dysfunction with the potential to also increase inflammation, both of which are key players in the pathogenesis of liver disease.

5. Modulation of inflammatory signalling in HepG2 cells by dicarbonyl stress

Introduction

Recently, in a clinical trial conducted by Michel et al., a positive correlation between elevated levels of serum MGO, worsening liver disease prognosis and liver cirrhosis, and widespread inflammation was revealed (Michel et al., 2021).

As previously discussed, emerging evidence suggests NAFLD and T2DM share pathogenetic features, including aberrant immune activity in the form of chronic, low-grade inflammation in the liver, that can also lead to systemic effects (Gehrke & Schattenberg, 2020; Targher et al., 2021). It is not only considered an additional risk factor for associated inflammatory complications, but also a major contributory factor, particularly in liver disease (Mohamed et al., 2016; Wellen & Hotamisligil, 2005). T2DM patients with NAFLD, tend to have more severe metabolic disturbances, including greater visceral adiposity and insulin resistance, more severe dyslipidaemia and higher levels of inflammatory markers such as IL-6 and TNF-α, compared with those without (Banerji et al., 1995; Kelley et al., 2003; Smith & Adams, 2011b; Toledo et al., 2006).

The liver is indispensable in the regulation of normal physiology and metabolism via the production and secretion of various plasma proteins, including albumin, complement factors, transport proteins, and other factors (Watt et al., 2019). The role of liver as a major secretory organ has long been appreciated, and in recent years, hepatokines in particular have drawn increasing attention due to their capacity to actively mediate metabolic regulation between the liver and other organs. Several, including fibroblast growth factor 21 (FGF21) and growth differentiation factor 15 (GDF15), have been identified and investigated for their roles in the regulation of various biological processes such as glucose and lipid metabolism, in an autocrine, paracrine, and endocrine fashion, and in the development of obesity, insulin resistance, and NAFLD (Han et al., 2016; Jensen-Cody & Potthoff, 2021; Ke et al., 2019; Dariusz M Lebensztejn et al., 2016; Oh et al., 2017; Watt et al., 2019).

The hepatic NF-κB pathway is a central mediator of immune and stress responses, and it is critical for liver tissue homeostasis (Gehrke & Schattenberg, 2020). It is considered a major transcription factor in the mechanisms underlying inflammation as it is involved in the transcription of a number of genes encoding pro-inflammatory cytokines, and it has been widely found to be activated in the liver of diabetic patients (Daniel et al., 2013). Activation of NF-κB in liver and appears to contribute to obesity-induced insulin resistance (Wellen & Hotamisligil, 2005) and inappropriate and persistent activation of NF-κB in T2DM patients with liver damage may play a pivotal role in the progression of hepatic steatosis to cirrhosis. As previously mentioned in chapter 1, as NAFLD progresses, inflammation becomes overt. Lipid metabolites, pro-inflammatory cytokines, such as IL-8, and hepatokines (for example, fetuin A, fetuin B, AGF, FGF21, GDF15 or selenoprotein) are observed (Targher et al., 2021) and in the liver and extrahepatic organs, this can contribute cellular dysfunction, cell death, and detrimental tissue remodelling in an effort to preserve structural and functional organ integrity. Sustained inflammation may lead to hepatic fibrosis and cirrhosis, resulting in an increased risk of developing more severe stages, including non-alcoholic steatohepatitis (NASH) and hepatocellular carcinoma (Gehrke & Schattenberg, 2020; Hijona et al., 2010; Mantena et al., 2008; Marchesini et al., 2003; Starley et al., 2010).

A large body of evidence also demonstrates how chronic liver tissue inflammation includes multi-organ cross-talk, leading to IR, impaired insulin secretion and glucose intolerance (Rohm et al., 2022). Numerous cross-sectional studies have associated insulin resistance and T2DM with higher serum levels of C-reactive protein (CRP), IL-6 and TNF-α, and Farhan et al. reported that the serum IL-8 levels in T2DM patients were markedly increased compared to healthy subjects, which also positively correlated with higher HbA1C (Farhan Mohammed et al., 2018). In patients with NAFLD, patients with abnormal ALT were found to have higher serum levels of IL-6 and statistically higher levels of serum IL-8, indicating that some degree of liver damage is correlated with increasing levels of circulating proinflammatory cytokines (Chu et al., 2007) . In addition, plasma MGO-derived CML accumulation was found to be significantly associated with the grade of hepatic steatosis and the grade of hepatic inflammation in obese patients, reflected by increased hepatic gene expression levels of IL-8 and CRP (Gaens et al., 2012). IL-8 is also strongly associated with fibrosis and increased fibrotic liver injury in patients with NAFLD (Glass et al., 2018). Furthermore, elevated levels of key hepatokines FGF21 and GDF15 are also both strongly linked with the occurrence of diabetes, (Adela & Banerjee, 2015; Schmid et al., 2022), NAFLD (Valenzuela-Vallejo et al.,

2023; Yilmaz et al., 2010) and obesity (Asrih et al., 2023; Berti et al., 2015) and are positively correlated with the presence of steatosis, hyperglycaemia, insulin resistance and inflammation (Dolegowska et al., 2019; Eddy & Trask, 2021; Meex & Watt, 2017; Mutanen et al., 2014; Schmid et al., 2022; Vila et al., 2011).

In chapter 3, the characterisation of the effects of BBGC-mediated dicarbonyl stress revealed an increase in oxidative stress and the release of the pro-inflammatory cytokine IL-8 in HepG2 cells, which mimics the effects of MGO. Therefore, we wanted to determine the underlying processes mediate IL-8 release. MGO has been previously shown to induce inflammation through increased pro-inflammatory gene expression (S.-H. Cha et al., 2019; Di Loreto et al., 2004; Kuntz et al., 2010), likely as a result of enhanced NF-κB activation. Although dicarbonyl stress has been linked to increased hepatic inflammation, and considering the close association between T2DM, NAFLD and hepatokines in the context of metabolic disease progression, to date, no research has been done to determine an association between dicarbonyl stress and hepatokine expression and release. Therefore, the aims of this chapter are to determine whether BBGC causes NF-kB activation to induce inflammation in HepG2 cells, and to determine the effects of dicarbonyl stress on hepatokines FGF21 and GDF15 expression and secretion.

Experimental protocol and methods

In the first series of experiments, HepG2 cells were treated with 20µM BBGC for 30 minutes, 1, 2, 3, 4, 5 and 6 hours to determine the course of IL-8 release upon treatment before measuring IL-8 secretion using ELISA. HepG2 cells were then treated for 1, 2, 4 and 6 hours with 20µM BBGC to determine the optimal time point for the nuclear translocation of NF-κB, assessed using confocal microscopy as described below. It was then determined that 4 hours yielded the best results. HepG2 cells were also incubated for 24 hours with 20µM BBGC to determine changes in IL-8, IL-6 and TNF-α mRNA expression by RT-PCR.

In a second series of experiments, HepG2 cells were treated with 20µM BBGC for 24, 48 and 72 hours to examine GDF15 and FGF21 mRNA expression and protein secretion. Following treatment, samples were collected as previously described in chapter 2 and analysed using RT-PCR to determine mRNA expression, and to measure hepatokine release, treatment supernatants were collected and analysed using their respective ELISA kits.

Detection and localisation of NF-κB by immunocytochemistry (ICC)

HepG2 cells were treated for 4 hours with 20 µM BBGC. The cells were also treated with Palmitic Acid 300 µM for 1 hour to serve as a positive control.

Buffers and solutions:

- Wash buffer: 3% BSA in PBS.

- 3.7% formaldehyde fixative solution: 1mL of 37% formaldehyde in 9 mL of PBS.

- Permeabilization solution 0.5% Triton® X-100 in PBS.

- Blocking buffer: 1% BSA + 0.1% Triton® X-100 in PBS.

Following treatment, the cells were washed twice with PBS then fixed to the coverslip using formaldehyde fixative solution for 10 minutes at room temperature (~25°C). The fixative solution was then removed, and the cells were washed twice with wash buffer. The cells then underwent permeabilization by applying permeabilization solution to the coverslips for 20 minutes at room temperature (~25°C). After the cells were permeabilised, they were washed twice with wash buffer, and this was followed by a one-hour incubation with blocking buffer at 4°C. They were then incubated overnight with the rabbit anti-NFκB antibody (Abcam) at a 1:200 dilution in blocking buffer at 4°C.

The next day, the coverslips were washed 10 times in 3 changes of wash buffer before being incubated with the secondary anti-rabbit antibody Alexa Fluor® 488, diluted 1:1000 in blocking buffer for 2 hours at room temperature (~25°C). This was followed by another 10 washes in 3 changes of wash buffer and one wash with PBS. The coverslips were mounted using a DAPI-containing anti-fade mounting medium and visualised using a confocal microscope with filter sets appropriate for the label used.

The slides were scanned using confocal microscopy (ZEISS) at 20x resolution, and images captured with the using the ZEN software. The images were then analysed using the ImageJ software to determine the intensity of the NF-κB stain and its proportion within the cell nuclei.

Results

Measurement of time-dependent IL-8 secretion following GLO-1 inhibition

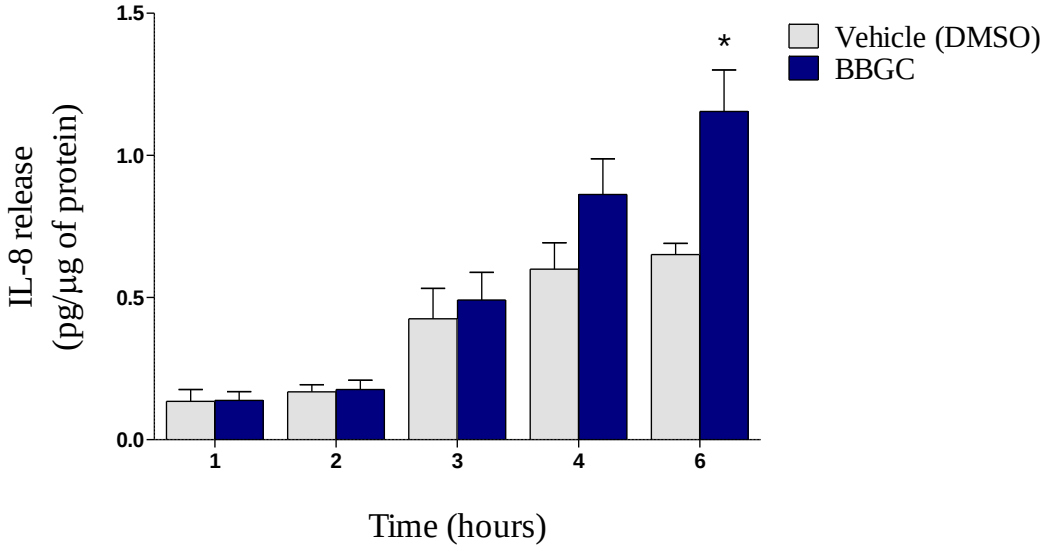


Figure 5.1. Time course analysis of dicarbonyl stress mediated IL-8 secretion in HepG2 cells. IL-8 secretion was measured at 1, 2, 3, 4 and 6 hours after treatment with 20 µM BBGC. Inhibition of Glyoxalase 1 caused a time-dependent increase in IL-8 release. Data are expressed as the mean ± SEM from n = 3 experiments (3 replicates per experiment). Statistical analysis was carried out using one-way ANOVA with Bonferroni post hoc, where p-value $p < 0.05$ was considered significant; *$p < 0.05$ vs. untreated cells.

Following the increased release in IL-8 secretion that is observed in response to BBGC mediated dicarbonyl stress (Figure 3.2.B), we examined the timing of its secretion. We have identified that IL-8 release following GLO-1 inhibition is time dependent reaching significance after 6 hours of BBGC treatment (Figure 5.1).

Measurement of changes in IL-8, TNF-α and IL-6 mRNA expression in HepG2 cells in response to dicarbonyl stress

To verify the transcriptional induction of pro-inflammatory cytokines by GLO-1 inhibition, we have measured IL-8, IL-6 and TNF-α mRNA expression following BBGC treatment in HepG2 cells. Indeed, the treatment induced an increase in the transcription of IL-8 and TNF-α (Figure 5.2.A and 5.2.B), indicating that MGO triggers the production of pro-inflammatory cytokines, leading to inflammation in HepG2 cells. The data for IL-6 is weaker but it is likely that IL-6 expression is also induced in response to the treatment (Figure 5.2.C).

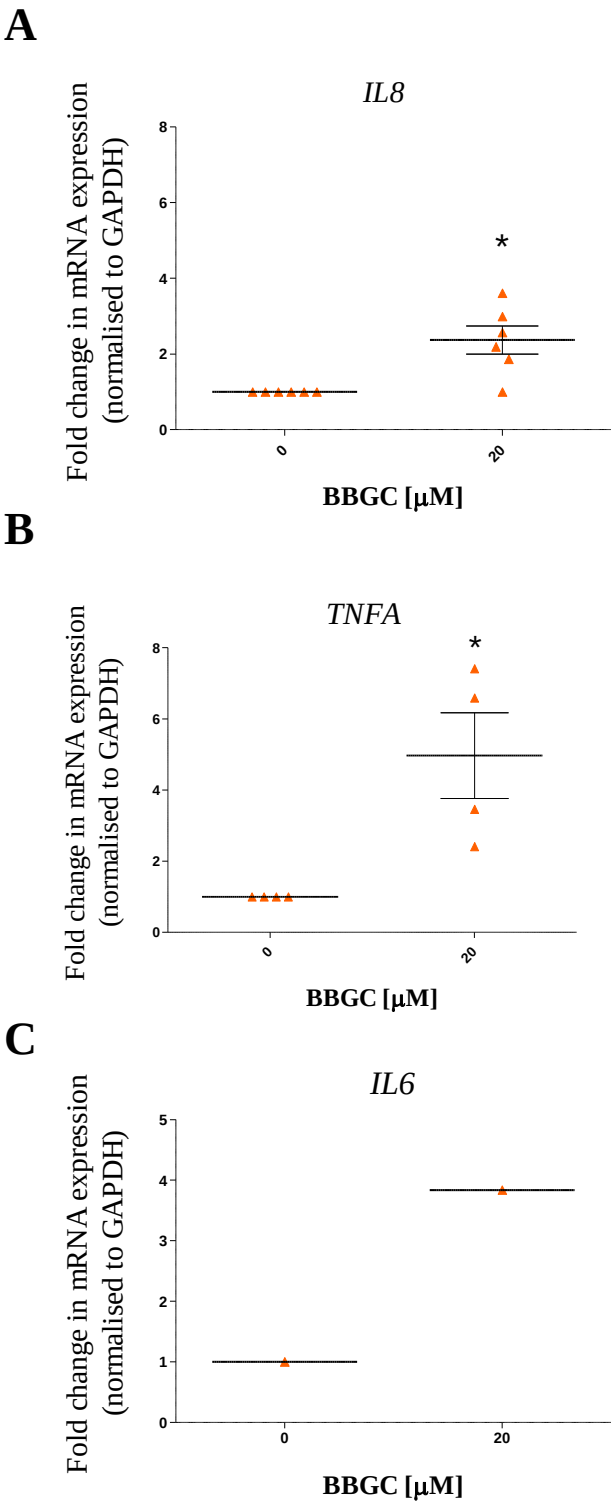


Figure 5.2. 24-hour exposure to BBGC increases IL-8 and TNF-α mRNA expression in HepG2 cells. mRNA expression of IL-8 (A), TNF-α (B) and IL-6 (C) was assessed by RT-qPCR and normalised to GAPDH following a 24-hour treatment with BBGC 20 µM. Data are expressed as $2^{(-\Delta\Delta Ct)}$ ± SEM *n* = 6 independent experiments for IL-8, n = 4 independent experiments for TNF-α and *n* = 1 independent experiment for IL-6. Statistically significant differences were determined using Kruskal-Wallis with Dunn's post hoc test. * $P < 0.05$ as compared to vehicle-treated control group.

Detection and localisation of NF-κB p65 in HepG2 cells following GLO-1 inhibition

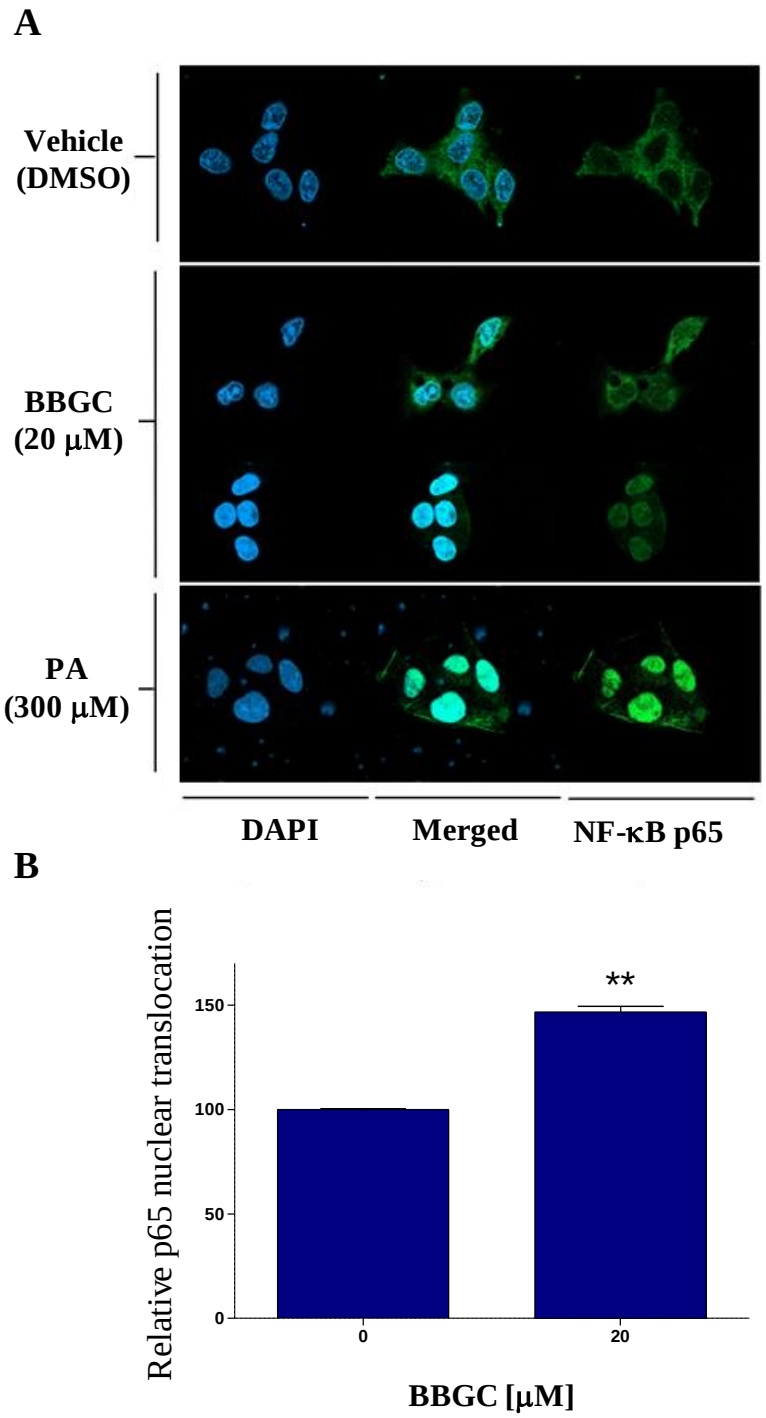


Figure 5.3. GLO-1 inhibition results in NF-κB p65 nuclear translocation in HepG2 cells. (A) Representative images of immunofluorescence staining showing NF-kB p65 (green) nuclear translocation in HepG2 cells treated with 20 μM BBGC for 4 hours. Palmitate (PA) 300μM was used as a positive control. Cell nuclei were detected by DAPI (blue). (B) Quantification of NF-kB p65 nuclear translocation in HepG2 cells after 4-hour BBGC treatment. Results are expressed as the percentage of the p65 nuclei-positively stained cells to the total cells. DMSO was used as a solvent control. Data obtained from n=3 experiments. Statistical analysis was carried out using Kruskal-Wallis with Dunn's post hoc test. **P<0.05 versus vehicle control.

To further understand the increase in inflammation as evidenced by the increased IL-8 secretion (Figure 3.2.B) and proinflammatory cytokine gene expression (Figure 5.2) in response to GLO-1 inhibition and subsequent MG-H1 accumulation, we looked at NF-κB p65 nuclear translocation. This was looked at various time points (data not shown) and it was found that NF-κB activation occurs at the 4-hour time point.

We can observe through immunolabelling the translocation of the active NF-κB p65 from the cytoplasm into the nucleus, indicating its activation and the induction of its transcriptional function (Figure 5.3). The cells were treated with 300µM palmitate to serve as a positive control for this set of experiments, as this compound has been established to activate NF-κB (Joshi-Barve et al., 2007).

ER stress increases IL-8 secretion in HepG2 cells

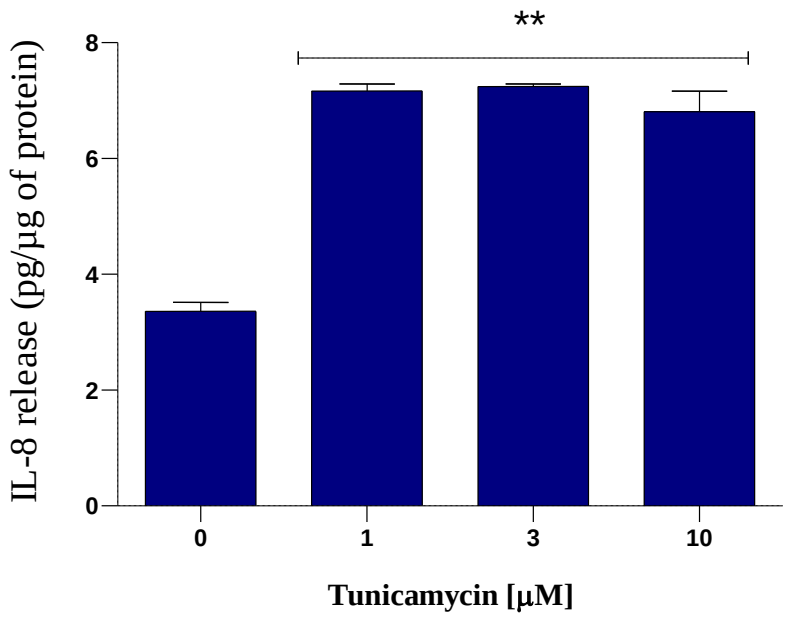


Figure 5.4. Exposure to tunicamycin increases IL-8 secretion in HepG2 cells. IL-8 secretion was measured after a 24-hour treatment with 1-10 µM tunicamycin to induce ER stress. The treatment caused a significant increase in IL-8 release. Data are expressed as the mean ± SEM from n = 3 experiments (3 replicates per experiment). Statistical analysis was carried out using one-way ANOVA with Bonferroni post hoc test, where p-value $p < 0.01$ was considered significant; **$p < 0.01$ vs. untreated cells.

To verify whether ER-stress leads to increased IL-8 production in HepG2 cells, we treated the cells with 1-10 µM tunicamycin, which causes an accumulation of unfolded glycoproteins in the ER, leading to ER stress (Oslowski & Urano, 2011). Indeed, tunicamycin triggers

significant IL-8 secretion in HepG2 cells (Figure 5.4), indicating that ER stress is involved in the modulation of inflammation via IL-8 in hepatocytes.

Dicarbonyl stress increases the expression and secretion of hepatokines GDF15 and FGF21 in HepG2 cells

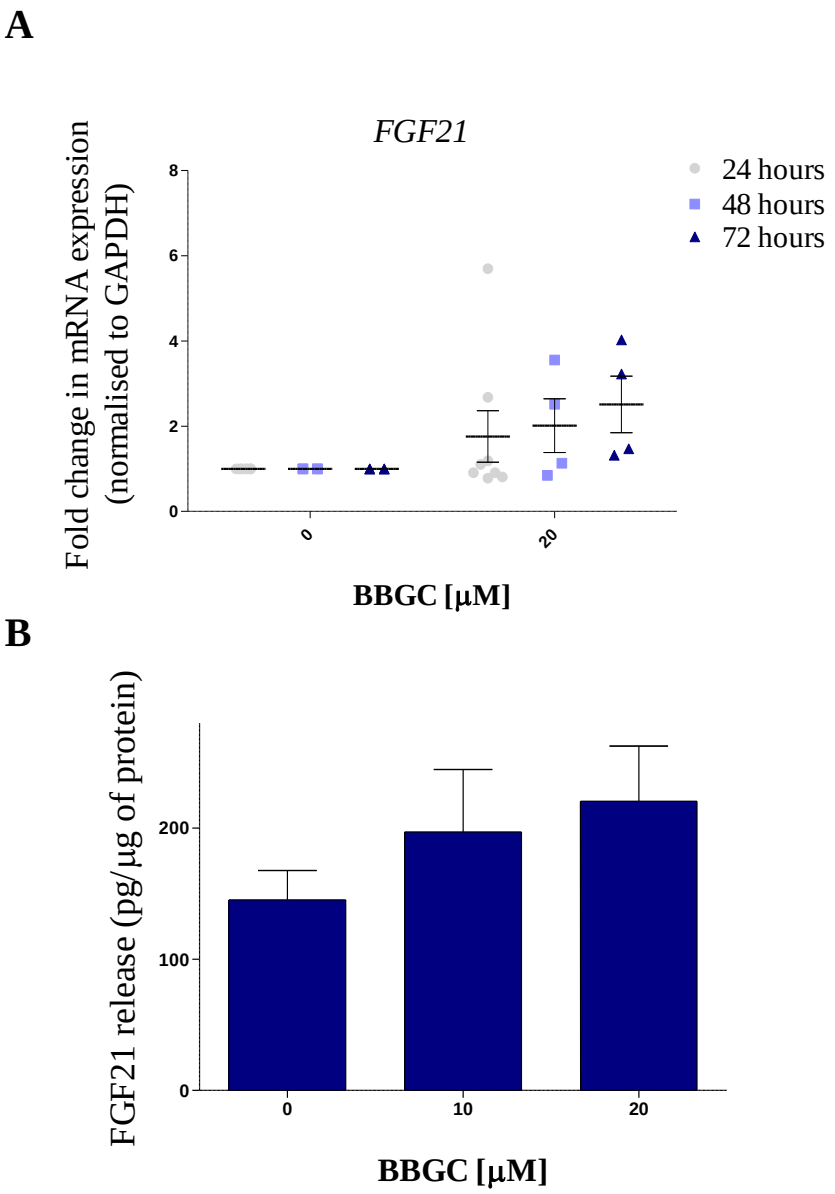


Figure 5.5. Exposure to BBGC mediated dicarbonyl stress increases FGF21 mRNA and secretion in HepG2 cells. mRNA expression of FGF21 (A) was assessed by RT-qPCR and normalised to GAPDH following a 24-, 48- and 72-hour treatment with BBGC 20 µM. Data are expressed as $2^{(-\Delta\Delta Ct)}$ ± SEM *n* = 4-5 independent experiments. FGF21 protein release (B) was measured following a 24-hour treatment with 10 µM and 20 µM BBGC using a specific FGF21 ELISA kit. Data are expressed as the mean ± SEM from n = 3 experiments (3 replicates per experiment). Statistically significant differences were determined using Kruskal-Wallis with Dunn's post hoc test and one-way ANOVA with Bonferroni post hoc test, where p-value $p < 0.05$ was considered significant.

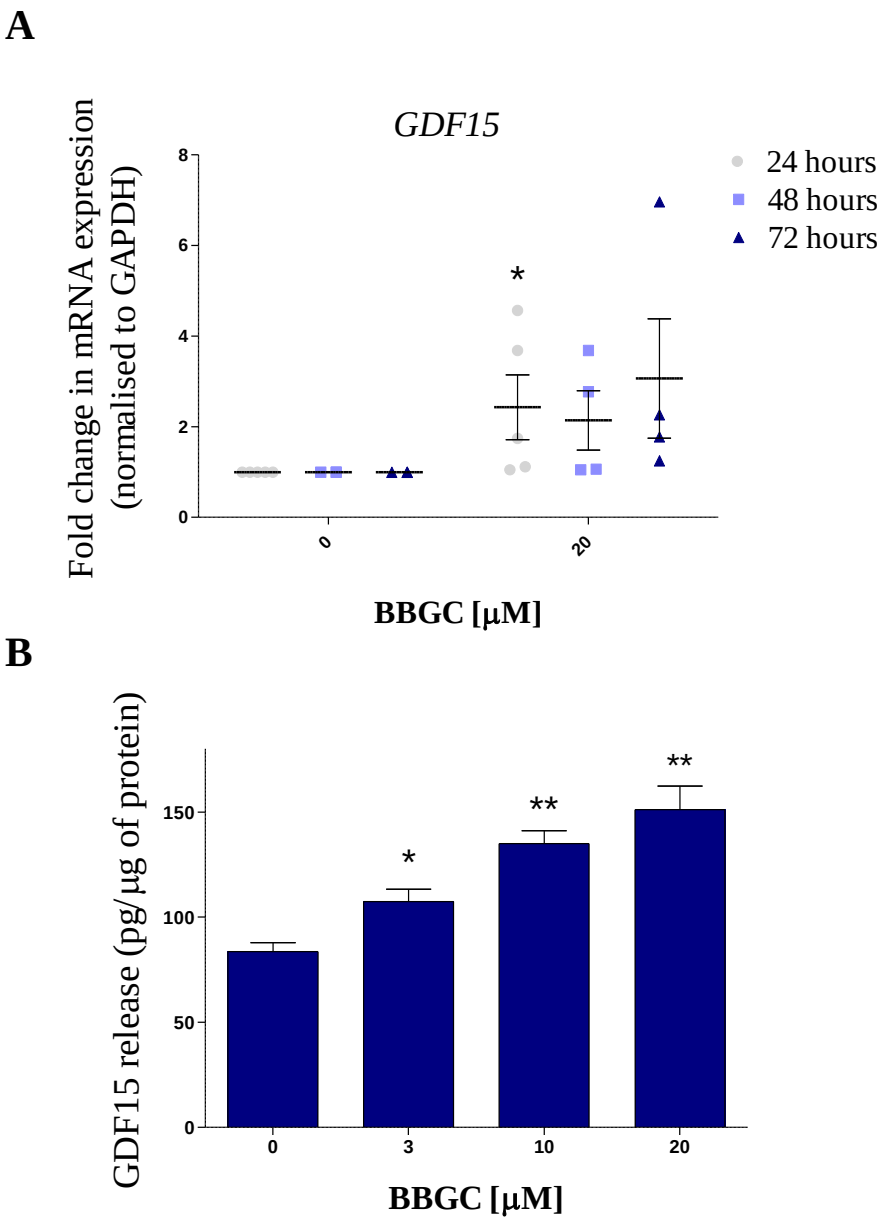


Figure 5.6. Exposure to BBGC mediated dicarbonyl stress increases GDF15 mRNA and secretion in HepG2 cells. mRNA expression of GDF15 (A) was assessed by RT-qPCR and normalised to GAPDH following a 24-, 48- and 72-hour treatment with BBGC 20 µM. Data are expressed as $2^{(-\Delta\Delta Ct)}$ ± SEM *n* = 4-5 independent experiments. GDF15 protein release (B) was measured following a 24-hour treatment with 10 µM and 20 µM BBGC using a specific GDF15 ELISA kit. Data are expressed as the mean ± SEM from n = 3 experiments (3 replicates per experiment). Statistically significant differences were determined using Kruskal-Wallis with Dunn's post hoc test and one-way ANOVA with Bonferroni post hoc test, **p < 0.01 and *p < 0.05 vs. vehicle treated cells.

Furthermore, as MGO increases oxidative stress and modulates the inflammatory response, we wanted to investigate its effects on hepatokine release, as this has not previously been done. Interestingly, BBGC mediated dicarbonyl stress significantly induces HepG2 transcription of both FGF21 (Figure 5.4.A) and GDF15 (Figure 5.5.A), however though GDF15 secretion was

significantly increased (Figure 5.5.B) there was only a trend to increase FGF21 secretion (Figure 5.4.B). These results expand upon our knowledge of MGO mediated inflammatory responses and carries significant weight regarding its implication in metabolic disorders.

Discussion

In our series of experiments, treatment with GLO1 inhibitor BBGC, revealed an increase in IL-8 release after 6 hour suggesting a transcriptional mechanism of dicarbonyl stress-mediated inflammation, confirmed by increases mRNA and NF-κB activation. This corroborates previous studies where MGO was found to increase the expression and release of pro-inflammatory cytokines and NF-κB activation (S.-H. Cha et al., 2019; Kuntz et al., 2010), and further supports the use of GLO1 inhibition as a physiological model for dicarbonyl stress. We have also identified for the first time that dicarbonyl stress increases both the expression and release of the hepatokines GDF15 and FGF21.

While the IL-8 data was clearer, unfortunately we were not able to effectively measure TNF-α or IL-6 secretion by ELISA. Although ELISA sensitivity is reliable and other groups were able to detect these cytokines using HepG2 cells, we can only assume that the kits at our disposal showed poor sensitivity to the cytokine levels secreted by our cells in particular. A way to overcome this may have been achieved using columns, to concentrate the sample prior to assaying, as described in 2.2.4 or with a cytokine array experiment, which is more sensitive. Nevertheless, the increased gene expression for all three cytokine is a good indicator that inflammation is triggered in response to intracellular MGO.

IL-8 has previously been reported to be elevated in the serum of T2DM and NAFLD patients, who have also been established to have increased serum and tissue levels of MGO and AGEs. Similarly, other compounds that are abundant in states of metabolic stress have been reported to increase hepatocyte IL-8 mRNA and secretion in vitro, such as glucose (Srinivasan et al., 2004; Srinivasan et al., 2003; Temaru et al., 1997) and palmitic acid, which was suggested by the authors to be through the activation of NF-κB and c-Jun N-terminal kinase/AP-1 (Joshi-Barve et al., 2007). Interestingly, HepG2 cells treated with palmitic acid and oleic acid both increased cellular MGO content, suggesting that fatty acid metabolism may drive increased MGO formation (Dillon et al., 2015). Similar results were obtained in HepG2 cells treated

with oleic and linoleic acid to induce lipid accumulation, which lead to an increase of endogenous MGO-derived CML levels coupled with an increase in IL-8 mRNA expression (Gaens et al., 2012). This suggests that high levels of fatty acids together with hyperglycaemia in T2DM may work in synergy to produce increased MGO generation in the liver, both leading to increased pro-inflammatory gene expression. In response to Kupffer cell-derived cytokine stimulation, which is also abundant in the liver in states of metabolic dysfunction, HepG2 cells and primary hepatocytes also produce large amounts of IL-8 (Thornton et al., 1991).

IL-8 is considered as an important cytokine in the inflammatory process. It is a multifunctional member of the CXC chemokine family that has potent chemoattractant and mitogenic effects on neutrophils and leukocytes and promotes neutrophil and macrophage infiltration (Desbaillets et al., 1997; Osawa et al., 2002; Rafaqat et al., 2024). ELR-CXC chemokines, including IL-8, also exert antiapoptotic effects (Osawa et al., 2002). However, IL-8 overexpression enhances oxidative stress in the mouse liver, and neutrophil-driven NASH progression in mice (Cho et al., 2023). Neutrophils express chemokine receptors, such as CXCR1 and CXCR2. CXCR1 functions as a receptor for CXCL6 and IL-8, while CXCR2 interacts with CXCL1, CXCL2, CXCL6, and IL-8 (Liu et al., 2016). Compared with the livers of patients with fatty liver, the livers of patients with NASH exhibit upregulated expression of neutrophil-recruiting chemokines their receptors CXCR1 and CXCR2 (Bertola et al., 2010). Increased plasma IL-8 levels were also detected in NAFLD and NASH patients (Ajmera et al., 2017; Auguet et al., 2020; Bahcecioglu et al., 2005; İlyas et al., 2003; Torer et al., 2007) and the presence of the A/A genotype in the IL-8 gene is associated with disease progression (Cengiz et al., 2014). Increased serum IL-8 correlate with other markers of liver damage and overall inflammation (Birerdinc et al., 2014). Histological data from paediatric NALFD patients revealed that IL-8 levels correlate with the degree of steatosis fibrosis severity (Perito et al., 2017), and another study found a significant correlation between circulating IL-8 levels and hepatic toll-like receptor 2 expression in obese women diagnosed with NASH (Auguet et al., 2020). Therefore, it may play a crucial role in the disease in which inflammation is a substantial pathophysiological feature (Birerdinc et al., 2014).

In addition, significant correlations for IL-6 and TNF-α with NAFLD have also been observed (Das & Balakrishnan, 2011; Duan et al., 2022). IL-6 and TNF-α have been extensively studied in metabolic disorders and have been established to be key mediators in chronic low-grade systemic inflammation and IR resistance, by interfering with insulin signalling pathways

(Rafaqat et al., 2024). TNF-α has been implicated in not only the early stages of NAFLD but also the transition to more advanced stages of liver damage and NASH in animals and humans (Crespo et al., 2001; Ding & Yin, 2004; Manco et al., 2007). In HepG2 cells, IL-8 is produced in response to stimulation by TNF-α (Dong Wumin, 1998). In Hc normal human foetal hepatocytes, among the genes upregulated by TNF-α treatment, IL-8 showed the highest increase in expression level, which was reversed by the inhibition of the NF-κB and PI3K/Akt pathways (Osawa et al., 2002).

IL-8 production is reported to be controlled by a variety of signals, for there are several potential binding sites for nuclear transcription factors such as AP-1, a downstream target of JNK, and NF-κB, in the IL-8 promoter region (Iguchi et al., 2000; Okamoto et al., 1994; Osawa et al., 2002). This highlights the overlap of metabolic and inflammatory pathways. Akt, a downstream effector of PI3K, is also involved in IL-8 production, although its activation alone is not sufficient to induce IL-8 production. More importantly, NF-κB has been shown to be essential for expression of IL-8 in several types of cells (Osawa et al., 2002; Osawa et al., 2001). Significant IL-8 secretion appears to occur after 6 hours, which suggests the overall increase in IL-8 production in HepG2 cells is a result of the activation of transcription mechanisms as it occurs shortly after NF-κB activation (at 4 hours), rather than direct secretion. We also observe that BBGC treatment induces the expression of TNF-α and possibly IL-6 mRNA in HepG2 cells. This increase may represent both a causal factor and a consequence of the NF-kB activation that occurs as a result of the treatment. Many studies have corroborated MGO and MGO-AGE-induced NF-kB activation (Hollenbach, 2017; Lai et al., 2022; Lee et al., 2021), including in HepG2 cells (S. H. Cha et al., 2019), while others have also implicated the role of MGO in increasing TNF-α and IL-6 in the liver and in HepG2 cells(S. H. Cha et al., 2019). In mice, a significant increase in hepatic TNF-α and IL-1β levels was shown after MGO treatment (Cheng et al., 2015), and lower TNF-α expression was found in cardiac tissue from STZ diabetic mice over expressing GLO1, compared to their WT-STZ diabetic counterparts (Branka Vulesevic et al., 2016).

NF-κB is ubiquitously expressed, including in hepatocytes, where it takes on the role of proliferation and survival regulation during cell regeneration and development (Mohamed et al., 2016). NF-κB activation is mediated by diverse signals including AngII, AGEs, oxidized LDL, ROS, pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and C-reactive protein (Basta et al., 2004; Brasier, 2010; Han et al., 1999; T. Liu et al., 2017). These pro-inflammatory

compounds activate IκB kinase (IKK) to phosphorylate inhibitor of NF-κB (IκB), leading to the nuclear translocation of NF-κB (Rohm et al., 2022; Wellen & Hotamisligil, 2005). The findings demonstrating its activation in the liver of diabetic patients (Daniel et al., 2013) can be explained by the increased serum levels of, AngII, AGEs, TNF-α, IL-6, and C-reactive protein in these patients (Sena et al., 2013). In addition, as discussed in the previous chapter when ER stress occurs, UPR is activated. Notably, elements of the UPR converge on many inflammatory pathways including JNK and IKK, and the ER is a critical site for inflammasome activation and thus may constitute another mechanism that amplifies the activation of NF-kB during cellular stress (Hung et al., 2004; Yang et al., 2015).

Increased low grade inflammation has been suggested to be a consequence of activation of the UPR. All three major UPR pathways, ATF6, PERK, and XBP-1, have been implicated in inflammatory signalling (Janssens et al., 2014; Yamazaki et al., 2009). The histone-lysine N-methyltransferase SETD7 pathway, which is activated in response to XBP-1, is a pathway that may particularly be linked to dicarbonyl stress (Rabbani et al., 2021). When XBP-1 is activated, it induces SETD7 expression, which in turn which increases methylation of lysine 4 of histone 3 on NF-kB p65 promoter, p65 expression and binding activity. This was associated with the upregulation of the NF-kB inflammatory cascade and subsequent transcription of inflammatory genes such as IL-8, cell adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein-1 (MCP-1) as well as RAGE and GRP78. It was reported that this proinflammatory signalling mediated by SETD7 was activated in a transient and persistent model of hyperglycaemia and, interestingly, was inhibited by overexpression of GLO1 (El-Osta et al., 2008). In our experiments, inducing ER stress in HepG2 cells by using tunucamycin triggers a strong response in IL-8 secretion, which is likely indicative of the importance of ER-stress in the inflammatory response and in particular, the modulation of IL-8 production in hepatocytes.

ER stress and increases in TNF-α and ROS production within the cell, as observed in our experiments, constitute inflammatory and stressful stimuli, that are known to activate IKK (Rohm et al., 2022; Wellen & Hotamisligil, 2005). Indeed, an important means by which MGO may contribute to inflammation is by modulating oxidative stress, as covered in chapter 3, which would then activate NF-κB. IL-8 mRNA expression and secretion were also induced in HepG2 cells exposed to H_2O_2, and the presence of antioxidant agents reduced this response (Dong Wumin, 1998), emphasising the link between oxidative stress and IL-8 induction. Many

studies have provided insight into the mechanisms involved, implicating mitochondrial dysfunction and oxidative stress, leading to repetitive liver inflammation, as major drivers of chronic liver disease development (Hotamisligil, 2006), both of which are present in our studies. In a rat cirrhosis model, decreased liver GLO1 expression was observed, which led to MGO accumulation, inflammation and stress (Hollenbach et al., 2017). This is also consistent with findings of a study where the overexpression of GLO1 in mouse models reduces MGO-induced inflammation and prevents ventricular dysfunction (Nigro et al., 2019).

The activation of NF-κB may trigger different types of responses depending on stimuli received, such as pro-apoptotic gene activation or cell regeneration (T. Liu et al., 2017). Activation of IKK in liver and myeloid cells appears to contribute to obesity-induced insulin resistance (Wellen & Hotamisligil, 2005) and inappropriate and persistent activation of NF-κB in T2DM patients with liver damage may play a pivotal role in the progression of hepatic steatosis to cirrhosis. It was reported that the activation of NF-κB may play a part in hyperglycaemia induced apoptosis, which can synergise with fatty acid induced inflammation, to increase liver tissue damage through indirect and direct production of ROS by NF-κB, yielding additional oxidative damage via LPOs, AGEs and POPs (Hollenbach, 2017; Lai et al., 2022), which can further activate NF-κB. Pro-inflammatory cytokines were also found to induce the production of free radicals, initiating a vicious cycle and further increases overall inflammation (Chatterjee, 2016; Elmarakby & Sullivan, 2012). Thus, the activation of NF-κB by MGO may initiate a series of deleterious events in the liver, as NF-κB further stimulates the secretion of pro-inflammatory mediators, via increasing the expression of genes encoding cytokines such as TNF-α, IL-8 and IL-6 (T. Liu et al., 2017; Rohm et al., 2022), cell adhesion molecules, chemokine receptors and inducible enzymes such as NADPH oxidase, COX2 and iNOS, collectively contributing to neutrophil infiltration, increased inflammation, and liver injury. In mice, the increase of NADPH oxidase 2 complex components implicated in the neutrophil oxidative burst was linked to IL-8-induced liver damage (Cho et al., 2023).

Furthermore, important determinants of IR are inflammatory mediators released from a fatty liver (Gehrke & Schattenberg, 2020) and circulatory cytokines have been linked to impaired insulin sensitivity through inhibitory phosphorylation of serine residues of IRS-1, which reduces both tyrosine phosphorylation of IRS-1 in response to insulin and the ability of IRS-1 to associate with the insulin receptor, and thereby inhibits downstream signalling and insulin action (Qian et al., 2019; Wellen & Hotamisligil, 2005). Thus, IKK plays a large role in

counteracting insulin action, by directly phosphorylating IRS-1 on serine residues (Yin, Yamamoto and Gaynor 1998, Gao, Hwang et al. 2002) as well as through activating NF-κB and stimulating production pro-inflammatory cytokines (Shoelson, Lee and Yuan 2003).

In addition to increased NF-κB activation and an increase in the expression of pro-inflammatory cytokines, we show for the first time that BBGC-induced dicarbonyl stress increases the expression and secretion of key hepatokines FGF21 and GDF15 in HepG2 cells. Several stimuli can lead to increased FGF21 and GDF-15 expression in hepatocytes, including glucose (K. Iizuka et al., 2009; Karczewska-Kupczewska et al., 2012; Schernthaner-Reiter, Kasses, Tugendsam, Riedl, Peric, Prager, Krebs, Promintzer-Schifferl, Clodi, & Luger, 2016; Schernthaner-Reiter, Kasses, Tugendsam, Riedl, Peric, Prager, Krebs, Promintzer-Schifferl, Clodi, Luger, et al., 2016) and ROS, the former known to be a source of endogenous MGO production. In line with this, both MGO and ROS accumulation are seen in HepG2 cells treated with BBGC. In male rats fed a high-fat high-fructose diet, which is a known precursor of MGO, FGF21 was also increased, although this effect was not seen in female rats in the same study. This was associated with marked liver damage and similarly to our findings, inflammation, and oxidative stress (Chukijrungroat et al., 2017). Furthermore, in a murine model of early onset diabetes, Akita mice, severe hyperglycaemia higher HbA1c correlated with up-regulated mRNA expression of GDF15 and FGF21. In this study, MGO levels were shown to positively and strongly correlate to HbA1c levels. They also reported elevated mitochondrial oxidative stress in cardiac tissue, consistent with hyperglycaemia-induced mitochondrial dysfunction (Park et al., 2020).

As mitochondrial dysfunction is a feature of MGO mediated cellular toxicity, it is worth noting that the effects in our study observed may be mitochondrial dysfunction related. Both FGF21 and GDF15 are strongly induced in genetically modified mouse models of mitochondrial dysfunction (Dostálová et al., 2009; Ehses et al., 2007). Mechanistically, mitochondrial dysfunction is largely associated with local activation of the integrated stress response (ISR) and mitochondrial UPR (Lefer et al., 1990) that involves the activation of the transcription factors and signalling molecules such as transcription factor associated with stress-1 (ATFS-1), ATF4, p53, target of rapamycin complex 1 and increased phosphorylation of eIF2α via several possible upstream regulatory mechanisms (Hausenloy & Yellon, 2004; Liu et al., 2019; X. Wang et al., 2016). This promotes not only a reduction of overall protein synthesis while selectively favouring translation of specific proteins required for the cellular stress response,

such as GRP78/BiP encoding ER chaperone proteins (Rampidis et al., 2019), but also the secretion of hepatokines, especially FGF21 and GDF15. A key effector of this mechanism is ATF4. Consequently, mitochondrial stress–induced ISR pathway activation is sufficient to increase the expression of FGF21 and GDF15, with the eIF2α–ATF4 axis regulating FGF21 expression (Kempf et al., 2012) and the eIF2α–ATF4–CHOP axis activating GDF15 expression (Eddy & Trask, 2021; Keipert & Ost, 2021; Welsh et al., 2003). It is important to note that the ISR pathway can also be stimulated by ER stress (Asrih et al., 2023). Both mRNA and protein expression CHOP expression were seen to be upregulated in HepG2 cells treated with BBGC, which suggests the increases may also be ER-stress (mitochondrial UPR) related.

As discussed previously, GDF15 belongs to the transforming growth factor-β (TGF-β) superfamily proteins and is considered an inflammatory marker (Jensen-Cody & Potthoff, 2021). A large number of data confirms it plays a major role in the development of inflammation, and metabolic disorders such as T2DM and NAFLD (Rochette et al., 2020). Overall, the epidemiological data consistently identifies elevated GDF15 serum levels as an independent risk factor for insulin resistance or diabetes or a marker of increased risk of developing these conditions (Dostálová et al., 2009). In both those with diabetes or NAFLD, serum or liver levels of GDF15 also independently predict end-organ damage and other complications and is considered a potential biomarker for assessment of the severity of the disease (Asrih et al., 2023; Breit et al., 2021; Bo Kyung Koo et al., 2018; Schmid et al., 2022). Serum GDF-15 concentrations were also markedly higher in patients with NAFLD and T2DM than in those without T2DM, and positively correlated with fasting glucose and HbA1c concentrations in these patients. Moreover, other correlations were observed with liver IL-6, IL-8 and hs-CRP concentrations, suggesting that chronic hyperglycaemia and inflammation have a role in increasing hepatic GDF15 secretion and thus circulating concentrations of GDF15 (Bilson et al., 2021).

GDF15 is significantly induced in cells as a result of stressful stimuli (Rochette et al., 2020) such as hypoxia, proinflammatory cytokines, oxidative stress, and ER stress (Asrih et al., 2023) (Adela & Banerjee, 2015; Yakut et al., 2021). Numerous regulators of GDF15 expression have been identified. NF-κB, HIF-1a and AP-1, regulate GDF15 expression (Asrih et al., 2023) and as mentioned above, following mitochondrial UPR and ISR activation, ATF4, CHOP and the stress-responsive transcription factors p53 can induce GDF15 expression, as well as early growth response 1 (EGR1) and AMPK, potentially. EGR1 levels correlate with steatotic

injuries in patients with NAFLD, and likely contributes to NAFLD likely by promoting insulin resistance and inflammation and suppressing fatty acid oxidation (Guo et al., 2023; Z. Li et al., 2019). EGR1 is reported to being upregulated in diabetes (Karthikkeyan et al., 2018), and has been shown to be induced by oxidative stress and inflammation, hyperglycaemia, and AGE-RAGE interaction (Karthikkeyan et al., 2018; Pagel & Deindl, 2012; Xu et al., 2010). Our results are in in line with a previous study where, in mouse cardiac endothelial cells treated with MGO for 24 hours, GDF15 mRNA expression was strongly induced (Bulkescher et al., 2021). In our group, we also examined the effects of palmitic acid on GDF15 expression and release (data not shown). Although palmitic acid is known to trigger NF-κB activation, the treatment showed no effects on GDF15 in HepG2 cells.

Thus, GDF15 is considered to be a stress-responsive cytokine that plays a significant role as a metabolic regulator, although its precise mechanism of action and its exact role in the pathophysiology of diabetes, an NAFLD remains to be elucidated. Similarly to FGF21, it carries out a beneficial role which has been supported by several experimental and clinical studies. It is involved in lipid and glucose metabolism, increased insulin sensitivity, and protection from chronic inflammation in adipose tissue (Adela & Banerjee, 2015), so it may be hypothesised that T2DM/NAFLD may induce a state of resistance to endogenous GDF15. GDF15 induction in the liver and elevated serum levels are also considered a part of the anti-inflammatory response and a compensatory mechanism during metabolic disorders, as evidenced by aggravated hepatic steatosis, inflammation, fibrosis and metabolic deterioration in *Gdf15*-knockout mice, all of which were attenuated in *Gdf15*-transgenic mice (Kim et al., 2018). Li et al. proved that increased GDF-15 protects endothelial cells against high glucose induced cellular injury by activating PI3 K/AKT/eNOS signalling pathway and attenuating NF-κB/JNK activation. Nitric oxide production was significantly lower in GDF-15 siRNA transfected HUVEC cells, emphasizing its protective role against cell apoptosis through PI3 K/Akt/eNOS pathway (Li et al., 2013).

The underlying mechanisms likely involve the modulation of hepatic β-oxidation genes by GDF15, leading to increased fatty acid oxidation and increased triglyceride export in addition to reduced ROS generation (Asrih et al., 2023). In conjunction with this, cultured myoblasts and hepatocytes treated with recombinant GDF-15 were found to have increased respiration and fatty acid oxidation in a dose dependent manner (Chung et al., 2017). These findings suggest that GDF-15 may be important in maintaining cellular respiration, particularly under

conditions of stress (Eddy & Trask, 2021). Furthermore, recent studies indicate that the antidiabetic and anorexic effects of metformin, are mediated by an increase in GDF15 serum levels possibly as a result of AMPK activation, although the mechanisms are still unclear (Aguilar-Recarte et al., 2023; Coll et al., 2020; Day et al., 2019).

However, the consensus of the studies suggests that exogenous GDF15 exerts protective effects against obesity and insulin resistance, while the role of endogenous GDF15 in these diseases is more ambiguous and therefore the consideration of GDF15 as a promising therapeutic compound in the management of NAFLD should be revisited. One study in particular has identified GDF15 as a pro-fibrotic factor by activating of stellate cells in mouse cirrhotic livers and primary hepatic stellate cells and in (Bo Kyung Koo et al., 2018; Qi et al., 2021) and in humans, patients with advanced cirrhosis or chronic liver failure exhibit a strong correlation of GDF15 with macrophage activation, and cytokine and chemokine production (I. W. Zhang et al., 2022). However, this effect seems to be associated with more severe stages of disease. Conflicting results were also revealed in animal models of cardiovascular disease, where some studies suggest that GDF15 may worsen disease (Tsai et al., 2018) while Li et al. proved that increased GDF-15 protects endothelial cells against high glucose induced cellular injury by activating PI3 K/AKT/eNOS signalling pathway and attenuating NF-κB/JNK activation. GDF15's intended action may initially be adaptive to help restore homeostasis in an systemically autocrine manner, but in disorders where significantly elevated levels are observed, GDF15's actions may become maladaptive (Breit et al., 2021).

Currently, our understanding of the physiological regulation of GDF15 and the mechanisms underlying GDF15 resistance are limited. The beneficial effects of GDF15 are thought to be mediated through the interaction of GDF15 with one of its identified receptors, glial-derived neurotropic factor family receptor-αlike (GFRAL), as demonstrated in the lack of response to GDF15 in GFRAL knockout animal models (Mullican et al., 2017; Yang et al., 2017) and the positive effects of GFRAL receptor agonists (Benichou et al., 2023). When GDF15 binds to GFRAL, it forms a complex with a co-receptor, tyrosine kinase RET. Then, RET is phosphorylated, which leads to downstream signalling via AKT, ERK1/2, and phospholipase C-γ (Asrih et al., 2023; Baek & Eling, 2019). Moreover, as GDF15 belongs to the TGF-β family, it may potentially signal through the Smad pathway (Asrih et al., 2023; Rochette et al., 2021). Recombinant GDF15 promoted AMPK activation and reduced the levels of phosphorylated SMAD3 and the markers of gluconeogenesis and fibrosis in mouse primary

hepatocytes, suggesting that the metabolic alterations observed in *Gdf15*$^{-/-}$ models may be mediated by the TGF-β1/SMAD3 pathway, independently of GFRAL (Jurado-Aguilar et al., 2024).

Nevertheless, a recent study has implicated the matrix metalloproteinase MT1-MMP (also known as MMP14) in the negative regulation of GDF15–GFRAL signalling in the context of obesity (Tysoe, 2022), by directly cleaving GFRAL to reduce its expression level, thereby suppressing the activation of GFRAL by GDF15. Gene polymorphisms of *MMP14* have been associated with obesity and diabetes (Chun et al., 2010) and MT1-MMP is seemingly increased in obesity, ageing, and in age-related conditions (Asthana & Wong, 2024). Some suggest it plays an important regulatory role in lipid metabolism and insulin resistance by directly inhibiting insulin signalling (Asthana & Wong, 2024; Guo et al., 2022; Xia et al., 2021). Importantly, inhibition of MT1-MMP effectively protects against obesity and diabetes, making it an attractive target in the management of T2DM and other metabolic disorders (Asthana & Wong, 2024). However, the full extent of GDF15 downstream signalling has not yet been fully elucidated. Several recent studies suggest there may be other receptors local that are yet to be studied (Dogon et al., 2024; Lasaad & Crambert, 2024). This line of study is crucial to help fully understand the mechanisms behind GDF15 signalling and how it may contribute to liver disease at its different stages.

The results of our study indicate that in response to dicarbonyl stress, both and GDF15 and FGF21 mRNA expression and secretion in HepG2 cells is induced. FGF21 expression is regulated by nutrient signals to subsequently moderate nutrient homeostasis. Different FGF21 transcription factors (including peroxisome proliferator-activated receptor (PPAR) α, ATF4, carbohydrate response element-binding protein (ChREBP), and CCR4-NOT transcription complex subunit 6-like (CNOT6 L)) can be regulated under different states to promote FGF21 expression (Inagaki et al., 2007; Jensen-Cody & Potthoff, 2021; Ke et al., 2019; D. M. Lebensztejn et al., 2016; von Holstein-Rathlou et al., 2016; Watt et al., 2019). Under nutrient-deficient states, fatty acids activate the transcription factor PPARα, inducing FGF21 to increase hepatic gluconeogenesis, beta oxidation, and ketogenesis (Badman et al., 2007; Spann et al., 2022). ATF4 is another effector that induces FGF21 expression under nutrient deficiency (De Sousa-Coelho et al., 2012), ER and oxidative stress (Schaap et al., 2013; Wan et al., 2014). However, under conditions of carbohydrate load, FGF21 is predominately induced by ChREBP in the liver, to regulate *de novo* lipogenesis (Lundsgaard et al., 2017; Spann et al., 2022). Some

studies suggest hepatic PPARα is required for the carbohydrate load-induced induction of FGF21 by ChREBP and vice versa (Iroz et al., 2017) while others conclude ChREBP mediates glucose-induced repression of PPARα gene expression in pancreatic β-cells and brown adipocytes (Boergesen et al., 2011; Iizuka et al., 2013).

Nonetheless, ChREBP has been shown to directly bind to the *Fgf21* promoter in hepatocytes in response to high glucose concentrations (Katsumi Iizuka et al., 2009; Poungvarin et al., 2015). Models of hepatic ChREBP overexpression or activation, show increased liver FGF21 expression and circulating levels, reflecting a systemic ChREBP response with beneficial effects on glucose and energy metabolism (Abdul-Wahed et al., 2014; Benhamed et al., 2012) through a liver-brain axis (Talukdar et al., 2016; von Holstein-Rathlou et al., 2016). However, in disease, ChREBP activity in the liver is increased and contributes to hepatic steatosis through direct stimulation of the lipogenic pathway (Abdul-Wahed et al., 2017). The enhanced and aberrant hepatic ChREBP-induced release of an array of hepatokines could affect local or systemic insulin resistance (Abdul-Wahed et al., 2017; Hannou et al., 2018) and a recent study directly linked ChREBPβ to fructose-induced "fatty liver" and hepatic insulin resistance (Kim et al., 2019).

Regarding the effect of FGF21 on improving liver inflammation, FGF21 can regulate hepatic metabolic gene expression and hepatic mitochondrial function to improve inflammation through decreased phosphorylation of NF-kB p65 among other stress signalling kinases (Lee et al., 2016; Lee et al., 2012; Tan et al., 2023; Yu et al., 2015). Yu et al. also found that FGF21 could reduce mRNA expression of polyol pathway enzymes which facilitate the production of sorbitol and fructose, and in turn MGO formation (Yu et al., 2015). The beneficial effects of FGF21 seem to be predominately mediated by the PPARα/FGF21 axis. In NAFLD, PPARα exerts its protection through the upregulation of lipid oxidation and mitochondrial biogenesis and the transcriptional repression of inflammatory genes via the direct inhibition through binding to the JNK-responsive part of c-Jun and NF-κB p65 (Delerive et al., 1999; Lin et al., 2022; Mogilenko et al., 2013), and further evidence also shows that PPARα can bind and transactivate IκBα in hepatocytes, thereby increasing its activity to prevent the activation of NF-κB (Delerive et al., 2000; Zhang et al., 2014). PPARα agonism improved lipid and glycaemic parameters in patients with diabetic dyslipidaemia (Kaul et al., 2019), and may alleviate steatosis and injury of the liver, counteract insulin resistance (Shao et al., 2019), inflammation and NASH disease progression, and it reduces activated macrophages, pro-

inflammatory cytokine levels, and improves histological evidence of liver dysfunction as shown in rodent models (Larter et al., 2012; Lefere et al., 2020; Pawlak et al., 2014). The activation of PPARα also prevents the progress of diabetes complications (Lin et al., 2022). In humans, the expression of PPARα negatively correlates with the presence of NAFLD and the severity of steatosis (Francque et al., 2015). PPARα expression is also downregulated during diabetes (Lin et al., 2022) and variations in the PPARα gene are linked to higher plasma lipid concentrations in T2DM (Flavell et al., 2000). Aside from glucose, reduced PPARα expression is believed to be a result of JNK activation (Cao et al., 2023; Roduit et al., 2000; Vernia & Cavanagh-Kyros, 2014). In line with this, hepatic JNK1/2 ablation promotes PPARα activation and results in increased FGF-21, which was believed to partially mediate the beneficial metabolic effects of hepatic JNK1/2 deficiency (Vernia & Cavanagh-Kyros, 2014). On the other hand, AMPK was shown to induce PPARα expression (Joly et al., 2009), and as briefly mentioned in the previous chapter, MGO was shown to directly inhibit AMPK.

FGF21 has been suggested to serve as a potential diagnostic biomarker and therapeutic agent for metabolic diseases such as obesity, type 2 diabetes mellitus (T2DM), and fatty liver (Geng et al., 2020). Many studies helped establish the important role FGF21 plays in lipid and carbohydrate metabolism as well as energy and nutrient homeostasis (Su et al., 2019). Altogether, a plethora of evidence suggests that FGF21 itself plays a beneficial and protective role through increasing hepatic insulin sensitivity, decreases lipogenesis, triggers fatty acid β-oxidation, reduces hepatic ER stress and mitochondrial dysfunction, and diminishes VLDL delivery to the liver (through down-regulation of VLDL receptor expression in hepatocytes) (Flippo & Potthoff, 2021; Geng et al., 2020; Tezze et al., 2019). FGF21 could also decrease postprandial triglycerides (TGs) and facilitate fatty acid storage in adipose tissue (Dariusz M Lebensztejn et al., 2016; Schlein et al., 2016; Watt et al., 2019). A review by Tan et al. provides an exhaustive insight on the role FGF21 plays in metabolic dysfunction, notably in T2DM and NAFLD (Tan et al., 2023). These observations lead to the conclusion that these elevations in FGF21 may reflect an impaired endocrine signalling state and a compensatory response designed to attenuate the resulting lipo- and glucotoxicity (Watt et al., 2019). The aforementioned findings also seem to suggest its increased levels found in disease state may likely be due to AT4 activation as a result of ER stress and an overactivated ChREBP, rather than PPARα and so, the beneficial effects of the PPARα/FGF21 pathway may be lost or undermined as evidenced by the observable reduction in PPARα. This may be mediated by dicarbonyl stress, as it affects many of the pathways involved in its regulation. The increase in

the hepatic expression of FGF-21 was also suggested to reflect impaired endocrine signalling that results in FGF21 resistance, which may play a significant role in the pathogenesis and progression of both T2DM and NAFLD, similar to insulin resistance (Gehrke & Schattenberg, 2020; Dariusz M Lebensztejn et al., 2016).

FGF21 mediates its biological functions in target tissues by forming a cell surface receptor complex with FGF receptors (FGFR) and another coreceptor, β-Klotho. FGF21 directly binds to β-Klotho, which then facilitates binding to FGFR1c which initiates downstream signalling events (Cao et al., 2023; Jensen-Cody & Potthoff, 2021). While the specific signalling cascade of FGF21 has not been fully determined, activation of the FGF21/FGFR1c/ β-Klotho receptor complex results in the phosphorylation of ERK1/2 and FGF receptor substrate 2α (FRS2α) (Kharitonenkov et al., 2005). Its importance is further emphasized in murine models where the deletion of *Fgf21*, its receptor FGF receptor-1c, or the coreceptor *β*-klotho, results in greater adiposity, hepatic steatosis, and liver insulin resistance, increased hepatic glucose production, and hyperglycaemia (Watt et al., 2019).

Interestingly, human embryonic kidney (HEK)293T cells treated with 200μM MGO for 24 hours down-regulated FGFR1 and upregulated FGFR2 as well as diminished phosphorylation of FGFR1 and FRS2 and the phosphorylation of its substrate protein, FRS2α. NAC, aminoguanidine, or metformin reversed this effect. It is worth noting that mRNA expression of FGFR1 gene was not perturbed by MGO, so this effect may be a result of post translational modifications by MGO (Miao et al., 2016). Previous reports also revealed that renal Klotho mRNA and protein were significantly decreased in *db/db* mice, and a similar decline was observed in the primary cultures of mouse tubule epithelial cells treated with MGO-modified albumin (Zhao et al., 2011). Interestingly, the data of this study show that Klotho suppresses nuclear factor NF-κB activation and the subsequent production of inflammatory cytokines in response to TNF-α stimulation in kidney cells, including primary cultures of mouse tubular epithelium, HK-2, and HEK293 cells. The authors suggest that Klotho serves as an anti-inflammatory factor, regulating the production of NF-κB–linked inflammatory cytokines, chemokines, and growth factors via a noncanonical NF-κB activation pathway. States of FGF21 resistance, which often co-exist with states of inflammation, may be explained by dysfunctional FGF21/FGFR1c/KLB receptor complex activity, mediated by methylglyoxal induced damage. Further research is required to investigate this theory.

In conclusion, the use of BBGC appears to be a suitable model to study MGO-induced inflammation in hepatocytes, and the available evidence strongly suggests that MGO is involved in the inflammatory response in hepatocytes, via increasing IL-8 release, activating NF-κB and causing ER stress. We were also able to link for the first time dicarbonyl stress to the modulation of hepatokine release. MGO-induced inflammation and particularly hepatokine release may not only constitute a central mechanism by which dicarbonyl stress increases the incidence and severity of liver disease, but also affect other peripheral tissue signalling (Darakjian et al., 2021) by establishing destructive inflammatory and endocrine signalling and dysregulated glucose and lipid metabolism, which can further sustain and exacerbate insulin and hepatokine resistance. Targeting inflammation, by the identification of pathways that connect inflammation and hepatokine signalling to T2DM, can help manage and prevent T2DM complications including development of NASH and fibrosis. It may also improve risk stratification by using these inflammatory markers as potential indicators of the severity of the disease (Darakjian et al., 2021; Goldfine et al., 2011).

6. The impact of glyoxalase 1 inhibition on drug-metabolising enzymes in the liver

Introduction

T2DM patients tend to show large variability in drug treatment outcomes, including glycaemic control, and in the incidence of ADEs among patients receiving the same treatment (Manolopoulos, 2007; Pacanowski et al., 2008; Pearson, 2009). The progression of the disease and the treatment itself both can disrupt the expression and activity of drug transporters and drug-metabolizing enzymes (DMEs), potentially triggering pharmacokinetics-based drug interactions due to disruptions in drug absorption, distribution, metabolism (F. Chen et al., 2018). Clinical studies examining the impact of NAFLD on pharmacotherapy are fewer, but the available evidence strongly highlights the potential of NAFLD to equally lead to alterations of drug pharmacokinetic profiles, causing variable drug response, ADEs and possibly toxicity (Cobbina & Akhlaghi, 2017).

As briefly covered in chapter1, a key function of the hepatocyte is xenobiotic metabolism, which is coordinated by many various enzymes, of which cytochrome P450 (CYP) 3A4 and carboxylesterase 1 (CES1). Many studies indicate that diabetes is associated with a significant decrease in hepatic CYP3A4 enzymatic activity and protein level (Angiolillo et al., 2014; Dostalek et al., 2011; Dostalek et al., 2012; Gravel et al., 2019b; Hryniewiecka et al., 2019; Marques et al., 2002), where a link was possibly identified between the disruption of glucose homeostasis, i.e. hyperglycaemia, to the changes observed. Studies in T2DM animal models have shown both increased (Barnett et al., 1990; Kudo et al., 2010; Verrecchia & Guaitani, 1993) and more recently, decreased (Maximos et al., 2017; Yao et al., 2019; Zhou et al., 2016) levels of CYP3A4 expression and activity. It must be noted that the animal models used may not reflect accurately human CYP P450 mRNA and protein expression, but the clear dysregulation indicates this may be a feature of diabetic pathology. The discrepancies may be explained by the differences in the models used and/or the mode by which diabetes was induced (Maximos et al., 2017). Many studies also report a reduction in the expression and activity of CYP3A4 in NAFLD (Donato et al., 2006; Jamwal et al., 2018; Kostrzewski et al., 2017; Rey-Bedon et al., 2022; Woolsey et al., 2015), which correlated with severity of steatosis

and the progression of the disease (Kolwankar et al., 2007), as well as increased inflammatory markers (Fisher et al., 2009). Similar reductions were observed in steatotic hepatocytes *in vitro (Donato et al., 2006)*. In view of the high prevalence of NAFLD in the diabetic population, it is likely that liver injury associated with NAFLD could be involved in the downregulation of CYP3A4 activity in diabetes. Moreover, it has been demonstrated that CYP3A4 expression, protein level and enzymatic activity are selectively changed in chronic diseases such as liver cirrhosis (Yang et al., 2003) or hepatocellular tumours of the non-cirrhotic liver (Haas et al., 2009).

Although CES1 plays a critical role in the metabolism of many clinically important medications, as well as lipid homeostasis, CES1 pharmacogenetics are understudied compared other major drug metabolizing enzymes e.g. CYP P450s (Her & Zhu, 2020). Several discrepancies can be found among studies investigating CES1 expression in metabolic disease. Liver biopsies from patients with simple steatosis and NASH revealed elevated CES1 expression (Ashla et al., 2010) and CES1 mRNA expression level in adipose tissue was positively associated with body mass index, fasting glucose level, insulin, and triglycerides (Friedrichsen et al., 2013). Recent studies have also found that CES1 function was positively correlated with increased liver lipid storage and plasma lipid concentrations, indicating that CES1 might be heavily involved in lipid metabolism and is a potential drug target for the treatment of human metabolic disorders (Kaddurah-Daouk et al., 2018; Lian et al., 2018). In mice, it was reported that hepatic CES1 expression was decreased by fasting but elevated in a diabetic model, and glucose was able to induce CES1 expression in vitro and in vivo (Xiong et al., 2014; J. Xu et al., 2014).

However, in a study looking at 5 female obese T2DM patients vs. 5 female non-diabetic obese patients, decreased hepatic levels of CES1 were observed (Valle et al., 2012). In a study looking at 15 patients with T2DM vs 10 healthy subjects, CES1 mRNA expression in adipose tissue was downregulated in female T2DM patients and old male T2DM patients, vs. control, but upregulated in young male T2DM patients. These alterations were seen among other genes' expression relating to stress, cell differentiation, cell cycle and immune regulation. The differences seen suggest age and sex may have an impact on the pathogenesis of these dysregulations (Xiao et al., 2012; Yang et al., 2009) and appear to be tissue dependent. In a study carried out by Chen et al. aiming to investigate alterations of carboxylesterases in T2DM mice models, it was found that rodent orthologs to human CES1, CES1d and CES1e, had

decreased expression and capacity of hydrolytic activity in the liver and intestine. These alterations were also seen after high insulin treatment of primary mouse hepatocytes and HepG2 cells kept in a high-glucose DMEM medium, which was aimed to mimic T2DM conditions (R. Chen et al., 2015). In liver tissue obtained from STZ-induced diabetic mice , mRNA and protein expression of CES1 were also found to be significantly decreased (Ge et al., 2020) and a lower CES1 expression and activity in liver microsomes of the same model was reported (Yao et al., 2020). Other studies also corroborated these findings in hepatic CES1 mRNA, protein and activity in ZDF rats (Yao et al., 2019) and in the liver of diabetic db/db mice (Han et al., 2023). Hepatic CES1 is also decreased in an NAFLD murine model (He et al., 2019),.

The presence of oxidative stress and inflammatory mediators like TNF-α and IL-6 have been implicated in the alterations of nuclear factors in diabetes and NAFLD, including transcription factors encoding the genes for DMEs, which may eventually alter their expression. These alterations could be potential sources of drug variability in patients and could have serious consequences on safety and efficacy. As BBGC induces oxidative stress and in hepatocytes, we wanted to investigate the effects of BBGC mediated MGO accumulation on the expression of key DMEs CYP3A4 and CES1.

Experimental protocol and methods:

In a first series of experiments, HepG2 cells were exposed to 10 and 20 µM BBGC in HepG2 medium supplemented with 3% FCS and incubated for 24 and 48 hours to determine CYP3A4 and CES1 activity. Then, the cells were treated with 20 µM BBGC in HepG2 medium supplemented with 3% FCS and incubated for 24 and 48 hours to determine CYP3A4 and CES1 mRNA and protein expression. The BBGC-containing treatment medium was removed and replaced with fresh every 24 hours.

Ina second series of experiments, HepG2 cells were treated with a CES1 pharmacological inhibitor, WWL113 (0.003 to 3µM) before measuring HepG2 cell viability, cellular ATP levels, IL-8, GDF15 and FGF21 secretion and mRNA expression.

CYP3A4 Activity assay

A lytic P450-Glo™ CYP3A4 Assay (Promega) was performed to determine CYP3A4 activity in our cells. In summary, HepG2 cells were grown in a white-walled culture plate with clear-

bottom wells to avoid luminescent cross talk between wells. Following treatment protocol, BBGC containing culture medium was removed and the cells were washed once with PBS. The treatment was replaced with fresh culture medium containing 3µM Luciferin IPA (proluciferin), a luminogenic CYP3A4 substrate and incubated for 45 minutes at 37°C. To determine background luminescence, 3µM Luciferin-IPA was added to to a set of empty wells. After incubation, 50µl of the Luciferin Detection Reagent provided (prepared as per manufacturer's instructions) was added to each well, and the plate was placed briefly on a plate shaker, to mix the components. Inside the cell, a CYP3A4 enzyme converts the substrate to a luciferin product, and luciferin is formed and detected with Luciferin Detection Reagent. The light output of the luciferase reaction is proportional to CYP3A4 activity. The plate was equilibrated at room temperature for 15 minutes prior to reading luminescence with an integration time of 1 second per well. The net signals were determined by subtracting background luminescence values (no-cell control) from test compound-treated and vehicle-control values. CYP3A4 activity was expressed as the % luminescence relative to vehicle treated control samples.

Carboxylesterase 1 activity assay

To determine CES1 activity following GLO1 inhibition, a specific quantitative CES1 activity assay kit (Abcam) was used. Following treatment protocol, the samples to be assayed were prepared. BBGC or WWL113 containing medium was removed and the cells were washed with PBS prior to mechanically harvesting the cells in PBS. Each sample was centrifuged to obtain a pellet, which was resuspended in the extraction buffer provided. The samples were the incubated-on ice for 20 minutes. Then, the extracts were centrifuged at 12000 × g, 4°C, for 20 minutes and the supernatant collected, and the pellet was discarded. The protein content in each sample was determined using the Bradford protein assay and diluted in incubation buffer (provided), equally load each well with 300 µg of cell extract protein. Then, the first set of the assay was conducted according to the manufacturer's instructions, to measure CES1 activity as a kinetic absorbance reading at 402 nm for one hour. The second set of the assay was then performed to quantitate CES1 content in each well as absorbance readings at 600 nm. Thus, we were able to derive net and relative CES1 activity values from the respective end-point absorbance readings

RESULTS

Measurement of CYP3A4 mRNA expression, protein levels and activity in HepG2 cells following GLO-1 inhibition

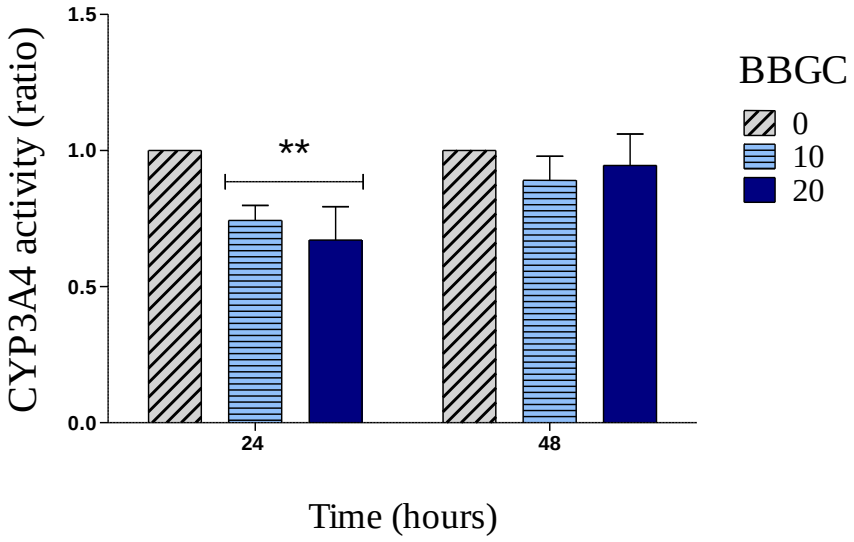


Figure 6.1. GLO-1 inhibition by BBGC decreases CYP3A4 activity in HepG2 cells at 24 hours but not 48 hours. CYP3A4 was assessed using a luminometric specific activity assay kit following treatment with 10 and 20 µM BBGC for 24 and 48 hours. CYP3A4 activity was significantly decreased 24 hours in a dose dependent manner but after 48 hours, it had no effect. Data are expressed as the ratio between luminesce units of the treated sample vs vehicle treated control ± SEM from *n* = 3-4 independent experiments (3 replicates per experiment). Statistically significant differences were determined using one way ANOVA with Bonferroni post hoc test. ** P < 0.01 as compared to vehicle-treated control group.

To examine whether key hepatocyte drug metabolism enzyme is affected by dicarbonyl stress, we first measured CYP3A4 enzyme activity. The results have shown that BBGC mediated dicarbonyl stress only decreases CYP3A4 activity at the earlier time point of 24 hours (Figure 6.1). At 48 hours of treatment, HepG2 CYP3A4 activity is recovered to that observed in untreated cells. To further understand CYP3A4 regulation in response to dicarbonyl stress, we analysed CYP3A4 mRNA and protein expression at 24, 48 and 72 hours. This revealed a rather more complicated picture than activity. CYP3A4 mRNA expression is unchanged at 24h however is significantly increased after 48 and 72 hours (Figure 6.2.A). CYP3A4 protein expression was unchanged at 24 and 48 hours however surprisingly (Figure 6.2.B). Therefore, there seems to be an apparent disconnect between CYP3A4 activity and expression following GLO-1 inhibition.

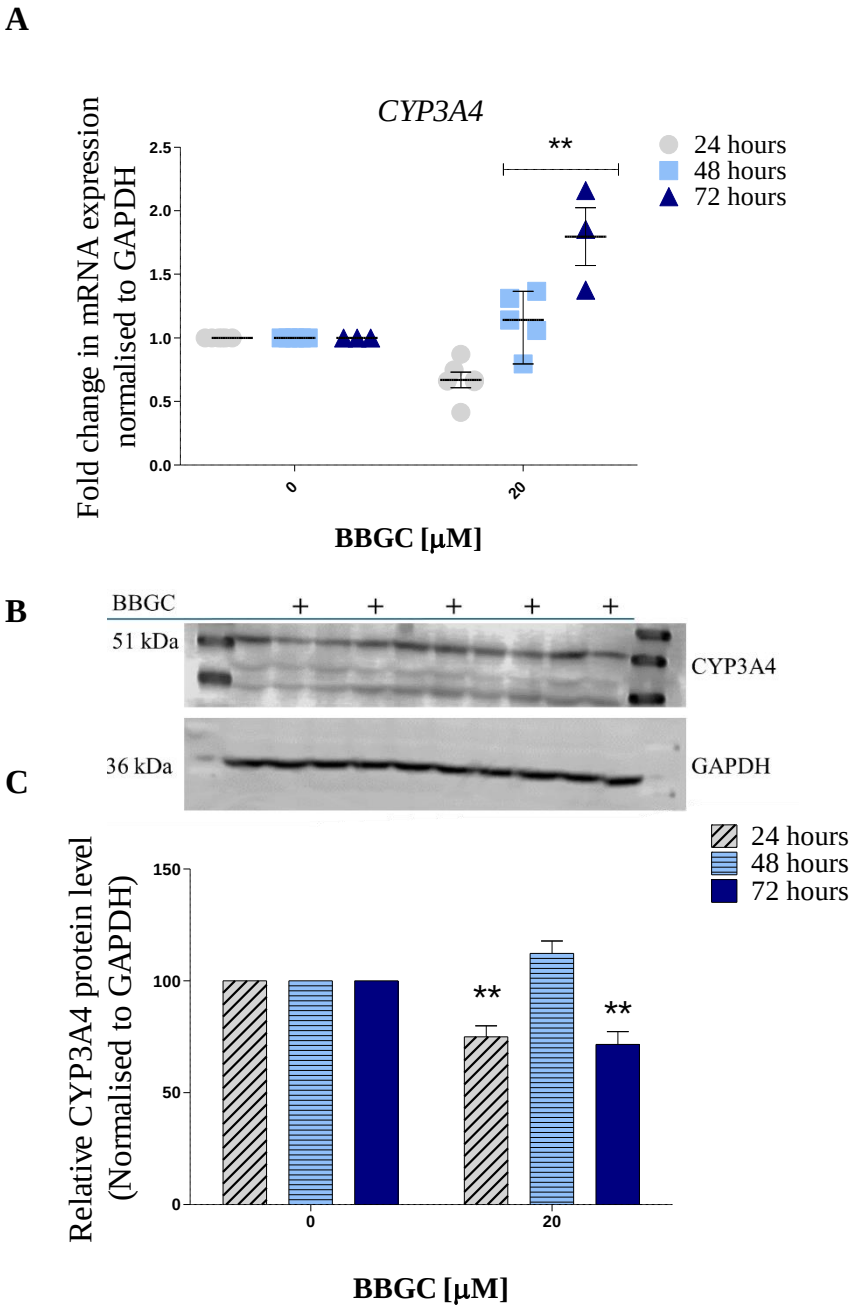


Figure 6.2. The effects of BBGC mediated dicarbonyl stress causes changes in CYP3A4 mRNA and protein expression in HepG2 cells. (A) CYP3A4 mRNA expression of was assessed by RT-qPCR and normalised to GAPDH following treatment with 20 µM BBGC for 24, 48 and 72 hours. CYP3A4 mRNA expression was found to be decreased only at 24 hours. The expression is restored at 48 hours and continues to increase at 72 hours. Data are expressed as $2^{(-\Delta\Delta Ct)}$ ± SEM *n* = 3-5 independent experiments. (B) Representative western blot of CYP3A4 protein expression from five independent experiments after 24-hour treatment with BBGC. The cell lysates were prepared after treatment with 20 µM BBGC for 24, 48 and 72 hours. 80µg of lysates was used to conduct Western blot analyses using antibodies against CYP3A4 and GAPDH. (C) GLO-1 inhibition by BBGC decreases CYP3A4 protein levels in HepG2 cells at 72 hours. The relative protein level was expressed as CYP3A4/GAPDH arbitrary units. Data are expressed as mean ± SEM *n* = 2-5 independent experiments Statistically significant differences were determined using Student's t-test, Kruskal-Wallis with Dunn's post hoc test and one-way ANOVA with Bonferroni post hoc test, where p-value $p < 0.05$ was considered significant. ** $P < 0.01$ as compared to vehicle-treated control group.

Measurement of CES1 mRNA expression, protein levels and activity in HepG2 cells following GLO-1 inhibition

Another important enzyme mediating key hepatocyte functions is CES1. Unlike the kit that was used to measure CYP3A4, the CES1 activity assay enables us to not only equally load the sample based on protein amount, but also quantify the level of CES1 protein that is present in the sample at the time of the assay. This allows to accurately assess whether the activity is changed in response to dicarbonyl stress. BBGC mediated dicarbonyl stress does result in decreased net activity (Figure 6.3.A) in a significant dose and time dependent manner, but not through inhibiting CES1 activity as demonstrated by the lack of effect in the CES1 activity data that was normalised against CES1 protein content (Figure 6.3.B). This was rather a result of a dicarbonyl stress mediated decrease in CES1 protein content in HepG2 cells (Figure 6.3.C). The use of a CES1 pharmacological inhibitor, WWL113, which inhibits its enzymatic activity was used as a control in these experiments. The results confirm that it CES1 activity is almost entirely abrogated in response to the treatment (Figure 6.3.B).

To further corroborate these observations, we measured CES1 mRNA and protein expression in HepG2 cells following treatment with BBGC. The findings confirm that in HepG2 cells, CES1 protein levels are significantly decreased by intracellular MGO (Figure 6.4.B), in a more consistent fashion compared to CYP3A4, possibly due to decreased *CES1* transcription levels. CES1 mRNA is indeed significantly decreased in a time dependent manner (Figure 6.4.A).

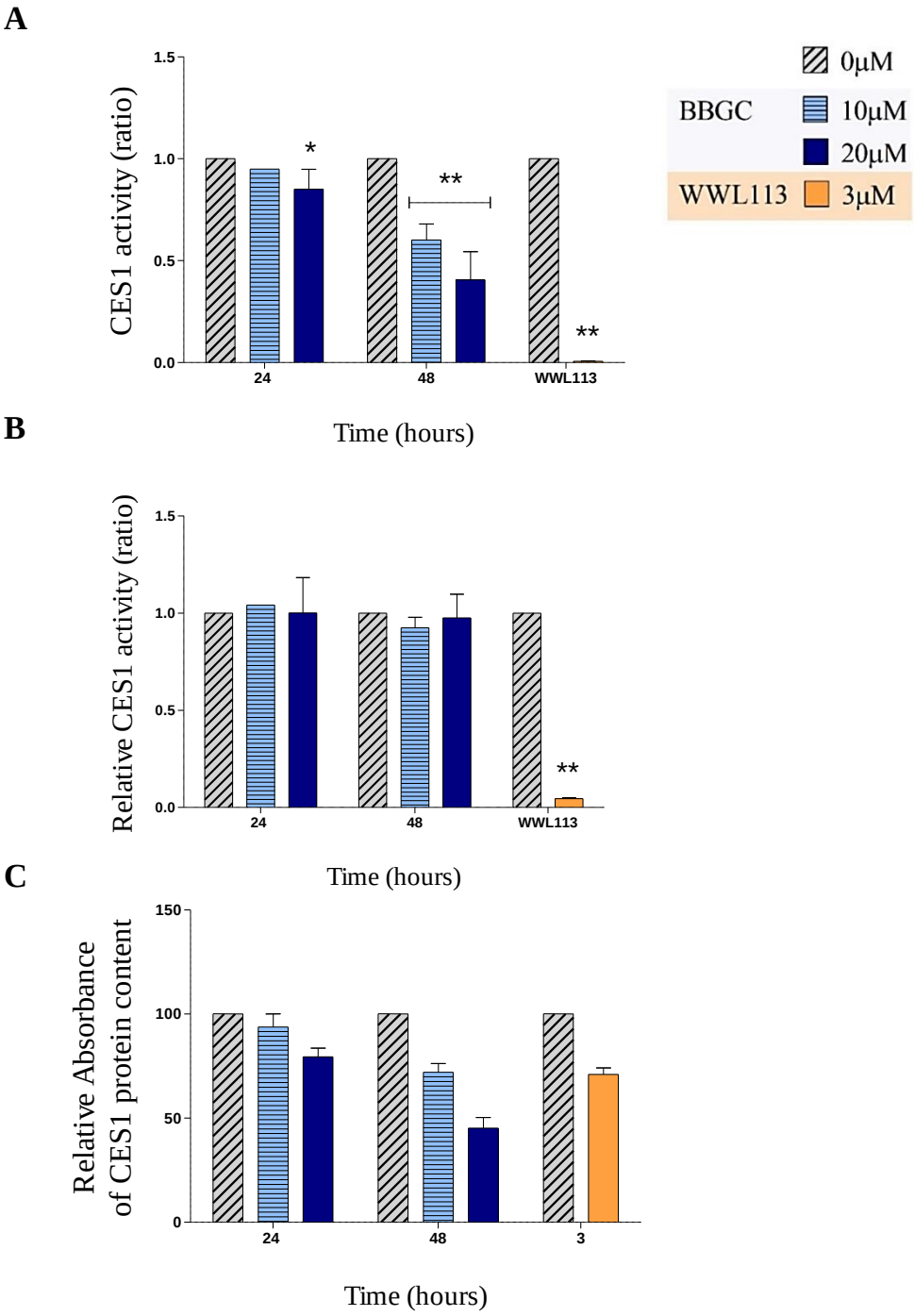


Figure 6.3. GLO-1 inhibition by BBGC decreases CES1 activity in HepG2 cells as a result of decreased protein expression. CES1 was assessed using a specific kinetic activity assay kit following treatment with 10 and 20 µM BBGC for 24 and 48 hours, in two different steps. (A) In a first set of measurements, a kinetic assay was carried out on equally loaded samples (300 µg of protein) to determine net CES1 activity for each sample. (C) In a second set of experiments, quantitative measurements of CES1 content for each sample were performed in order to determine the relative sample CES1 activity to its respective protein level, as shown in graph (B). This revealed that CES1 protein expression is downregulated as a result of dicarbonyl stress in HepG2 cells. Treatment with CES1 inhibitor WWL113 was used as a control experiment, which showed effective CES1 activity inhibition (B). Data are expressed as the ratio between absorbance measurements of treated sample vs vehicle treated control ± SEM from n = 3-5 independent experiments (1 replicate per experiment). Statistically significant differences were determined using one way ANOVA with Bonferroni post hoc test. ** $p < 0.01$ and * $p < 0.05$ as compared to vehicle-treated control group.

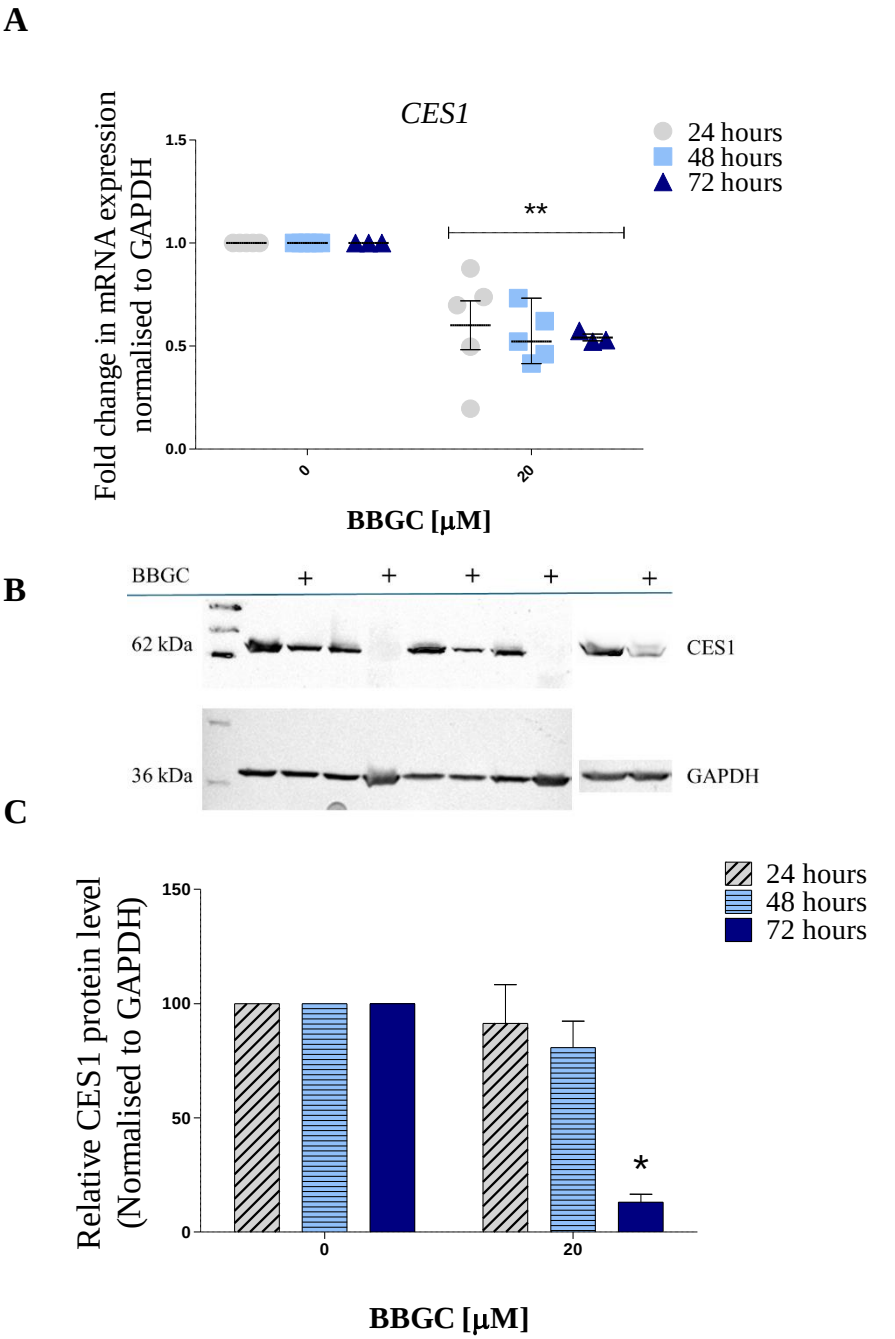


Figure 6.4. BBGC mediated dicarbonyl stress decreases CES1 mRNA and protein expression in HepG2 cells. (A) CES1 mRNA expression of was assessed by RT-qPCR and normalised to GAPDH following treatment with 20 µM BBGC for 24, 48 and 72 hours. CES mRNA expression was significantly decreased in a time dependent manner. Data are expressed as $2^{(-\Delta\Delta Ct)}$ ± SEM *n* = 3-5 independent experiments. B) Representative western blot of CES1 protein expression from five independent experiments after 72-hour treatment with BBGC. The experiments in which CES1 protein was not detected were not used in the analysis. The cell lysates were prepared after treatment with 20 µM BBGC for 24, 48 and 72 hours. 80µg of lysates was used to conduct Western blot analyses using antibodies against CES1 and GAPDH. (C) GLO-1 inhibition by BBGC decreases CES1 protein levels in HepG2 cells significantly at 72 hours. The relative protein level was expressed as CES1/GAPDH arbitrary units. Data are expressed as mean ± SEM *n* = 2-5 independent experiments Statistically significant differences were determined using Student's t-test, Kruskal-Wallis with Dunn's post hoc test and one-way ANOVA with Bonferroni post hoc test, ** $p < 0.01$ and * $p < 0.05$ as compared to vehicle-treated control group.

GLO-1 inhibition causes a reduction in PXR mRNA expression

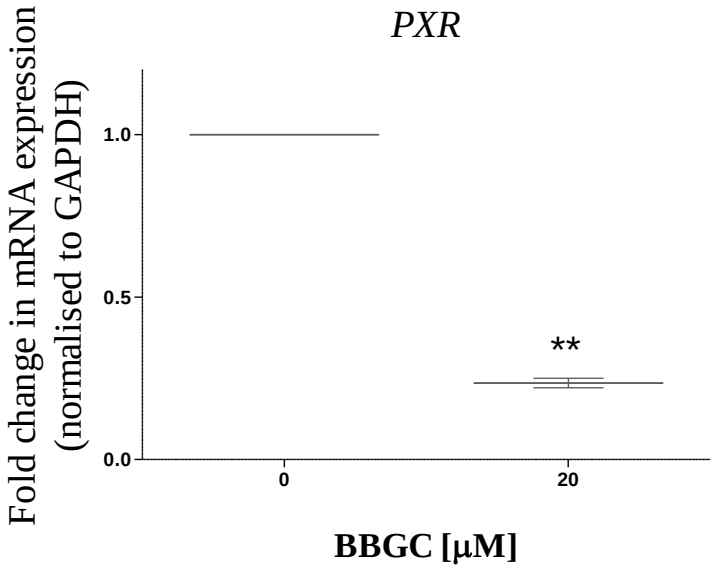


Figure 6.5. 24-hour exposure to BBGC decreases PXR mRNA expression in HepG2 cells. mRNA expression of PXR assessed by RT-qPCR and normalised to GAPDH following a 24-hour treatment with BBGC 20 µM. Data are expressed as $2^{(-\Delta\Delta Ct)}$ ± SEM n = 2 independent experiments. Statistically significant differences were determined using Kruskal-Wallis with Dunn's post hoc test. * $p < 0.05$ as compared to vehicle-treated control group.

To further understand the mechanisms behind the downregulation in CES1 expression, we examined several key transcription factors that regulate its expression. We attempted to measure PXR, FXR, CAR and PPARα expression after BBGC treatment but unfortunately, the primers for FXR, CAR and PPARα did not work during the RT-qPCR analysis and because of time restrictions, we were unable to carry on studies on these transcription factors. Although this was carried out in n=2 independent experiments as part of a screening, we are able to show that PXR may be downregulated in response to dicarbonyl stress in HepG2 cells (Figure 6.5).

The effects of pharmacological CES1 inhibition on HepG2 cell viability and cellular ATP levels

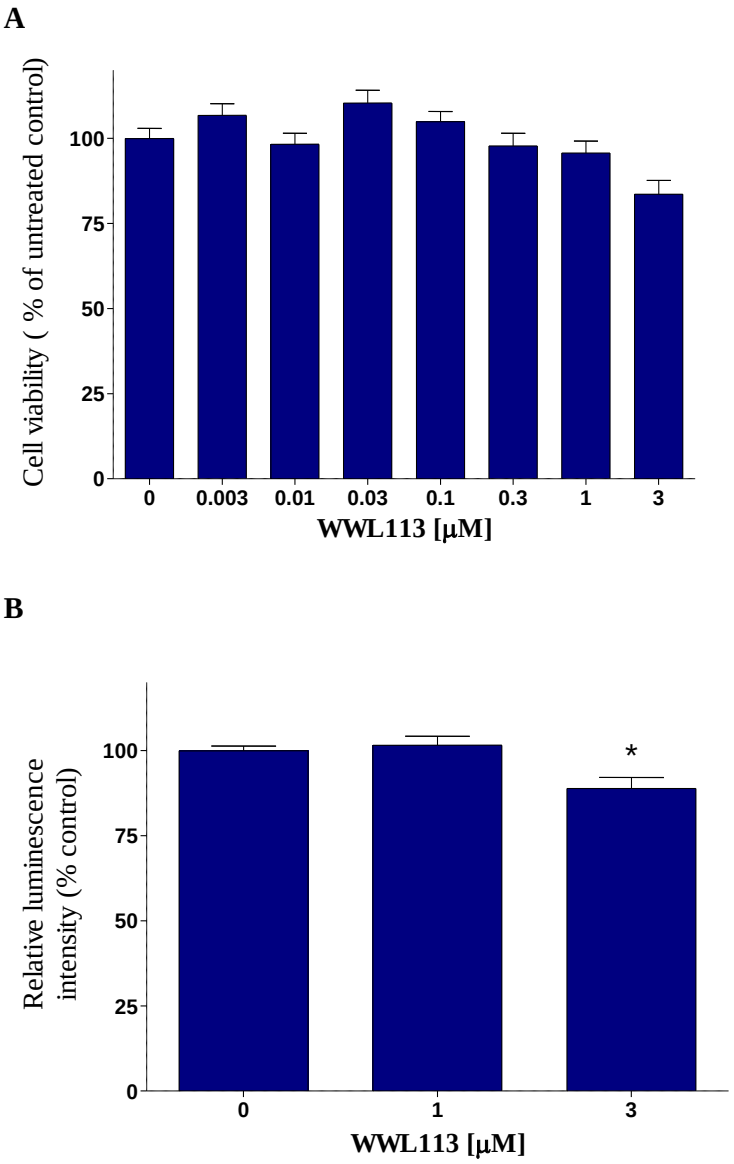


Figure 6.6. The effects of WWL113 treatment on HepG2 cell viability and cellular ATP levels. (A) 24-hour WWL113 treatment does not cause any significant effect on HepG2 cell viability compared to vehicle-treated control. Data are expressed as the mean ± SEM from n = 5 experiments (6 replicates per experiment). Statistical analysis was carried out using one-way ANOVA where p-value $p < 0.05$ was considered significant; *$p < 0.05$ vs. untreated cells. (B) WWL113 treatment causes a small but significant decrease in cellular ATP levels in HepG2 cells. HepG2 cells were exposed to WWL113 for 24 hrs before quantifying cellular ATP levels using the luciferase reaction followed by luminometry. Data are expressed as mean ± SEM from $n = 4$ independent experiments. Statistically significant differences were determined using Kruskal-Wallis with Dunn's post hoc test where $p < 0.05$ was considered as significant. * $p < 0.05$, significantly different from vehicle-treated control cells.

Due to the importance of CES1 on important hepatocyte functions that surpass drug metabolism, we wanted to focus on the effects of CES1 inhibition in HepG2 cells, especially

considering dicarbonyl stress in involved in its downregulation. The treatment of HepG2 cells with a CES1 pharmacological inhibitor, WWL113, had little to no effect on cell viability (Figure 6.6.A) but lead to a small decrease in cellular ATP levels at 3 µM WWL113 (Figure 6.6.B).

The effects of pharmacological CES1 inhibition on HepG2 inflammation.

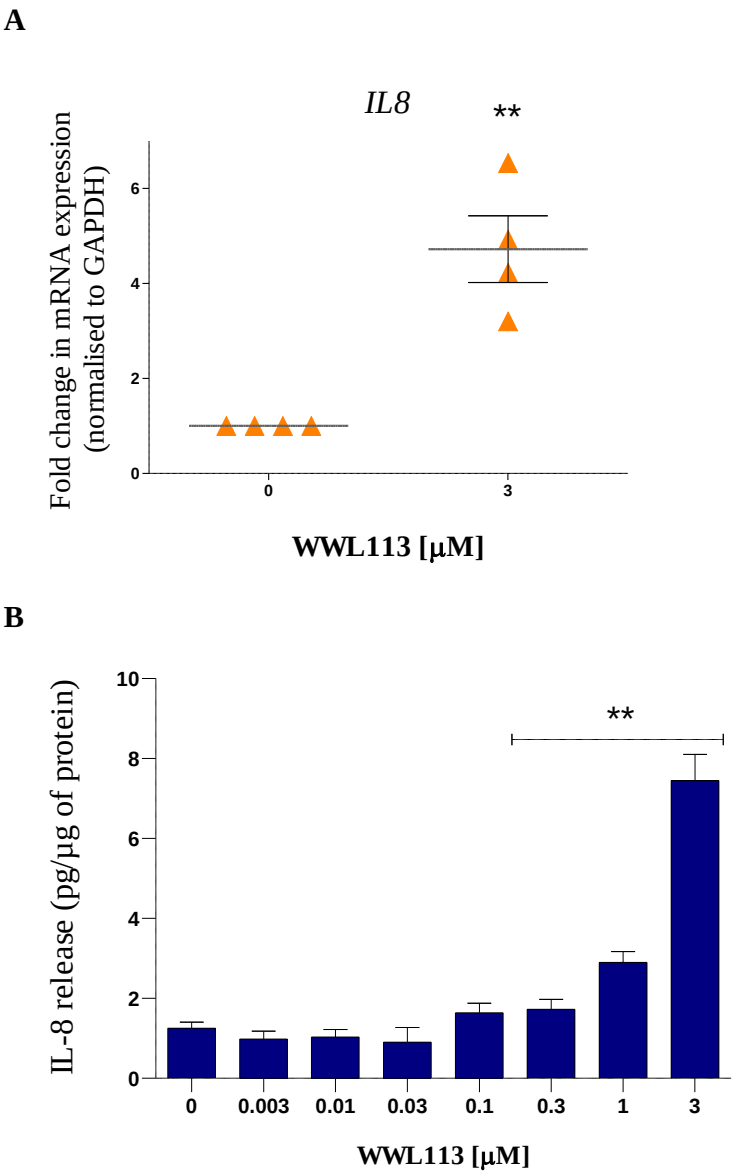


Figure 6.7. 24-hour exposure to WWL113 increases IL-8 secretion and mRNA expression in HepG2 cells. (A) CES1 inhibition causes a significant induction in IL-8 mRNA expression. mRNA expression of IL-8 was assessed by RT-qPCR and normalised to GAPDH following a 24-hour treatment with WWL113 (3µM). Data are expressed as $2^{(-\Delta\Delta Ct)}$ ± SEM *n* = 4 independent experiments. This was coupled with a dose-dependent increase in IL-8 release following 24-hour WWL113 treatment. (B) Data are expressed as the mean ± SEM from n = 4 experiments (3 to 6 replicates per experiment). Statistically significant differences were determined Kruskal-Wallis with Dunn's post hoc test and one-way ANOVA with Bonferroni post hoc test. **p < 0.01 and *p < 0.05 vs. vehicle treated cells.

Recently, increasing studies have brought attention to the role of CES1 in the modulation the inflammatory response. Therefore, we wanted to measure the effects of WWL113 on IL-8 secretion in HepG2 cells, which is an important proinflammatory cytokine considering its role in NAFLD and NASH. We have found that despite the lack of effect on cell viability, the loss of CES1 activity strongly and significantly triggers IL-8 mRNA expression (Figure 6.7.A) and secretion (Figure 6.7.B) especially at concentrations over 1 µM WWL113.

CES1 inhibition modulates the expression and release of hepatokines GDF15 and FGF21 in HepG2 cells.

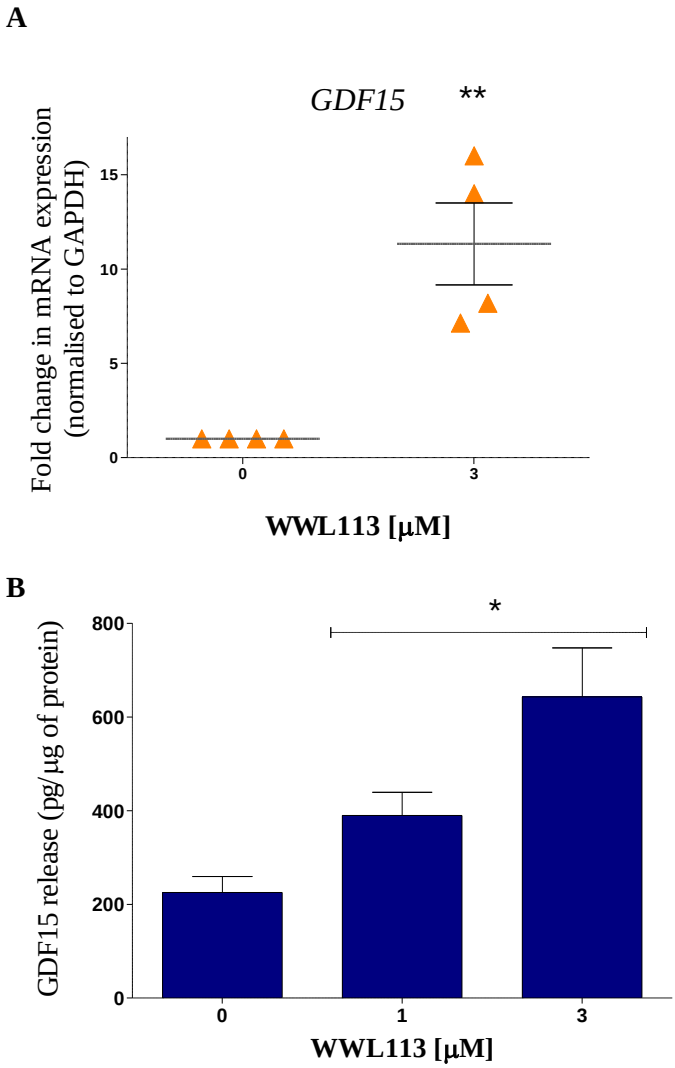


Figure. 6.8. Exposure to WWL113 increases GDF15 mRNA and secretion in HepG2 cells. mRNA expression of GDF15. (A) was assessed by RT-qPCR and normalised to GAPDH following a 24-hour treatment with WWL113 (3µM). Data are expressed as $2^{(-\Delta\Delta Ct)}$ ± SEM *n* = 4 independent experiments. GDF15 protein release (B) was measured following a 24-hour treatment with 1 and 3 µM WWL113 using a specific GDF15 ELISA kit. Data are expressed as the mean ± SEM from n = 3 experiments (3 replicates per experiment). Statistically significant differences were determined using Kruskal-Wallis with Dunn's post hoc test and one-way ANOVA with Bonferroni post hoc test. **p < 0.01 and *p < 0.05 vs. vehicle treated cells.

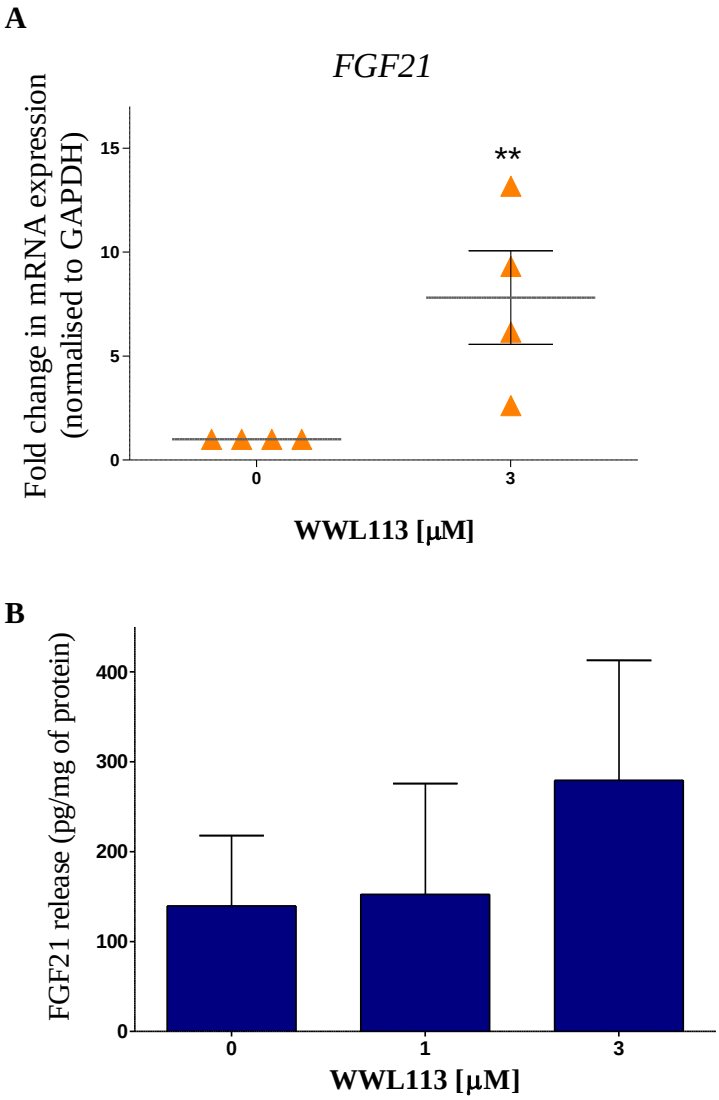


Figure. 6.9. Exposure to WWL113 increases FGF21 mRNA and secretion in HepG2 cells. mRNA expression of FGF21. (A) was assessed by RT-qPCR and normalised to GAPDH following a 24-hour treatment with WWL113 (3µM). Data are expressed as $2^{(-\Delta\Delta Ct)}$ ± SEM *n* = 4 independent experiments. FGF21 protein release (B) was measured following a 24-hour treatment with 1 and 3 µM WWL113 using a specific FGF21 ELISA kit. Data are expressed as the mean ± SEM from n = 3 experiments (3 replicates per experiment). Statistically significant differences were determined using Kruskal-Wallis with Dunn's post hoc test and one-way ANOVA with Bonferroni post hoc test. **p < 0.01 and *p < 0.05 vs. vehicle treated cells.

Similar to the increase in IL-8 mRNA expression and secretion, CES1 inhibition strongly and significantly triggers GDF15 mRNA expression and release in HepG2 cells (Figure 6.8). 3 µM WWL113 results on an over 10-fold increase in GDF15 mRNA expression at 24 hours (Figure 6.8.A). FGF21 mRNA expression is also strongly and significantly upregulated following CES1 inhibition (Figure 6.9.A) in HepG2 cells. Although FGF21 release did not reach significance, there is an important increase in its secretion at 3 µM WWL113 compared to vehicle treated control (Figure 6.9.B).

DISCUSSION

Our findings suggest that in HepG2 cells, BBGC treatment leads to a loss in the activities of DMEs CYP3A4 and CES1, at 24 hours. CYP3A4 activity is restored at 48 hours, but CES1 activity continues to decrease at this time-point. Interestingly, as the kit used to measure CES1 activity also quantifies CES1 protein levels in the loaded sample, we were able to normalise the activity to the amount of CES1 for each sample, to produce the relative CES1 activity, reflecting the true functional activity of this enzyme. This normalisation revealed that the activity was unaffected by BBGC, but in fact, a result of reduced CES1 protein expression as a result of the treatment. The kit to measure CYP3A4 did not have this feature, but it was interesting to observe a rebound effect in CYP3A4 activity. In order to expand upon these findings, we measured the mRNA expression and protein levels for both of these enzymes. mRNA expression of both CYP3A4 and CES1 were downregulated at 24 hours, but significance was not achieved for protein expression. For CYP3A4, this inhibitory effect on mRNA expression is absent after 48 hours and we see an increase of CYP3A4 mRNA expression at 72 hours (Figure 6.2.A.), suggesting a possible reinduction in transcription levels. The protein levels appear to increase after 48 hours but are however, reduced at 72 hours (Figure 6.2.C). This may explain why CYP3A4 activity was unaltered by BBGC at 48 hours. CES1, on the other hand, showed a time-dependent decrease in activity and both mRNA and protein expression (Figure 6.3 and 6.4), suggesting there may be distinct mechanisms involved in the changes in DME expression following by GLO1 inhibition,

CYPs P450 enzymes catalyse different oxidation and some reduction reactions to metabolise exogenous (xenobiotics) and endogenous compounds (Guengerich, 2017). The CYP 3A4 is the most expressed CYP in the liver and intestine and may represent about 60% of total CYP in liver (Dostalek et al., 2011). It belongs to the CYP3A subfamily (other members include CYP 3A5, 3A7 and 3A43) that metabolise more than 50% of currently used medications (Darakjian et al., 2021; Dostalek et al., 2011; Martignoni et al., 2006). CYP3A4 substrates include statins (except pravastatin), antiarrhythmics amiodarone and quinidine, calcium channel blockers nifedipine, diltiazem, beta blocker propranolol, apixaban, and anti-diabetic drugs such as sulfonylureas glibenclamide and glipizide, thiazolidinedione pioglitazone, DPP-4 inhibitors alogliptin saxagliptin and sitagliptin, sodium-glucose transport protein 2 inhibitor canagliflozin, meglitinides nateglinide, and repaglinide (F. Chen et al., 2018; Flockhart DA, 2021; Klyushova et al., 2022; May & Schindler, 2016). Any disruptions to the metabolism of

these drugs, which are all commonly prescribed in the diabetic population, can alter pharmacokinetic profiles, and possibly create additional challenges when tackling diseases and key systems that are related to diabetic complications. Furthermore, the bioactivation of clopidogrel is mediated by the conversion of 2-oxo-clopidogrel by CYP enzymes into a thiol metabolite, which is eventually converted into the active metabolite via CYP3A4/5 oxidation. 2-oxo-clopidogrel is the result of the CES1 mediated hydrolysis of clopidogrel (Alkattan & Alsalameen, 2021; Lau et al., 2004; Xu et al., 2022) which accounts to approximately 85% of clopidogrel and thus only 15% is subsequently metabolised by CYPs (Zhu et al., 2013).

In addition to xenobiotic metabolism, CYP3A enzymes participate in physiological functions by taking part in processes including steroid catabolism, and the metabolism of various endogenous compounds such as cholesterol, bile acids, long -chain polyunsaturated fatty acids (PUFAs) (arachidonic acid), and vitamin D (Klyushova et al., 2022). Cholesterol is metabolized to 4β-hydroxycholesterol by CYP3A4, which was proposed to serve as an endogenous marker of CYP3A activity in patients (Diczfalusy et al., 2011; Nitta et al., 2018). In bile acid homeostasis, CYP3A4 catalyses intermediates in the biosynthesis of primary bile acids and the hydroxylation of secondary bile acids (Furster & Wikvall, 1999; Zhang et al., 2019). CYP3A4 catalyses the 6α-hydroxylation of both taurochenodeoxycholic acid and the toxic secondary metabolite of bile acids lithocholic acid in a process designed to eliminate it (Araya & Wikvall, 1999).

Disruptions to these processes as a result of diminished CYP3A4 activity may lead to cholestasis, characterised by bile acid accumulation in the liver, resulting in bile duct damage, inflammation and hepatocyte death following their exposure to high concentrations of cytotoxic bile acids (Klyushova et al., 2022). Moreover, epoxyeicosatrienoic acids (EETs) are a class of functionally bioactive lipid mediators derived from the metabolism of long-chain PUFAs, such as arachidonic acid, by multiple enzymes among which, CYPs. One the main CYP enzymes involved in the biosynthesis of EETs in the liver is CYP3A4 (Colombero et al., 2020; Snider et al., 2007) and EETs have been shown to exert anti-inflammatory effects via downregulating TNF-α and IL-1β expression (Klyushova et al., 2022; Node et al., 1999; Zhang et al., 2013). Furthermore, it has been reported that CYP3A4 (Ile118Val) polymorphism, which is related to the activity of CYP3A4, is associated with increased susceptibility to T2DM in Japanese populations (Yamada et al., 2007). Although CYP3A4 demonstrated its ability to recover its expression levels after 48 hours in our experiments, it is still important to consider the ability

of MGO to interfere with its regulation, even if it's only at an earlier time-point, which coincides with the downregulation of PXR, its main regulator in the cell. This mechanism may still be of value to study the observed downregulation of CYP3A4 in T2DM and NAFLD, but further studies in different models are required. As an essential metabolising enzyme, CYP3A4's inducibility is known to be sensitive to numerous compounds, and the preservation of its expression may reflect greater resilience compared to CES1 in our experimental setting.

Indeed, similar to CYP3A4, the potential for alterations in CES expression and activity should be fully appreciated as altered CES1 xenobiotic metabolism may diminish drug efficacy and lead to unfavourable outcomes in the treatment of various ailments related to diabetes (F. Chen et al., 2018; Her & Zhu, 2020). CES1 is considered a phase I drug-metabolizing enzyme, and a recent proteomics study has shown that CES1 is the most abundant drug-metabolizing enzyme in human liver microsomes, responsible for 80%–95% of total hydrolytic activity in the liver (Achour et al., 2017; Imai, 2006). CES1 is responsible for the metabolism of a variety of ester-containing drugs, prodrugs, and environmental toxins. Such compounds include anticancer prodrugs like capecitabine, angiotensin-converting enzyme inhibitors (ramipril, enalapril, temocapril, imidapril, perindopril, quinapril, etc.), prodrug-type angiotensin II type 1 receptor blocker candesartan cilexetil, and many other clinically important medications and other drugs with ester moieties, such as fenofibrate, simvastatin, dabigatran and methylphenidate (Hosokawa, 2008; Imai, 2006; Ishizuka et al., 2013; Ross & Crow, 2007; Satoh & Hosokawa, 1998; Shi et al., 2016; Xu et al., 2019). This suggests the need to consider the influence and the progression of the disease on drug enzyme metabolic capacity as well as genetic and non-genetic factors when prescribing medicine in the diabetic population. This could improve drug response, tailor individualised treatment plans, and prevent ADEs as cases of CYP3A- mediated disease–drug– interactions have been observed in patients with inflammation before (Gravel et al., 2019a; Schmitt et al., 2011).

Mechanistically, the regulation of hepatic CES and CYP P450 mRNA and protein expression and the interactions with their gene regulators is complex and involves a wide variety of ligand-activated transcription factors and mediators. The expression of target genes such as CYP P450 3A4 and CES1 enzymes, among others, are regulated by microsomal enzyme inducers that exert their effects through various key transcription factors; such as PXR, FXR, LXR, CAR, PPAR, Nrf2 and DEC1 (Cobbina & Akhlaghi, 2017; Collins et al., 2022; de Jong et al., 2020;

Klyushova et al., 2022; J. Liu et al., 2022; Morgan, 2009; Y. Shen et al., 2019; D. Wang et al., 2018; Xie et al., 2004).

Our study reveals that BBGC leads to a downregulation of *PXR* in HepG2 cells after 24 hours of treatment, although more experimental replicates are needed to confirm this result. PXR was also found to be significantly decreased by ER stress in in primary human hepatocytes and HepG2 cells, subsequently decreasing CYP3A4 induction (Vachirayonsti et al., 2015). This could also affect the expression of CES1, which is also a direct PXR target gene and found to be decreased during ER stress, as mentioned above. Vachirayonsti et al. also revealed responsive elements to IL-6 and ER stress in a PXR promoter region, indicating a functional relation between ER-stress stress and proinflammatory cytokine signalling in its regulation (Vachirayonsti et al., 2015). Nevertheless, different mechanisms could be proposed to account for the observed decrease in hepatic CES1. Their regulation can be impacted by several intrinsic and extrinsic factors, such as genetics, environment, concomitant medications, and inflammatory markers (Gravel et al., 2019a; Zanger & Schwab, 2013). These include the effects of pro-inflammatory cytokines, noncytokine components, oxidative stress, and the presence of obesity. It is now well recognized that T2DM and NAFLD represent a condition of low chronic inflammation and in our experiments, MGO accumulation in HepG2 cells, following the inhibition of GLO1 by BBGC, results in the release of proinflammatory cytokine IL-8, and an increase in the expression of IL-8 and TNF-α mRNA. This is coupled with the activation of NF-κB.

Cytokine-mediated alteration of gene transcription is thought to be the main regulatory mechanism accountable for changing CES expression during inflammation. Inflammatory signalling and cytokine-induced downregulation of CES1was reported in primary hepatocytes and HepG2 cells (Li et al., 2021; Mao et al., 2011; Y. Shen et al., 2019; Xiong et al., 2014; Yang et al., 2007) in studies that implicate NF-κB and MAPK activation in the downregulation, and the requirement of high glucose in the media.. In addition to DMEs, inflammation can alter the activities of numerous transcription factors affecting the regulatory pathways of various proteins including DMEs and drug transporters. High glucose increases PXR expression and activity in HepG2 cells, but high glucose combined with LPS, triggering inflammation, decreases it (Daujat-Chavanieu & Gerbal-Chaloin, 2020; Xiong et al., 2014). Similar to PXR, CAR has also been shown to be modulated by inflammation. In primary human hepatocytes, HepG2 cells and HepaRC cells, and in mouse liver, the expression of PXR and CAR mRNA

was reduced in response to inflammatory mediators such IL-6 (Ogura et al., 2012; Pascussi et al., 2000; Tanner et al., 2018; Teng & Piquette-Miller, 2005). Thus, the inflammation that is observed in our study as a result of GLO1 inhibition may explain the downregulation in PXR mRNA expression, and again, as CES1 inhibition also induces inflammation, this may further exacerbate the negative regulation of DMEs that is observed.

DEC1 signalling was also identified as a mediator of IL-6-mediated downregulation of PXR and CAR in PHHs and HepG2 cells (Ning et al., 2017). DEC1 is also able to interact with the dimerization partner RXRα, thereby interfering with their mechanistic function, and IL-6 enhances this interaction. DEC1 expression is induced in response to IL-6, and negatively correlates with CES1 expression in a T2DM murine model (R. Chen et al., 2015). In the same study, the knockdown of DEC1 abolished the decrease in CES1 expression induced by the high insulin, high glucose treatment in HepG2 cells, aiming to mimic diabetic conditions. IL-6 signalling protein kinases such as JNK and PKC can also repress the activity of the nuclear receptors PXR and CAR, thereby altering their function and impact on downstream transcriptional CES1 activity, possibly by altering the phosphorylation status of these nuclear receptor proteins (Ding & Staudinger, 2005; Koike et al., 2007; Lichti-Kaiser et al., 2009; Taneja et al., 2018).

NF-κB can also directly control the expression of various CYP P450 enzymes and CES enzymes via interaction with the promoters of their genes, leading to downregulation in most cases (Darakjian et al., 2021; de Jong et al., 2020; Zordoky & El-Kadi, 2009). Gu et al. have reported an interference of NF-κB with the dimerization of PXR to RXR and its subsequent binding to DNA, thereby inhibiting the activity of PXR. It was found that this inhibited transcriptional activity of PXR in HepG2 cells (Gu et al., 2006), and the use of an inhibitor of NF-κB significantly attenuated LPS-mediated inhibition of PXR mRNA and protein expression (Beigneux et al., 2002). Zhou et al. also demonstrated a reciprocal inhibitory cross-talk between PXR and NF-κB proinflammatory pathways demonstrating mutual repression , implicating that NF-κB activation in the liver eventually leads to the suppression of hepatic CES mRNAs by inflammatory stimuli (Zhou et al., 2006). Furthermore, they reported that PXR knockout mice showed increased inflammatory markers, and that they found lower mRNA levels of TNF-α in liver samples obtained from patients treated with phenytoin, a known PXR inducer. PXR silencing also increased pro-inflammatory cytokine expression including TNF-α and IL-8 (Mencarelli et al., 2011). Indeed, pro-inflammatory NF-kB and AP-1 signalling are

significantly inhibited following PXR activation through a direct functional interaction (Deuring et al., 2019; Gu et al., 2006; Okamura et al., 2020). Thus, activation of the NF-κB pathway may modulate the activity of nuclear transcription factors, and thereby CES1 expression, through pro-inflammatory signalling, changes in dimerization, translocation, or chromatin remodelling (de Jong et al., 2020).

In addition to modulating the antioxidant response through binding to antioxidant response element (ARE), Nrf2 induces the expression of DMEs, and drug transporters as previously mentioned. NF-κB also antagonizes the transcriptional activity of Nrf2 (Wardyn et al., 2015) whereas MGO administration, decreased gene expression of transcription factor Nrf2 in hereditary hypertriglyceridaemic rats (Hüttl et al., 2020). In a murine NAFLD model, Vitamin E-mediated improvements in glucose tolerance and liver steatosis correlated with an upregulation of Nrf2, and CES1 expression, which were decreased in the control group. These effects were abrogated with the use of a Nrf2 inhibitor (He et al., 2019).

Many studies have also reported the relevance of CES to the development of metabolic disease, indicating these enzymes might be potential targets for treatment (J. Liu et al., 2022; D. Wang et al., 2018). Findings indicate a role for CES in endogenous lipid and glucose metabolism and inflammation, and thus CES may be significantly involved in diseases that exhibit dysregulation of these processes, such as diabetes and NAFLD (Ghosh et al., 2010; Lian et al., 2018; J. Liu et al., 2022; Ross et al., 2012; Ross et al., 2010; D. Wang et al., 2018; Wei et al., 2010). CES1 protein is far more abundant than CES2 protein in human liver (Godin et al., 2007), and analysis of the human liver proteome indicates that CES1 is the tenth most abundant protein (out of >6000) expressed in this tissue (Sun et al., 2010). More specifically, CESs actively participate in lipid homeostasis and regulate the formation and accumulation of liver lipid droplets (LDs), which is an important feature of NAFLD (Lian et al., 2018). Changes in fatty acid composition caused by CES hydrolysates can not only activate/inhibit nuclear receptors but also stimulate/suppress ER stress, thus affecting the progression of NAFLD (J. Liu et al., 2022).

LDs are complex and metabolically active organelles implicated in many cellular functions, including lipid storage and mobilization, protein storage and degradation and lipid mediated cell signalling (Walther & Farese Jr, 2012). They are composed of a neutral lipid core (predominantly triglycerides (TGs) and cholesterol esters) contained by a monolayer

of phospholipids, proteins and enzymes responsible for neutral lipid synthesis and metabolism (Olzmann & Carvalho, 2019; Scorletti & Carr, 2022). Abnormalities in LD dynamics have been observed in cardiovascular disease and metabolic disorders such as obesity, T2DM and NAFLD. LD accumulation in hepatocytes is a distinctive characteristic of NAFLD (Lian et al., 2018; Mashek et al., 2015), as a result of increased free fatty acid (FFA) flux and *de novo* lipogenesis in hepatocytes. Dysregulation of systemic lipid metabolism contributes to the development of metabolic diseases as lipid homeostasis has a profound impact on insulin sensitivity and glucose metabolism, reflected by the negative correlation between plasma FFA levels and the degree of insulin sensitivity (Rachek, 2014). Disrupted or dysregulated LD formation may lead to increased intracellular lipid and lipid (TG) metabolite accumulation, steatosis, ER stress, oxidative stress, and inflammation in hepatocytes (J. Liu et al., 2022; Mashek, 2021). Moreover, the increase in LDs causes the activation of hepatic stellate cells, leading to liver fibrosis and hepatocellular carcinoma (Scorletti & Carr, 2022).

Naturally, most studies investigating the effect of CES1 deletion or overexpression were conducted in animal studies, more specifically mice, which have eight *Ces1* genes (*Ces1a-Ces1h*). Ces1d deficiency proved to be protective against high-fat diet induced steatosis, reduced liver inflammation, oxidative stress and fibrosis (Lian et al., 2016; Lian et al., 2012), suggested by the authors to be a result of decreased hepatic *de novo* lipogenesis, increased fatty acid oxidation, and improved insulin sensitivity. More recent studies also demonstrated that Ces1d directly targets lipid droplets, where it hydrolyses TG and promotes FFA production in adipocytes (Li et al., 2022). Ces1g, on the other hand, shows opposite functional effects to Ces1d. Ces1g deletion in mice results in increased hepatic lipogenesis leading to hyperlipidaemia and increased secretion of VLDL and fat deposition in peripheral tissues. The mice developed obesity, steatosis, hyperinsulinemia, and insulin resistance (Quiroga et al., 2012; Jiesi Xu, Yuanyuan Li, Wei-Dong Chen, et al., 2014). These effects are effectively reversed by restoring hepatic Ces1g expression in Ces1g knockout mice (Bahitham et al., 2016). Increased lipid accumulation as a result of Ces1g ablation is believed to result from the increased activation of hepatic SREBP1c processing leading to increased lipogenesis. This is because of the high affinity Ces1g exhibits for PUFAs-containing TG, which suppress the activity of SREBP1c promoter and *Srebf1* mRNA (Lian et al., 2018).

This is well demonstrated in the study by Quiroga et al., where they reported increased de novo lipogenesis was associated with decreased PUFA release from TG and sustained SREBP1c

activation in the liver (Quiroga et al., 2012). Furthermore, CES1g has been shown to prevent TG accumulation in rat hepatocytes exposed to oleic acid, and showed increased production of acid-soluble metabolites, indicating improved of β-oxidation (Ko et al., 2009). Xu et al. show that hepatic CES1g is essential for controlling both regular and FXR-controlled lipid homeostasis, and CES1g over-expression in mice promotes fatty acid oxidation and lowers hepatic TG and plasma glucose levels in both wild-type and diabetic mice while improving glucose tolerance in diabetic mice (Jiesi Xu, Yuanyuan Li, Wei-Dong Chen, et al., 2014), emphasizing the role of CES1 in glucose metabolism. This is in line with other findings that showed that glucose induces CES1g expression, and that the induction of CES1g expression, in turn, regulates plasma glucose level, likely through increasing insulin sensitivity (J. Xu et al., 2014).

The functional human ortholog for Ces1g has not yet been defined, although human CES1 is believed to be Ces1d and not Ces1g (Lian et al., 2018). According to these murine models, the role of human CES1 in NAFLD remains ambiguous. Because mice have eight *Ces1* genes (*Ces1a-Ces1h*) that have shown contradictory functions, interpretations to infer the role human CES1 may have should be approached with caution. More recently, a study by Xu et al. aimed to specifically examine the effects of human CES1 overexpression in mouse hepatocytes *in vivo* revealed that human CES1 protects against Western diet or alcohol-induced steatohepatitis and hyperlipidaemia, supported by the observed reduction in plasma TG levels, hepatic FFAs and free cholesterol levels, lipid peroxidation, ROS production, apoptosis and inflammation in hepatocytes (Xu et al., 2020b). This indicates that a repressed CES1 expression and activity in the liver may be one of the key reasons why patients with T2DM exhibit an increased risk of developing NAFLD, as the apparent CES1-mediated protection against insulin resistance, steatosis and hyperlipidaemia may be lost. CES1 gene copy number variations loss was significantly associated with greater risk of NAFLD in a Chinese Han population (Chen et al., 2021). Thus, the MGO-mediated decrease in CES1 mRNA and protein, and thereby its inductivity, reported herein may be of critical relevance. This decrease is also observed in HepG2 cells exposed to high insulin and high glucose, aimed to mimic diabetic conditions (R. Chen et al., 2015).

In view of the importance the evident role of CES1 in crucial hepatocyte functions, we decided to examine the effects of CES1 inhibition in HepG2 cells with the use of a pharmacological inhibitor, WWL113, which covalently binds to the catalytic serine residue in and, hence,

causing a mechanism-based inhibition of its enzymatic activity. The results revealed that a loss of CES1 activity leads to an increase in IL-8 protein and mRNA expression, a small decrease in ATP, and a strong induction in the expression and secretion of key hepatokines FGF21 and GDF15 in HepG2 cells, with no effect on cell viability. This suggests CES1 plays an important role in the modulation of the hepatocyte inflammatory response, although the exact underlying mechanisms remain elusive. Previous studies have shown that hepatocyte-specific overexpression of human CES1 inhibits hepatic mRNA levels of inflammatory genes (*Tnfα, Il-6, Il-1β,* and *Mcp1*) in both Western diet-fed and alcohol-fed mice (Xu et al., 2020a). In the liver of Ces1d knockout female mice, Il6 mRNA expression and lymphocyte numbers were significantly higher following LPS treatment compared to their WT counterparts (Szafran et al., 2022). In the same study, the use of another CES1 inhibitor, WWL229, in WT female mice was also shown to enhance neutrophil infiltration and Il1b mRNA in the lung. These findings seem pertinent considering IL-8 plays an important chemotactic role in neutrophil and lymphocyte recruitment and activation (Brennan & Zheng, 2007), and CES1 inhibition in our study increases IL-8 expression and release in HepG2 cells. Thus, the loss of CES1 induced by MGO may contribute significantly to deleterious hepatic inflammatory processes in NAFLD, including neutrophil infiltration, as discussed in the previous chapter.

The available evidence seems to suggest the effects observed may be a result of mitochondrial dysfunction, oxidative stress and ER stress. In addition to increased LD formation, CES1-deficient human pulmonary endothelial cells show increased ER stress markers, CHOP, BiP/GRP78, IRE1α, ROS production, mitochondrial fragmentation and apoptosis (Chakraborty et al., 2022). Similar results were obtained by another group, who were also examining the effects of pharmacological inhibition of CES1 activity in HepG2 cells, this time, using WWL229 as well as CES1-KO cells. WWL229-treated cells and CES1-KO cells were characterised by increased ROS and ER stress markers BiP/GRP78 and XBP1, decreased ATP levels, mitochondrial dysfunction, and dysregulated lipolysis, as evidenced by significantly larger LDs, TG accumulation and lower levels of PUFAs and FFAs. Furthermore, lipid components involved in the plasma, mitochondrial and ER membranes were reduced by WWL229, which also caused a large decrease in the mRNA and protein expression of acylcarnitine, an important factor in the transport of activated long-chain FAs into mitochondria for β-oxidation. This may also explain the downregulation in the expression of mitochondrial of fatty acid β-oxidation-related enzymes and biogenesis. Thus, altered lipid profiles as a result of CES1 inhibition are directly linked to impaired mitochondrial function. The decrease in

PUFAs was also suggested to explain the downregulation of the expression of stearoyl-CoA desaturase 1, a direct target of PPARα/γ, via reduced PUFA-mediated PPARα/γ activation, as well as *SOD1*, *SOD2*, *GPX1*, and *CAT1* expressions, revealing a new mechanism by which CES1 may actively participate in genetic transcriptional regulation (Li et al., 2023).

These findings may shed light on the mechanisms underlying the effects on the reduction in ATP levels and the increase in IL-8 expression and release that are observed in our study, as mitochondrial dysfunction, ROS accumulation and ER stress are known inducers of inflammation. Furthermore, as CES enzymes are mainly membrane-bound and located on the lumen side of the ER (Di, 2019), a down regulation of CES1 under ER stress in LO2 cells and an acetaminophen-induced acute liver injury animal model can be observed (Han et al., 2023). Furthermore, hyperactivated IRE1α is known to possess ribonuclease activity to induce the degradation of mRNAs through regulated Ire1 dependent decay (RIDD), including mRNAs encoding certain CYP enzymes (Hur et al., 2012) and CES1 (So, Hur, Tarrio, Ruda, Frank-Kamenetsky, Fitzgerald, Koteliansky, Lichtman, Iwawaki, Glimcher, et al., 2012), and as previously discussed, MGO is known to induce the UPR and IRE1α hyperactivation (Xue et al., 2024), and we showed that it induced ER stress in our studies. This leads us to conclude that MGO-mediated decreases in CES1 expression observed may partly be due to ER stress, and that the inflammation observed as a result of CES1 inhibition is not responsible for the BBGC induced inflammation, as CES1 protein and activity is downregulated at 48 hours whereas BBGC exerts its effects much earlier. Nevertheless, once CES1 expression and activity is lost, it may establish a vicious cycle as, together with dicarbonyl stress, it would contribute to mitochondrial dysfunction, inflammation and an increased risk of hepatotoxicity and liver disease, as described above. The effects of CES1 inhibition on mitochondrial dysfunction and ER stress may also explain the strong induction of hepatocytes FGF21 and GDF15, further emphasizing the role of CES1 in the regulation of inflammatory and endocrine signalling.

In conclusion, this suggests that the results obtained may be the negative regulatory effects of MGO-induced mitochondrial dysfunction, ER-stress, and acute-phase inflammatory response and NF-κB activation on CES1 mRNA expression and protein levels, *in vitro*. This could be considered a limitation as the inflammatory response depends on a variety of cellular and cytokine communication, from different cells normally present physiologically, but absent in our current model. Nevertheless, the weight of evidence gathered, both in animal and clinical studies, suggests that the regulation of drug metabolizing proteins, such as CYP P450 and

CES1, and their nuclear transcription factors would be interconnected with T2DM and/or NAFLD- related inflammation and ER-stress. Hence, these alterations in conditions such as T2DM could be potential sources of drug variability in patients and treatment outcomes, which could have serious consequences on safety and efficacy (Darakjian et al., 2021). These results also suggest that, in metabolic disorders where increased hepatic MGO levels, chronic ER stress, low-grade inflammation and NF-κB activation is present, CES1 may not only in lose its xenobiotic metabolism capacity but could also contribute to the progression of the disease and diabetic complications, as CES1 was found to modulate inflammatory responses and hepatokine release. Many studies have confirmed its essential role in normal lipid and carbohydrate metabolism, and when induced, it's protective role against hyperlipidaemia, NAFLD, and even atherosclerosis (Jiesi Xu, Yuanyuan Li, Wei-Dong Chen, et al., 2014).

7. General Discussion

The increased risk of liver disease in patients with T2DM remains a growing health problem, and developing effective physiological models to study the mechanistic pathways that drive its pathogenesis is essential if we are to develop new therapeutic strategies to address this problem.

Altogether, the evidence gathered in this study helps support that the inhibition of GLO1 using BBGC increases dicarbonyl stress in HepG2 cells in a more physiological manner, through increasing intracellular MGO levels. This model sets up a more appropriate method to study the effects of dicarbonyl stress in experimental settings, as it mimics its natural occurrence. In our study, it enabled us to expand upon our knowledge of the mechanisms affected by MGO-induced toxicity in hepatocytes, which is currently relatively understudied.

The pathogenic mechanisms of NAFLD are yet to be fully understood, and multiple lines of evidence have indicated that the pathogenesis of NAFLD is a complicated and multifactorial process (Targher et al., 2021; Tilg et al., 2017). Understanding the pathways that mediate the progression of liver disease in T2DM patients is crucial to the identification of potential targets that can help mitigate the increased incidence and severity of liver complications in this population. In line with this, the role of dicarbonyl stress in diabetic pathology should be emphasised, as it has been ubiquitously linked the development and progression of both diabetes and its various microvascular and macrovascular complications.

A critical turning point in NAFLD is the transition from pure steatosis to fatty inflammation, during which many cellular stress pathways are activated, including oxidative stress, ER stress, inflammation, autophagy, and apoptosis (Xia et al., 2020). These stress processes may cause irreversible damage to cells and tissue integrity, which exacerbates the progression of the disease to irremediable stages.

We have found that BBGC mediated dicarbonyl stress in HepG2 cells not only mimics the cytotoxicity of direct MGO exposure but also seems to mimic the underlying cellular pathways mediating these damaging effects, such as increased oxidative stress, mitochondrial dysfunction, inflammation, increased the ER stress markers CHOP and Bip/Grp78 expression, and apoptosis. These pathways are all critical to hepatocyte dysfunction and are key players in the pathogenesis of liver disease. Notably, MGO is highly involved in the inflammatory response in hepatocytes, via increasing IL-8 release, activating NF-κB and causing ER stress.

We were also able to link for the first time dicarbonyl stress to the modulation of hepatokine GDF15 and FGF21 expression and release.

The role of the liver as a major secretory organ has long been appreciated, and proteomic analyses have enabled researchers to identify ~10,200 proteins produced in the human liver (M.-S. Kim et al., 2014), with up to 40% of hepatic transcripts encoding secretory proteins (Uhlén et al., 2015), suggesting there is clearly a large scope for significant and varied protein secretion from the liver. As previously discussed, the inflammation as a result hyperglycaemia induced synergises with fatty acid induced inflammation in the liver to promote systemic inflammation, IR (Gehrke & Schattenberg, 2020) and increase liver tissue damage through oxidative stress and oxidative damage (Chatterjee, 2016; Elmarakby & Sullivan, 2012; Hollenbach, 2017; Lai et al., 2022), and through the increase in secretion of pro-inflammatory mediators, such as TNF-α, IL-8 and IL-6, cell adhesion molecules, chemokine receptors and inducible enzymes such as NADPH oxidase, COX2 and iNOS, collectively contribute to neutrophil infiltration, fibrotic liver injury (T. Liu et al., 2017; Rohm et al., 2022).

Similarly to adipose tissue or skeletal muscle, where the secretion of respective organokines exerts their endocrine function, the liver has more recently been identified to contribute to the endocrine control of metabolism by producing liver-derived factors – hepatokines. In response to nutrient overabundance and deficiency, the liver secretes hepatokines, which convey energy status and mediate the regulation of nutrient availability to multiple peripheral tissues including adipose tissue, skeletal muscles and the central nervous system. A disruption or dysregulation of any of the many pathways involved may result in metabolic dysfunction that directly contributes to the development of insulin resistance T2DM, or fatty liver disease (Kirpich et al., 2011; R. C. Meex et al., 2015; Younossi et al., 2005; Zhang et al., 2010). Despite the potential beneficial properties that characterise FGF21 and GDF15, they are established markers of cell damage and inflammation, positively correlating to the severity of steatosis, hyperglycaemia, insulin resistance and end-organ damage in patients with diabetes or NAFLD (Adela & Banerjee, 2015; Dolegowska et al., 2019; Eddy & Trask, 2021; Meex & Watt, 2017; Mutanen et al., 2014; Schmid et al., 2022; Valenzuela-Vallejo et al., 2023; Vila et al., 2011; Yilmaz et al., 2010).

GDF15, a stress-induced cytokine/hepatokine and a member of the TGF-β superfamily, also called macrophage inhibitory cytokine-1, is associated with numerous biological and

pathological processes including obesity, diabetes, metabolic syndrome, heart failure, atherosclerosis, inflammatory diseases and cancer (Bauskin et al., 2006; BreitSN, 2011; Keipert & Ost, 2021; Mullican & Rangwala, 2018; Suriben et al., 2020). High serum GDF-15 levels were positively associated with microvascular and macrovascular complications in T2DM patients, with some groups of researchers suggesting that the increase in GDF15 levels in the plasma might be part of anti-inflammatory response for the onset of diabetes (Asrih et al., 2023; Breit et al., 2021; Carstensen et al., 2010; Bo Kyung Koo et al., 2018; Schmid et al., 2022). Liver biopsies of patients with NAFLD revealed that GDF-15 could be used as a molecular biomarker of NASH and liver fibrosis as it closely related to liver fibrosis score (B. K. Koo et al., 2018). Serum GDF-15 concentrations are also markedly higher in patients with NAFLD and T2DM than in those without T2DM, with correlations to liver IL-6, IL-8 and CRP levels, suggesting that chronic hyperglycaemia and inflammation have a role in increasing hepatic GDF15 secretion and thus circulating concentrations of GDF15 (Bilson et al., 2021).

FGF21 is primarily produced and expressed in the liver and secreted into the bloodstream to fulfil its function as a hepatokine, myokine, and adipokine. Due to its pleotropic effects on preserving energy balance in rodents and humans, it is of particular interest in the setting of metabolic disorders (Jensen-Cody & Potthoff, 2021; Oh et al., 2017). FGF21 levels have been proposed as an alternative to the glucose tolerance test as a biomarker for predicting prediabetes and T2DM (Woo et al., 2017). Another study examined the relationships between FGF21, HbA1c, β-cell function, and insulin resistance in 2 584 patients who were not receiving any anti-diabetic medication and discovered that patients with higher FGF21 levels have a higher risk of glycaemic progression over the course of five years (Ong et al., 2017), indicating a strong correlation between increased FGF21 and poor disease prognosis. Moreover, according to a number of studies, both juvenile and adult patients with NAFLD had considerably higher levels of hepatic FGF-21 mRNA, which was correlated with higher blood levels of FGF-21 (D. M. Lebensztejn et al., 2016; Watt et al., 2019). Studies in animal models further demonstrated that FGF-21 is generated in response to toxic lipid accumulation in the first stages of methionine- and choline-deficient NASH, but it was decreased as the severity of NASH worsened (Tanaka et al., 2015).

Considering this, the observations that we have made are particularly interesting, as it alludes to a new mechanism by which dicarbonyl stress regulates hepatocyte signalling in the absence of hyperglycaemia and fatty acids, and hepatokine modulation may represent a newly identified

manifestation of dicarbonyl induced hepatotoxicity. As mitochondrial dysfunction and ER stress are a feature of MGO mediated cellular toxicity, it is worth noting that the effects in our study observed may be mitochondrial dysfunction related and ER stress related. MGO-induced inflammation and particularly hepatokine release may not only constitute a central mechanism by which dicarbonyl stress increases the incidence and severity of liver disease, but also affect other peripheral tissue signalling (Darakjian et al., 2021) by establishing destructive inflammatory and endocrine signalling and dysregulated glucose and lipid metabolism, which can further sustain and exacerbate insulin and hepatokine resistance.

We were also able to show for the first time that MGO decreases the expression of CES1 mRNA and protein levels in HepG2 cells, while linking CES1 activity to strong inflammatory responses. The loss of CES1 activity increases proinflammatory cytokine IL-8 mRNA expression and secretion, and strongly induces GDF15 and FGF21 mRNA expression, despite having no observable effects on cell viability. Due to the decrease in hepatic levels of CES1 that are observed in an NAFLD murine model (He et al., 2019) and in diabetic patients and experimental diabetic models (R. Chen et al., 2015; Ge et al., 2020; Han et al., 2023; Valle et al., 2012; Yao et al., 2019; Yao et al., 2020), these findings are extremely relevant.

Recently, an increasing number of studies are examining the role CES1 may play in NAFLD development and hepatic inflammation as discussed in the previous chapter. Human CES1 overexpression in mouse hepatocytes *in vivo* inhibits hepatic inflammatory gene expression and improves dyslipidaemia, lipid peroxidation, ROS production and apoptosis (Xu et al., 2020b), whereas CES1 knockout or inhibition increases hepatic inflammation, mitochondrial dysfunction and ER stress (Szafran et al., 2022),, similar to our findings. This indicates that a repressed CES1 expression and activity in the liver may be one of the key reasons why patients with T2DM exhibit an increased risk of developing NAFLD, and thus, the loss of CES1 induced by MGO may contribute significantly to deleterious hepatic inflammatory processes in diabetes, including neutrophil infiltration and disturbed hepatokine signalling. Targeting inflammation, through the identification of pathways that connect dicarbonyl stress, inflammation and hepatokine signalling to T2DM, can help manage and prevent debilitating T2DM complications including NASH and fibrosis.

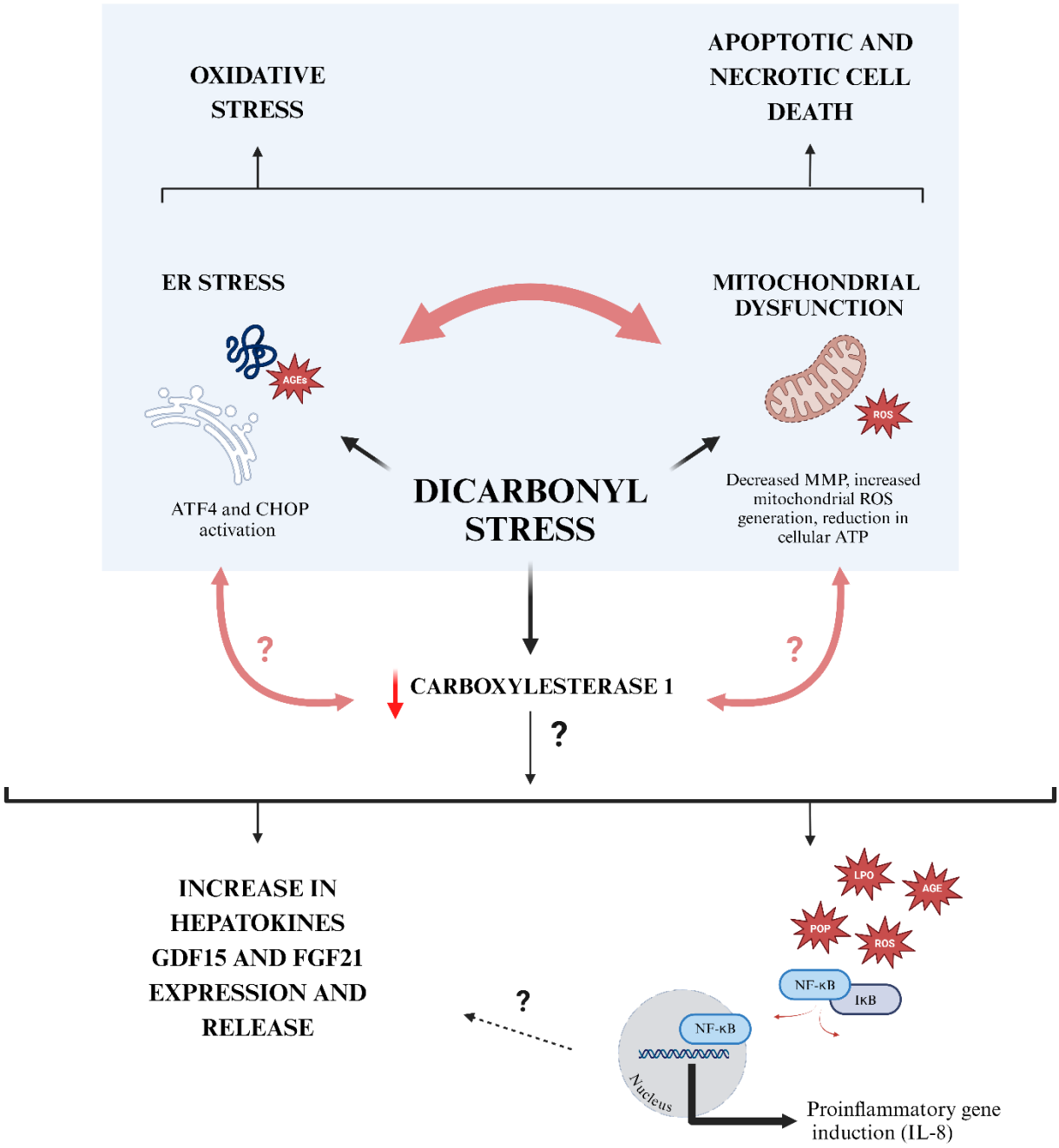


Figure 7. Graphical summary of the main findings of this study. BBGC mediated dicarbonyl stress in hepatocytes leads to mitochondrial dysfunction, likely through direct damage to mitochondrial integrity, and induces ER-stress leading to CHOP activation, likely through glycative and oxidative stress induced cellular damage. Dicarbonyl stress also leads to a decrease in CES1 gene and protein expression, and both dicarbonyl stress and a loss in CES1 activity leads to the modulation of inflammatory responses and hepatokines GDF15 and FGF21 expression and secretion. Further work is required to fully understand the underlying mechanisms of the dicarbonyl stress mediated downregulation of CES1 in hepatocytes, and the subsequent pathways that explain the inflammatory response that occurs as a result. Created with BioRender.com.

Moreover, several clinical observations can be made in other conditions outside of the scope of metabolic disease. For example, GLO1 expression and activity decreases with age, as observed in arterial, human lens and brain tissues in ageing phenotypes (Nigro et al., 2019), which correlates with increasing MGO levels as the loss of GLO1 function leads to MGO accumulation (Rabbani et al., 2016a). The balance between MGO formation and its detoxification by GLO1 can significantly contribute to the aging process. The overexpression of GLO1 protects against age-related disease in rodent models and improves senescence (Ikeda et al., 2011). Interestingly, Petersen et al demonstrated that elderly patients have decreased mitochondrial oxidative phosphorylation activity, which contributes to insulin resistance in old age (Petersen et al., 2003) and recently, GDF15 and FGF21 were identified as increased in ageing too, with no change in FGF21 tissue responsiveness which as discussed in chapter 5, could be a result of MGO-induced glycation (Pence, 2022; Villarroya et al., 2018). Furthermore, CES1 was found to decrease in (Ohura et al., 2014; Xiao et al., 2012; Yang et al., 2009). It is plausible that MGO may be involved in these observations and may be the root cause of these disturbances observed in conditions related to its impaired detoxification. Targeting dicarbonyl seems pertinent to promoting healthy ageing.

To mitigate dicarbonyl stress, several strategies have been proposed from dicarbonyl scavengers to GLO1 inducers, through Nrf2 agonists such as resveratrol (Cheng et al., 2012; Rabbani et al., 2021; Yılmaz et al., 2018). Studies have confirmed that MGO inhibits AMPK activity and leads to mitochondrial fission, inhibition of mitochondrial biogenesis and autophagy. In addition, they reported an AMPK-dependent upregulation of GLO1 expression via AMPK-Nrf2-ARE axis, consistent with previous findings (Matzinger et al., 2020; Xue et al., 2012). This may have been corroborated in human studies, where they observed T2DM patients receiving metformin treatment showed decreased plasma MGO levels and increased GLO1 activity in peripheral mononuclear cells, red blood cells and atherosclerotic lesions (Peters et al., 2018). Previous studies describe metformin is a MGO scavenger (Kinsky et al., 2016), but Sekar et al. suggest the results of their study show that the beneficial action of metformin against MGO toxicity is mainly through AMPK activation, especially given that metformin applied up to 4 h after-MGO treatment still exerts similar protection as during co-treatment (Sekar et al., 2023). Moreover, in human neuroblastoma cells, emodin, a cytoprotective agent, was able to protect against MGO-induced mitochondrial dysfunction, and this protection was abolished after inhibiting AMPK or the silencing of the Nrf2 or heme-

oxygenase-1 (HO-1) (de Oliveira et al., 2021). Therefore, these studies further emphasised the importance of AMPK/Nrf2/ signalling and suggests the inhibition of AMPK by MGO may constitute a substantial mechanism in its cytotoxic effects. Thus, AMPK inducers and metformin seem like promising candidates in not only preventing dicarbonyl stress mediated cellular dysfunction, but also in improving GLO1 expression to reduce dicarbonyl stress itself.

Interestingly, in db/db mice, the formation of a product of metformin and MGO reaction - imidazolinone metabolite (IMZ), correlated with increased hepatic SIRT1 expression and the downregulation of ChREBP at the protein and mRNA level. These results, achieved via scavenging of MGO by metformin, not only adds to the mechanism of metformin drug efficacy but could directly implicate MGO in the upregulation of ChREBP in disease states where MGO levels accumulate i.e. T2DM (Hargraves, 2019). Moreover, a study examining the effects of rosiglitazone when added to metformin regimen in T2DM patients yielded interesting results – significantly declined HbA1c, and HOMA-IR and interestingly, fasting plasma FGF-21 levels (Li et al., 2009), indicating that this results in reduced stress-induced FGF21 secretion from the liver. It was previously reported that rosiglitazone enhanced FGF-21 action to induce tyrosine phosphorylation of FGF receptor (FGFR)-2 through activating the PPAR-g pathway in 3T3-L1 adipocytes (Moyers et al., 2007). Thus, rosiglitazone may also improve FGF-21 action through the PPAR-g pathway, and consequently alleviate FGF-21 resistance, and reduce plasma FGF-21 levels (Li et al., 2009). Similar results were observed in a clinical trial in NAFLD patients treated with resveratrol, where resveratrol significantly decreased plasma FGF21 and TNFα levels, and improved insulin resistance, lipid profile and liver damage markers (S. Chen et al., 2015).

Further work is necessary to fully explore the pathways involved in dicarbonyl stress mediated regulation of inflammation and hepatokine signalling and the downregulation of CES1 in hepatocytes. In addition, it would be extremely important to identify the role CES1 plays in inflammation and hepatokine expression, as studies are now revealing it plays a major transcriptional role in hepatocytes (Li et al., 2023) and considering other stressors such as FFAs (palmitate) do not modulate GDF15 expression in hepatocytes. This was observed in our studies as well as by another group (Bo Kyung Koo et al., 2018). The use of antioxidants or ER stress inhibitors could prove useful and replicating and expanding the study in other models, such as primary human hepatocytes, 3D spheroid cell cultures and animal models for *in vivo* studies.

The limitations of our study are important to consider as the inflammatory response depends on a variety of cellular and cytokine communication, from different cells normally present physiologically, but absent in our current model. We also did not verify our results with the use of pharmacological antioxidants. The cell line used is also not ideal for studies investigating CYPP450 enzymes (Arzumanian et al., 2021), although among those, CYP3A4 is one of the highest expressed.

In the current global climate, the incidence of T2DM is still climbing and is more recently developing at an earlier age in patients. This may have a significant effect on the quality of life in patients including liver complications, and on their longevity, in addition to being a weighty health economic burden. As liver disease is key to a poorer prognosis, as well as being harder to reverse once it reaches more severe stages, understanding the full mechanistic effects and cellular pathways involved in mediating the increased incidence of liver disease in diabetic is an important priority for health care and research. Using this model of dicarbonyl stress may help achieve this and support the development of clinical strategies to combat this, which would have a major impact on global health and ensure healthy ageing in the affected population.

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