Letter to the Editors-in-Chief

Title: Direct Oral Anticoagulant (DOAC)-mediated vasodilation: role of nitric oxide.

Authors: Jon Mabley[a], Jignesh P. Patel[b,c], Abdelrahman Sayed[a], Roopen Arya[c], Greg Scutt[d,e]

a. School of Pharmacy and Biomolecular Sciences, University of Brighton, Brighton, UK
b. Institute of Pharmaceutical Science, King's College London, London, UK
c. King's Thrombosis Centre, Department of Haematological Medicine, King's College Hospital NHS Foundation Trust, London, UK
d. Brighton and Sussex Centre for Medicines Optimisation, University of Brighton, Brighton, UK
e. Brighton and Sussex University Hospitals NHS Trust, Brighton, UK

Corresponding author:
Dr Greg Scutt
School of Pharmacy and Biomolecular Sciences
University of Brighton
Brighton
United Kingdom
BN2 4GJ

Tel: +441273642123
Email: g.scutt@brighton.ac.uk

Word count: 1620 (including method)

Keywords

Endothelial, nitric oxide, Factor Xa inhibitors, vasodilation, apixaban, rivaroxaban

Abbreviations

Direct Oral Anticoagulants (DOACs)

Venous Thromboembolism (VTE)

Atrial Fibrillation (AF)

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Dear Editors-in-chief,

1 Introduction

Anticoagulant therapy is commonly prescribed for both the acute treatment, and long-term prevention of venous thromboembolism (VTE), and as primary and secondary prevention of stroke in the context of atrial fibrillation (AF) [1]. Until recently the majority of patients requiring chronic anticoagulant therapy were prescribed vitamin K antagonists (VKA), as these were the only oral anticoagulant agents available [1]. A requirement for regular monitoring and VKA -drug or-food interactions has meant that that not all patients that have an indication for anticoagulation have benefitted from these agents. To overcome these issues, the direct Xa inhibitor class of direct oral anticoagulants (DOACs, e.g. apixaban, edoxaban, rivaroxaban) were developed, which have the advantage of predictable pharmacokinetics and a minimal requirement for regular monitoring of anticoagulant effect [2].

A common side effect experienced by patients prescribed rivaroxaban in the landmark phase III clinical trial evaulating it against warfarin for stroke prophylaxis in AF was dizziness and headaches. This occured in up to 1 in 10 patients, and frequently led to discontinuation of the drug [3]. This side-effect is also being observed, albeit to a lesser extent, with other DOACs. At present, it is not known why this occurs, and why rivaroxaban appears to induce these effects in a greater proportion of patients than the other DOACs.

DOACs have recently been reported to have direct cellular effects which appear to be independent of their ability to inhibit Factor Xa [4]. A non-Factor Xa mediated effect

on vascular smooth muscle, producing vasorelaxation and a change in blood pressure in patients prescribed DOACs may explain the observed side effects of headaches and dizziness. A potential mechanism may be through facillitation of vascular cell nitric oxide release. We therefore hypothesise that direct Xa inhibitors have a direct vasodilatory effect on blood vessels, possibly through an endothelial cell dependent mechanism.

2 Methods

2.1 Reagents

Rivaroxaban and apixaban were obtained from Carbosynth Ltd. (Berkshire, UK). Acetylcholine chloride, dimethyl sulphoxide (DMSO), phenylephrine hydrochloride, and sodium nitroprusside were obtained from Sigma/Aldrich (Poole, UK). Sprague-Dawley rats used in the *ex vivo* studies were obtained from Charles River Laboratories (Kent, UK). All other chemicals were of reagent grade and obtained from Fisher Scientific (Loughborough, UK).

2.2 Ex vivo aortic ring preparation

Thoracic aorta from male Sprague-Dawley rats (180-220 g) were dissected and rings of 2-3 mm cut and mounted in organ baths filled with warmed (37°C) and gas-equilibrated (95% O_2, 5% CO_2) Krebs solution containing (in mmol/L) $CaCl_2$ 1.6, $MgSO_4$ 1.17, EDTA 0.026, NaCl 130, $NaHCO_3$ 14.9, KCl 4.7, KH_2PO_4 1.18, and glucose 5. Isometric tension of the rings was measured with force-displacement transducers (Danish Myo Technology), digitised using PowerLab. A preload tension of 1.5 g was applied, and the rings were equilibrated for 60 min, followed by measurement of the concentration-dependent contraction to phenylephrine (10^{-9} to 10^{-4} mol L^{-1}) before being washed with fresh Krebs buffer until the tension returned to that observed prior to the phenylephrine addition.

2.3 Experimental protocol

Rat aortic rings were precontracted with phenylephrine (10^{-6} mol L^{-1}) before being exposed to either rivaroxaban or apixaban (0.01-3 µmol L^{-1}). The tissue response was expressed as % relaxation from the maximum tension of the aortic ring prior to any

drug addition. The responses of the rings to rivaroxaban and apixaban were compared to the vehicle (DMSO) which was applied in the same volume as the drug with the resulting percentage of DMSO ranging from 0.0088 to 0.74% v/v. In a second series of experiments rat aortic rings either had their endothelial cells removed by gentle mechanical abrasion, or were treated with either the competative eNOS inhibitor L-N^G-nitroarginine methyl ester (L-NAME; 100 µmol L^{-1}) or the highly selective, irreversible inhibitor of soluble guanylyl cyclase (sGC) 1H-[1,2,3]oxadiazol[4,3-a]quinoxalin-1-one (ODQ; 10 µmol L^{-1}) for 10 minutes prior to the addition of DMSO, rivaroxaban or apixaban (0.01-3 µmol L^{-1}). Tissue response was expressed as % relaxation.

2.4 Statistical analysis

Results are presented as mean ± standard error of the mean (SEM). Two way repeated measures analysis of variance with Bonferroni's correction was used to compare mean values as appropriate. Differences were considered significant when $p<0.05$.

3 Results

3.1 Relaxant effect of rivaroxaban and apixaban on pre-contracted aortic rings

Exposure of phenylephrine pre-contracted rat aortic rings to either rivaroxaban or apixaban caused a statistically significant dose-dependent relaxation as compared to the vehicle DMSO (Fig. 1a). DMSO at the maximum 0.74% v/v caused a 16.5 ±4.7% relaxation as compared to 3 μmol L^{-1} rivaroxaban and apixaban which caused a 47.9±3.7% and 55.5±6.0% relaxation respectively ($p<0.05$ vs. DMSO).

3.2 Role of endothelial cells and nitric oxide in the aortic ring relaxant effect of rivaroxaban and apixaban

The relaxant effect of both rivaroxaban (Fig. 1c) and apixaban (Fig. 1d) was significantly attenuated by the removal of endothelial cells, with the relaxant response returned to that observed with vehicle alone. To determine the role of nitric oxide in the DOAC-mediated vasorelaxant effect we pharmacologically inhibited either eNOS or sGC and found that inhibiton of either of these enzymes blocked the relaxant effect of both rivaroxaban (Fig. 1c) and apixaban (Fig. 1d). Removal of endothelial cells, or inhibiton of either eNOS or sGC had no effect on the minor relaxant effect of the vehicle DMSO (Fig. 1b).

4 Discussion

The data presented here demonstrates that the DOACs rivaroxaban and apixaban have a direct relaxant effect on the vasculature in male Sprague-Dawley rats. We have also shown that this vasorelaxant effect of DOACs is both endothelial cell- and NO-dependent. The proposed mechanism may go some way to explain some of the side effects attributed to DOACs, including dizziness and headache. For example, DOAC-induced vasorelaxation of the vasculature may lead to hypotension, producing symptoms of dizziness as a result of decreased cerebral perfusion. DOAC-associated headaches on the otherhand may be attributable to NO-dependent vasorelaxant effects directly upon cerebral vascular smooth muscle. Both glyceryl trinitrate and isosorbide mononitrate are drugs which are well known to produce headaches through an NO-dependent mechanism [5]. This newly identified DOAC-mediated increase in NO release from endothelial cells may also contribute to the therapeutic effectivness of these drugs in VTE and stroke prophalaxis by not only inhibiting factor Xa, but also increasing NO release to reduce platelet coagulation.

Previous research has shown that apixaban enhances vasodilation [6]. Although no direct effect of apixaban on endothelial-mediated NO production was observed, vasodilation was mediated through protease-activated receptor (PAR)-2 by inhibiting its desensitization [6]. The group's results are in contrast to ours, but there are significant differences in the experimental design between the studies to explain these observations. For example, we used aortic rings, wheras Villari *et al*. used mesenteric arteries. Also, our maximum rivaroxaban concentration 3 μM was 3-fold lower than their lowest concentration of 10 μM [6]. Both we and Villari *et al*. identified that the

DMSO vehicle for DOACs has a confounding vasorelaxant effect, and it may be that this could mask any vasorelaxant effect but because we used lower concentrations of both rivaroxaban and apixaban we were able to keep the vehicle DMSO percentage below 1% while maintaining solubility of the DOACs, allowing the direct effect of DOACs on vasorelaxation to be observed.

The DOAC-mediated vasorelaxation was found to be both endothelial cell- and NO-dependent. Although this suggests that it is the endothelial cell NOS that is being activated by both rivaroxaban and apixaban to induce relaxation, we cannot rule out that other NOS isoform expressing cells of the vasculature, such as vascular smooth muscle cells, contribute to the observed DOAC effect [7]. The mechanism by which DOACs are increasing eNOS activity remains unknown. However, based on the side effect profile of DOACs, they are unlikely to be activating receptors that have large tissue distributions and wide-ranging physiological effects (e.g. muscarinic, oestrogen, purine, PAR, bradykinin, VEGF, thrombin, histamine) as the side effect profile associated with such activation would be more obvious from a clinical perspective. It is interesting to note that apixaban was found to modulate PAR-2 activity on endothelial cells [6] possibly indicating that this cellular pathway may be involved in the NO-mediated direct vasorelaxant effect. The role of PAR-2 in the DOAC-induced NO-dependent vasorelaxant effect is currently being determined using a specific pharmacological inhibitor.

DOACs may also be modifying eNOS activity through affecting its phosphorylation (eNOS has both stimulatory sites [Ser1177] and inhibitory sites [Thr495] whose phosphorylation status can affect enzyme activity [8]). Recently rivaroxaban has

been shown to increase nitric oxide synthesis in human arterial fibroblasts by dephosphorylating eNOS at the inhibitory site Thr495, while having no effect at the stimulatory site Ser1177 [9]. The underlying cellular signalling pathways responsible for this effect have yet to be elucidated, and whether DOACs can have similar effects on NOS phosphorylation status in endothelial or vascular smooth muscle cells remains unknown.

The concentrations of rivaroxaban and apixaban which caused the most pronounced NO-mediated vasorelaxation are an order of magnitude higher than those observed clinically (mean C_{max} of rivaroxaban is 0.5 µmol L^{-1} and median C_{max} of apixaban is 0.37 µmol L^{-1} [10]), and there may therefore be an argument that these experiments are not be clinically relevant. It is therefore important that future experiments are conducted on human tissue, over a range over doses to confirm clinical relevance. However, the requirement for these higher concentrations of DOACs to observe an experimental effect in these short term experiments may be related to their mechanism of action, for example if DOACs are affecting the endothelial cell eNOS phosphoylation status as previously shown in atrial fibroblasts [9] higher concentrations could be required to obtain the level of enzyme dephosphorylation to cause increased eNOS activity and NO production to mediate vasodilation. It may also be related to the difference in responsiveness of rat as compared to human endothelial cells, for example if the DOAC-induced vasodilation was mediated through the PAR-2 pathway it may be that the structure/activity relationship between DOACs and PAR-2 is species dependent.

DOAC-mediated dizziness and headaches are only seen in approximately 10% of patients, suggesting that there is a particular patient characteristic that may make them hypersensitive to the vasodilatory effects of DOACs. The most obvious is that the pharmacokinetics of DOACs may be altered in the the plasma of patients experiencing these side effects. These drugs are metabolised by both CYP-dependent and independent pathways (www.medicines.org.uk) and a polymorphism affecting metabolism could result in an increased C_{max} high enough to induce vasodilation. There is also the possibility of patients having polymorphisms in the cellular pathways which are activated by DOACs to cause vasodilation. Further studies to elucidate the specific DOAC-activated pathway that results in increased eNOS activity could help identify those patients who may go on to experience these side-effects.

In conclusion, we have identified a novel secondary effect of DOACs to directly affect endothelial cells and activate the NO-mediated vasorelaxant pathway which if affecting blood pressure may be the final component of the mechanism by which the side effects of dizziness and headaches occur. Identification of the specific endothelial cell pathways affected by DOACs will allow clinicians to appropriately optimise anticoagulant treatment and monitoring for patients.

5 References

[1] C. Kearon, E.A. Akl, A.J. Comerota, P. Prandoni, H. Bounameaux, S.Z. Goldhaber, M.E. Nelson, P.S. Wells, M.K. Gould, F. Dentali, M. Crowther, S.R. Kahn, Antithrombotic therapy for VTE disease: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines, Chest 141(2 Suppl) (2012) e419S-e496S.

[2] F. Dentali, N. Riva, M. Crowther, A.G. Turpie, G.Y. Lip, W. Ageno, Efficacy and safety of the novel oral anticoagulants in atrial fibrillation: a systematic review and meta-analysis of the literature, Circulation 126(20) (2012) 2381-91.

[3] M.R. Patel, K.W. Mahaffey, J. Garg, G. Pan, D.E. Singer, W. Hacke, G. Breithardt, J.L. Halperin, G.J. Hankey, J.P. Piccini, R.C. Becker, C.C. Nessel, J.F. Paolini, S.D. Berkowitz, K.A. Fox, R.M. Califf, R.A. Investigators, Rivaroxaban versus warfarin in nonvalvular atrial fibrillation, N Engl J Med 365(10) (2011) 883-91.

[4] T. Hashikata, M. Yamaoka-Tojo, S. Namba, L. Kitasato, R. Kameda, M. Murakami, H. Niwano, T. Shimohama, T. Tojo, J. Ako, Rivaroxaban Inhibits Angiotensin II-Induced Activation in Cultured Mouse Cardiac Fibroblasts Through the Modulation of NF-kappaB Pathway, International heart journal 56(5) (2015) 544-50.

[5] M. Ashina, L. Bendtsen, R. Jensen, J. Olesen, Nitric oxide-induced headache in patients with chronic tension-type headache, Brain 123 (Pt 9) (2000) 1830-7.

[6] A. Villari, G. Giurdanella, C. Bucolo, F. Drago, S. Salomone, Apixaban Enhances Vasodilatation Mediated by Protease-Activated Receptor 2 in Isolated Rat Arteries, Frontiers in pharmacology 8 (2017) 480.

[7] I.B. Buchwalow, T. Podzuweit, W. Bocker, V.E. Samoilova, S. Thomas, M. Wellner, H.A. Baba, H. Robenek, J. Schnekenburger, M.M. Lerch, Vascular smooth muscle and nitric oxide synthase, FASEB J 16(6) (2002) 500-8.

[8] V. Garcia, W.C. Sessa, Endothelial Nitric Oxide Synthase (eNOS): Perspective and Recent Developments, Br J Pharmacol (2018).

[9] C.C. Chung, Y.K. Lin, Y.C. Chen, Y.H. Kao, Y.H. Yeh, Y.J. Chen, Factor Xa inhibition by rivaroxaban regulates fibrogenesis in human atrial fibroblasts with modulation of nitric oxide synthesis and calcium homeostasis, J Mol Cell Cardiol 123 (2018) 128-138.

[10] D. Kubitza, M. Becka, W. Mueck, M. Zuehlsdorf, Rivaroxaban (BAY 59-7939)--an oral, direct Factor Xa inhibitor--has no clinically relevant interaction with naproxen, Br J Clin Pharmacol 63(4) (2007) 469-76.

Figure legends

Figure 1. Rivaroxaban or apixaban endothelial cell- and NO-dependently cause *ex vivo* aortic ring vasorelaxation. Rivaroxaban and apixaban dose-dependently caused vasorelaxation (A). Removal of endothelial cells or inhibiton of either eNOS or sGC significantly inhibited DOAC-mediated vasorelaxation (B-D). Key: (-E) After removal of endothelial cells, (L-NAME) after eNOS inhibition and (ODQ) after sGC inhibition. Data is expressed as mean ± SEM from 4-12 animals; †$p<0.05$ vs. DMSO-treated rings; **$p<0.01$ vs. DOAC alone.

Acknowledgments

None

Author's contributions

(1) substantial contributions to conception or design of the work (JM, JPP, AS, RA, GS);
(2) drafting of the work (JM, JPP, GS) or revising it critically for important intellectual content (AS, RA);
(3) all the Authors approved the submitted final version to be published and
(4) all the Authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriatelyinvestigated and resolved.

Disclosures

JPP has received investigator-initiated research funding from Bayer healthcare. RA has received investigator-initiated research funding from Bayer Healthcare and Covidien and honoraria from Bayer Healthcare, Boehringer-Ingelheim, Pfizer and Sanofi.