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Letter to the Editors-in-Chief

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

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55 Dear Editors-in-chief,

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57 **1 Introduction**

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

69

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

77

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

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

86

87 **2 Methods**

88 *2.1 Reagents*

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

95

96 *2.2 Ex vivo aortic ring preparation*

97 Thoracic aorta from male Sprague-Dawley rats (180-220 g) were dissected and rings
98 of 2-3 mm cut and mounted in organ baths filled with warmed (37°C) and gas-
99 equilibrated (95% O₂, 5% CO₂) Krebs solution containing (in mmol/L) CaCl₂ 1.6,
100 MgSO₄ 1.17, EDTA 0.026, NaCl 130, NaHCO₃ 14.9, KCl 4.7, KH₂PO₄ 1.18, and
101 glucose 5. Isometric tension of the rings was measured with force-displacement
102 transducers (Danish Myo Technology), digitised using PowerLab. A preload tension
103 of 1.5 g was applied, and the rings were equilibrated for 60 min, followed by
104 measurement of the concentration-dependent contraction to phenylephrine (10⁻⁹ to 10⁻
105 ⁴ mol L⁻¹) before being washed with fresh Krebs buffer until the tension returned to
106 that observed prior to the phenylephrine addition.

107

108 *2.3 Experimental protocol*

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

112 drug addition. The responses of the rings to rivaroxaban and apixaban were
113 compared to the vehicle (DMSO) which was applied in the same volume as the drug
114 with the resulting percentage of DMSO ranging from 0.0088 to 0.74% v/v. In a
115 second series of experiments rat aortic rings either had their endothelial cells removed
116 by gentle mechanical abrasion, or were treated with either the competitive eNOS
117 inhibitor L-N^G-nitroarginine methyl ester (L-NAME; 100 μmol L⁻¹) or the highly
118 selective, irreversible inhibitor of soluble guanylyl cyclase (sGC) 1H-
119 [1,2,3]oxadiazol[4,3-a]quinoxalin-1-one (ODQ; 10 μmol L⁻¹) for 10 minutes prior to
120 the addition of DMSO, rivaroxaban or apixaban (0.01-3 μmol L⁻¹). Tissue response
121 was expressed as % relaxation.

122

123 *2.4 Statistical analysis*

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

128

129 **3 Results**

130 *3.1 Relaxant effect of rivaroxaban and apixaban on pre-contracted aortic rings*

131 Exposure of phenylephrine pre-contracted rat aortic rings to either rivaroxaban or
132 apixaban caused a statistically significant dose-dependent relaxation as compared to
133 the vehicle DMSO (Fig. 1a). DMSO at the maximum 0.74% v/v caused a $16.5 \pm 4.7\%$
134 relaxation as compared to $3 \mu\text{mol L}^{-1}$ rivaroxaban and apixaban which caused a
135 $47.9 \pm 3.7\%$ and $55.5 \pm 6.0\%$ relaxation respectively ($p < 0.05$ vs. DMSO).

136

137 *3.2 Role of endothelial cells and nitric oxide in the aortic ring relaxant effect of*

138 *rivaroxaban and apixaban*

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

147

148

149

150 4 Discussion

151

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

166

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

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

180

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

196

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

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

206

207 The concentrations of rivaroxaban and apixaban which caused the most pronounced
208 NO-mediated vasorelaxation are an order of magnitude higher than those observed
209 clinically (mean C_{\max} of rivaroxaban is $0.5 \mu\text{mol L}^{-1}$ and median C_{\max} of apixaban is
210 $0.37 \mu\text{mol L}^{-1}$ [10]), and there may therefore be an argument that these experiments
211 are not be clinically relevant. **It is therefore important that future experiments are**
212 **conducted on human tissue, over a range over doses to confirm clinical relevance.**

213 However, the requirement for these higher concentrations of DOACs to observe an
214 experimental effect in these short term experiments may be related to their
215 mechanism of action, for example if DOACs are affecting the endothelial cell eNOS
216 phosphorylation status as previously shown in atrial fibroblasts [9] higher
217 concentrations could be required to obtain the level of enzyme dephosphorylation to
218 cause increased eNOS activity and NO production to mediate vasodilation. **It may**
219 **also be related to the difference in responsiveness of rat as compared to human**
220 **endothelial cells, for example if the DOAC-induced vasodilation was mediated**
221 **through the PAR-2 pathway it may be that the structure/activity relationship between**
222 **DOACs and PAR-2 is species dependent.**

223

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

236

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

243

244

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- 279

280 **Figure legends**

281

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

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295

296

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305 (3) all the Authors approved the submitted final version to be published and

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