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The vascular effects of rotigaptide *in vivo* in man

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ARTICLE INFO

Article history:
Received 10 July 2008
Accepted 18 August 2008

Keywords:
Gap junctions
Connexin 43
Blood flow
Endothelium
Fibrinolysis

ABSTRACT

Endothelium-derived hyperpolarising factor (EDHF) causes vasorelaxation and may contribute to the release of the endogenous fibrinolytic factor, tissue-plasminogen activator (t-PA). Rotigaptide enhances communication via the connexin 43 gap junction subunit and may potentiate the vascular actions of EDHF. The aims of the present study were therefore to determine whether rotigaptide influences basal and stimulated endothelium-dependent vasodilatation and t-PA release *in vivo* in man.

Using venous occlusion plethysmography, forearm blood flow was measured in 27 healthy volunteers during intra-brachial infusions of rotigaptide (0.25–25 nmol/min) alone, or co-administered with endothelium-dependent (acetylcholine [5–20 μg/min] and bradykinin [30–300 pmol/min]) and independent (sodium nitroprusside [2–8 μg/min]) vasodilators in the presence or absence of aspirin and the 'nitric oxide clamp'. The 'nitric oxide clamp' inhibits endogenous nitric oxide synthesis with L-N-monomethylarginine and restores resting blood flow with the exogenous nitric oxide donor, sodium nitroprusside.

Basal blood flow was unaffected by rotigaptide (P = NS). Acetylcholine, bradykinin and sodium nitroprusside all caused dose-dependent vasodilatation in the presence and absence of aspirin and the 'nitric oxide clamp' ($P \leq 0.005$ for all). These responses were unaffected by rotigaptide (P = NS). Bradykinin caused t-PA antigen and activity release ($P = 0.04$, $P < 0.0001$, respectively) that was unaffected by rotigaptide.

Augmentation of connexin 43 communication has no effect on basal vascular tone and does not enhance endothelium-dependent or independent vasodilatation, or t-PA release in the forearm arterial circulation of healthy men. It remains to be established whether augmentation of connexin 43 communication improves endothelial function in patients with vascular disease.

1. Introduction

The endothelium plays a major role in the regulation of vascular tone and is responsible for the release of the endogenous fibrinolytic factor, tissue-plasminogen activator (t-PA). The elucidation of their roles in vascular physiology and pathophysiology has been fundamental to recent advances in the treatment and prevention of many cardiovascular diseases.

After blockade of both nitric oxide and prostacyclin generation, a substantial degree of endothelium-dependent vasodilatation remains and is attributed to endothelium-derived

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0006-2952/$ – see front matter
doi:10.1016/j.bcp.2008.08.022

hyperpolarising factor (EDHF) [1]. EDHF activity has been demonstrated in the microcirculation of a variety of human vascular beds including the peripheral [2], mesenteric [3] and subcutaneous [4,5] circulations in addition to the coronary [6,7] and renal [8] vasculature. Consistently, its contribution is most prominent in the small resistance arteries [3,9] that regulate systemic blood pressure and local tissue perfusion. Furthermore, the release of t-PA from the endothelium is independent of both nitric oxide and prostacyclin production [10,11], and it has been suggested that EDHF may mediate its release [11,12]. Thus, alterations in EDHF activity may contribute to endothelial dysfunction and its manipulation presents an exciting opportunity to restore vascular health and reduce the burden of cardiovascular disease [13].

The transmission of hyperpolarisation from the endothelium to the underlying smooth muscle occurs via undefined mediators and pathways. Various mediators have been proposed but none has emerged as a universal EDHF in all species and vascular beds [14,15]. However, there is considerable evidence that gap junctions are required [15–20]. These aqueous pores are found at points of cell–cell contact and allow the intercellular transfer of small molecules (<1 kDa). Myoendothelial gap junctions are, therefore, ideally suited to the radial transfer of electronic charge or second messenger between the endothelium and underlying smooth muscle. Furthermore, gap junctions are particularly abundant in the microcirculation where the density of their expression correlates with an increasing contribution of EDHF to endothelial vasodilatation in these small resistance vessels [21]. Each gap junction hemichannel is composed of six connexin subunits including connexin (Cx) 37, 40 and 43 in the mammalian vasculature. Indeed, using specific connexin antagonist peptides, we have previously demonstrated the critical importance of Cx43 in EDHF-mediated vasodilatation of human resistance arteries *in vitro* [5].

It has not previously been possible to make a direct assessment of the role of gap junctions in the mediation of EDHF responses *in vivo* in man. However, rotigaptide (ZP-123) is a synthetic hexapeptide (Ac-D-Tyr-D-Pro-D-Hyp-Gly-D-Ala-Gly-NH_2) that alters the phosphorylation status of Cx43 to potentiate communication via gap junctions [22–25]. In addition to its clinical development as an anti-arrhythmic agent [26,27], it has been promoted as an important new tool to aid in the dissection of the physiologic role of gap junctions [22]. Here, we have conducted the first clinical assessment of the role of gap junctions, and specifically Cx43, in the peripheral vascular EDHF-mediated responses. We tested the hypothesis that, by increasing communication via gap junctions, rotigaptide would enhance EDHF-mediated vasodilatation and t-PA release in the forearm arterial circulation of healthy man.

2. Methods

2.1. Preliminary validation study

The biological activity of rotigaptide was assessed through *in vitro* measurements of its effect on transmural conduction velocity in rabbit ventricular myocardium. Hearts from four male New Zealand White rabbits were used for these experiments, which conform to the standards set out in the Animals (Scientific Procedures) Act 1986.

Rabbits ($n = 4$) were killed with a single intravenous injection of 100 mg/kg pentobarbital sodium (Rhône Mérieux, France). Hearts were excised, placed in chilled Tyrode's solution (containing [mmol/L]: Na 134.5, Mg 1.0, K 5.0, Ca 1.9, Cl 101.8, SO_4 1.0, H_2PO_4 0.7, HCO_3 20, acetate 20 and glucose 10) and perfused via the left coronary artery with oxygenated (95% O_2–5% CO_2) Tyrode's solution maintained at pH 7.4 and 37 °C. Perfused left ventricular free wall wedge preparations were dissected out and mounted in a custom built chamber which allowed access to the transmural surface for imaging. The preparation was stimulated using a bipolar electrode placed on the epicardial surface at 1.5× diastolic threshold using a 2 ms pulse at a basic cycle length of 350 ms. Perfusion pressure and electrocardiogram were monitored throughout. An optical mapping system was used to record optical action potentials as previously described [28]. Motion artifact was minimised using 15 mmol/L 2,3 butanedione monoxime (BDM; Sigma Aldrich, UK). Measurements were taken at 15-min intervals. After two control recordings the perfusate was changed to Tyrode's solution containing BDM and 1 μmol/L rotigaptide for a further 15 min and measurements were repeated.

2.2. Clinical study

This clinical study was performed with the approval of the local research ethics committee in accordance with the Declaration of Helsinki and with the written informed consent of each subject.

2.2.1. Subjects

Healthy non-smokers (mean age 22 years; range 19–25 years) were recruited into the study. Participants were excluded if they had clinically significant conditions including hypertension, hyperlipidemia, diabetes mellitus, asthma and coagulopathy. No participant had suffered a recent infective or inflammatory condition, or had taken any medications in the 7 days prior to the study. On the day of study, participants had fasted and abstained from caffeine and tobacco for at least 4 h and from alcohol for 24 h.

2.2.2. Drugs

Pharmaceutical grade bradykinin (Clinalfa AG, Läufelfingen, Switzerland), acetylcholine (Novartis Ltd., Middlesex, UK), L-N(G)-monomethyl arginine citrate (Clinalfa), sodium nitroprusside (Hospira Inc., CA, USA) and rotigaptide (American Peptide Inc., CA, USA) were dissolved in physiological saline. Aspirin was obtained from Dagra Pharma, Diemen, Netherlands.

2.2.3. Forearm venous occlusion plethysmography

All subjects underwent cannulation of the brachial artery with a 27G-standard wire steel needle under controlled conditions. All studies were performed with patients lying supine in a quiet, temperature controlled (22–25 °C) room. The intra-arterial infusion rate was kept constant at 1 mL/min throughout all

studies. Forearm blood flow was measured in the infused and non-infused arms by venous occlusion plethysmography using mercury-in-silastic strain gauges as described previously [29,30]. Supine heart rate and blood pressure were monitored at intervals throughout each study using a semi-automated non-invasive oscillometric sphygmomanometer.

2.2.4. Intra-arterial drug administration

2.2.4.1. PROTOCOL 1. Effect of rotigaptide on basal forearm blood flow. Five participants attended once each. They received intra-arterial rotigaptide at 0.25, 0.75, 2.5, 7.5 and 25 nmol/min for 6 min at each dose. Assuming basal forearm blood flow of 25 mL/min, this would give tissue rotigaptide concentrations of 0.01–1.0 μmol/L. Forearm blood flow was measured during the final 3 min of infusion of each dose.

2.2.4.2. PROTOCOL 2. Effect of rotigaptide on agonist-induced vasodilatation and tissue-plasminogen activator release. Twelve volunteers attended on each of three occasions. After a 20-min intra-arterial infusion of 0.9% saline, participants received either intra-arterial placebo (0.9% saline), 2.5 nmol/min rotigaptide or 25 nmol/min rotigaptide using a randomised double-blind cross-over design. Rotigaptide or placebo was administered alone for 20 min before being co-infused with ascending doses of bradykinin (an endothelium-dependent vasodilator that causes the release of tissue-plasminogen activator; 30–300 pmol/min), acetylcholine (an endothelium-dependent vasodilator that does not cause the release of t-PA; 5–20 μg/min) and sodium nitroprusside (an endothelium-independent vasodilator that does not cause the release of t-PA; 2–8 μg/min). Co-infused drugs were separated by a 20-min infusion of 0.9% saline. The order of agonist infusion was randomised between participants but maintained constant for each of the three visits.

2.2.4.3. PROTOCOL 3. Effect of rotigaptide on EDHF-mediated vasodilatation. Ten volunteers were recruited to attend on two occasions. EDHF activity was isolated by inhibiting the production of both prostacyclin and nitric oxide on each of the two visits. Cyclo-oxygenase activity was inhibited with a single 600 mg dose of oral aspirin 1 h prior to each study. Nitric oxide production was inhibited with L-N(G)-monomethyl arginine citrate (L-NMMA) in the 'nitric oxide clamp'. After a 20-min intra-arterial infusion of 0.9% saline, L-NMMA (8 μmol/min) was infused via the brachial artery. To compensate for L-NMMA induced basal vasoconstriction, forearm blood flow was restored to baseline using a titrated dose of exogenous nitric oxide in the form of intra-brachial sodium nitroprusside (SNP; 90–900 ng/min). The titrated dose of SNP was co-infused with L-NMMA throughout the study. This arrangement allows a constant 'clamped' delivery of exogenous nitric oxide whilst endogenous nitric oxide synthase activity is inhibited [31].

Either rotigaptide (25 nmol/min) or saline placebo was co-infused with the 'nitric oxide clamp' in a double-blind randomised cross-over design. Subsequently, ascending doses of bradykinin (30–300 pmol/min), acetylcholine (5–20 μg/min) and sodium nitroprusside (2–8 μg/min) were co-infused and separated by a 20-min infusion of 0.9% saline. The order of agonist co-infusion was randomised between participants but maintained constant between visits.

2.2.5. Blood sampling

Seventeen-gauge venous cannulae were inserted into left and right ante-cubital fossae during Protocol 2. Blood samples were drawn simultaneously from each arm before bradykinin infusion and after the maximum dose of bradykinin (300 pmol/min). Blood was collected into acidified buffered citrate (Stabilyte, Biopool International, UK; for t-PA assays) and into citrate (BD Vacutainer, BD UK Ltd., UK; for measurement of t-PA's major endogenous inhibitor, plasminogen activator inhibitor type 1 [PAI-1]). Samples were kept on ice before centrifugation at 2000 × *g* for 30 min at 4 °C. Platelet-free plasma was decanted and stored at −80 °C before assay. Plasma t-PA antigen and activity (t-PA Combi Actibind Elisa Kit; Technoclone, Austria) and PAI-1 antigen and activity (Elitest-PAI-1 Antigen and Zymutest PAI-1 Activity; Hyphen Biomed, France) concentrations were determined by enzyme-linked immunosorbant assays. Hematocrit was measured at baseline and at the end of the study.

2.3. Data analysis and statistics

2.3.1. Optical mapping validation study

Data analysis was performed using custom software. Activation time was determined at the midpoint between baseline and the peak of the action potential upstroke. Transmural conduction velocity was calculated for each time point using activation time from the earliest activation on the epicardial edge of the transmural surface to the earliest activation on the endocardial side.

2.3.2. Clinical study

Forearm plethysmographic data were analyzed as described previously [29]. Estimated net release of plasma t-PA and PAI-1 has been defined previously as the product of the infused forearm plasma flow (based on the mean hematocrit and the infused forearm blood flow) and the concentration difference between the infused and non-infused arms [32].

Variables are reported as mean ± S.E.M. and analyzed using repeated measures ANOVA with post hoc Bonferroni corrections and two-tailed Students *t*-test as appropriate. Statistical analysis was performed with GraphPad Prism (Graph Pad Software) and statistical significance taken at the 5% level.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

3. Results

3.1. Effect of rotigaptide on myocardial conduction velocity in vitro

In all experiments, transmural conduction velocity remained constant during control perfusion (20.8 ± 1.7 cm/s versus 21.4 ± 1.6 cm/s, P = NS). Following perfusion with rotigaptide, an increase in transmural conduction velocity was observed in rabbit ventricular tissue *in vitro* (29.4 ± 1.7 cm/s, $P < 0.001$, ANOVA; Fig. 1).

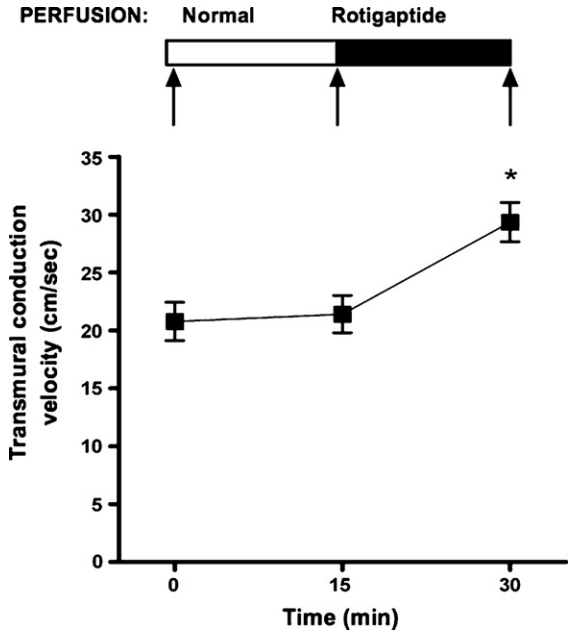

Fig. 1 – Effect of 1 μmol/L rotigaptide on transmural conduction velocity in perfused rabbit ventricular myocardium. *P < 0.001, 30 min versus 0 or 15 min, t-test.

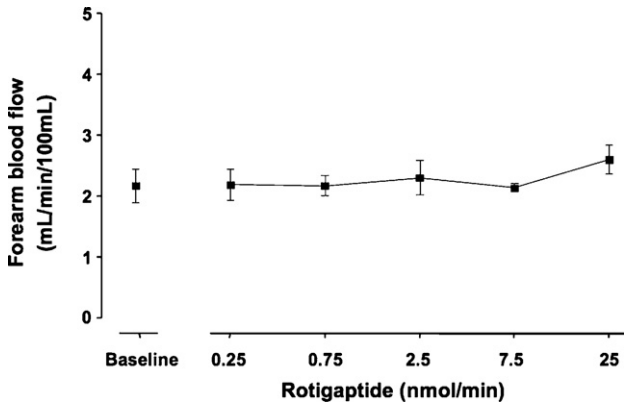

Fig. 2 – Forearm blood flow during intra-brachial infusion of rotigaptide (0.25–25 nmol/min).

3.2. *Effect of rotigaptide on basal forearm blood flow*

At doses of 0.25–25 nmol/min, rotigaptide had no effect upon basal forearm blood flow (P = NS, ANOVA: Fig. 2).

3.3. *Effect of rotigaptide on agonist-induced vasodilatation and tissue-plasminogen activator release*

Acetylcholine, bradykinin and sodium nitroprusside each caused dose-dependent arterial vasodilatation ($P < 0.0001$ for all, ANOVA) that was unaffected by either 2.5 or 25 nmol/min rotigaptide (P = NS for both, in the presence versus the absence of rotigaptide; ANOVA: Fig. 3).

At baseline, net t-PA antigen release was 0.09 ± 0.05 ng/100mL/min and net release of t-PA activity was 0.54 ± 1.4 IU/100 mL/min. This was unchanged by the presence of rotigaptide (P = NS for all; ANOVA). Bradykinin (300 pmol/min) caused the release of t-PA antigen (7.62 ± 5.56 ng/100 mL/min; $P = 0.04$, ANOVA) and activity (6.15 ± 2.20 IU/100 mL/min; $P < 0.0001$, ANOVA) but this was unaffected by either 2.5 or 25 nmol/min rotigaptide (P = NS for all, ANOVA).

Net release of PAI-1 antigen and activity was unaffected by bradykinin (300 pmol/min) in the presence or absence of rotigaptide (P = NS for all, ANOVA; data not shown).

3.4. *Effect of rotigaptide on EDHF-mediated vasodilatation*

Intra-arterial L-NMMA (8 μmol/min) reduced basal forearm blood flow by approximately 38% (from 2.40 ± 0.20 reduced to 1.50 ± 0.20 mL/100 mL tissue/min; $P < 0.0001$) and was unaffected by rotigaptide (P = NS, 2-way ANOVA). Forearm blood flow was restored to baseline levels by the titration of sodium nitroprusside (P = NS, ANOVA).

After the inhibition of endothelial nitric oxide and prostacyclin synthesis, dose-dependent vasodilatation was evoked by acetylcholine, bradykinin and sodium nitroprusside ($P \leq 0.005$ for all, ANOVA). This response was not altered by the presence of rotigaptide (P = NS, ANOVA; Fig. 4).

4. Discussion

For the first time, we have assessed the role of connexin 43 and gap junctions in the control of vascular tone and t-PA release *in vivo* in man. We have shown, in healthy volunteers, that potentiation of connexin 43-mediated intercellular communication with rotigaptide does not affect basal forearm arterial

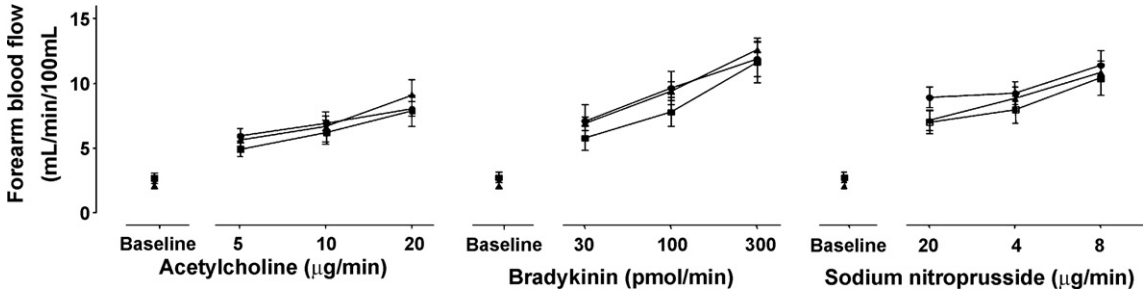

Fig. 3 – Forearm blood flow during intra-brachial infusion of acetylcholine (left hand panel), bradykinin (middle panel) and sodium nitroprusside (right hand panel) in the presence of placebo (squares), 2.5 nmol/min rotigaptide (triangles) or 25 nmol/min rotigaptide (circles). P = NS for rotigaptide versus placebo, ANOVA.

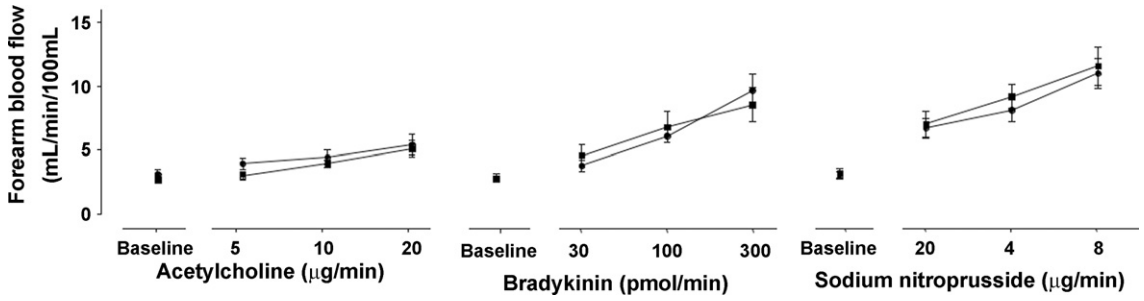

Fig. 4 – In the presence of the 'nitric oxide clamp' and oral aspirin, forearm blood flow during intra-brachial infusion of acetylcholine (left hand panel), bradykinin (middle panel) and sodium nitroprusside (right hand panel) in the presence of placebo (squares) or 25 nmol/min rotigaptide (circles). *P* = NS for rotigaptide versus placebo, ANOVA.

blood flow or agonist-induced vasodilatation and t-PA release in the presence or absence of concomitant inhibition of prostacyclin and nitric oxide production.

To date, there has been no direct assessment of the role of gap junctions, nor individual connexin subtypes, in the regulation of vascular function *in vivo* in man. The use of connexin antagonists has been limited by concerns about the potential for toxicity and older putative gap junction blockers exert a range of non-endothelial effects and alter ion channel permeability [33,34]. However, rotigaptide offers a novel means for the assessment of gap junction-dependent phenomena *in vivo*. Not only has it been safely administered to healthy humans [26,27] but its actions are specific to Cx43. Indeed, it augments communication via gap junctions in the absence of changes in membrane conduction or basal current [35], and it exhibits no binding to a large array of receptors including numerous ion channels [36]. It enhances intercellular dye transfer between HeLa cells expressing Cx43 but not between those expressing Cx26 or Cx32 [23] and promotes electrical coupling between ventricular myocytes via alterations in the phosphorylation status of Cx43 [25,37]. Indeed, phopshorylation of Cx43 maintains it an open state [38] and Kjølbye et al. have recently demonstrated that the anti-arrhythmic effects of rotigaptide are associated with the inhibition of Cx43 dephosphorylation [25].

We have previously demonstrated that EDHF-mediated vasodilatation of subcutaneous resistance arteries from pregnant women depends upon the presence of functional Cx43 whilst Cx37 and Cx40 are not required [5]. Indeed, in comparison to Cx37 and Cx40, the vascular expression of Cx43 appears to be particularly labile. It is up-regulated by oestrogen [39], at the leading edge of atherosclerotic plaques [40], after vascular injury [41] and at sites of increased shear stress [42,43]. Furthermore, type I diabetes mellitus is associated with an impairment of EDHF-mediated vascular responses [13,44], and elevated glucose concentrations cause the isolated down-regulation of Cx43 expression [45] and permeability [46] *in vitro*. The examination of Cx43-mediated responses is, therefore, of particular relevance not only to our understanding of vascular physiology, but might also represent a major therapeutic target to attenuate endothelial dysfunction and improve vascular health.

Despite theoretical indications, we have failed to demonstrate a major vascular effect of rotigaptide even during inhibition of both nitric oxide and prostacyclin production. Was the rotigaptide inactive or used at an inadequate dose? Our preliminary *in vitro* electrophysiological optical mapping study provided important information confirming the biological activity of our preparation of rotigaptide at 1 μmol/L. The maximum dose of rotigaptide assessed in the clinical study was 25 nmol/min which, based upon forearm blood flow of 25 mL/min, equates to an estimated tissue concentration of approximately 1 μmol/L. This concentration is in excess of the effective anti-arrhythmic concentration employed in previous animal models [24,47–49] and is similar to the maximum tissue concentration of rotigaptide achieved in clinical trials [27]. Furthermore, it is in excess of the concentration recently shown to be required for the prevention of Cx43 dephosphorylation [25]. We therefore do not believe that the preparation was inactive or used at the wrong dose.

Connexin 43 is expressed abundantly in a wide range of resistance arteries obtained from a variety of species [15] including humans [5]. Whilst our previous assessment of the role of connexin subtypes in human EDHF-mediated responses demonstrated a critical role for connexin 43 and not connexin 37 or 40 [5], it is possible that, in the non-pregnant state, the vasomotor and EDHF mechanism requires a contribution from all of these subtypes. Rotigaptide specifically augments connexin 43-mediated signalling but the augmentation of vascular gap junction mediated communication may therefore require the combined potentiation of all three of the major vascular connexin subtypes.

The present study made an examination of vascular responses in healthy young men. It is conceivable that vasomotor and endogenous fibrinolytic responses cannot be augmented because the endothelium is already maximally active with a high baseline open-state probability for connexin 43. Indeed, the anti-arrhythmic activity of rotigaptide is particularly potent in the context of acidosis and metabolic stress during which the open-state probability of Cx43 is relatively low [36]. Furthermore, we were careful to dissect out EDHF-mediated activity by the inhibition of prostacyclin and nitric oxide with oral aspirin and the nitric oxide clamp respectively. EDHF has activity that is reciprocal to nitric oxide [50] and becomes up-regulated to compensate for impaired nitric oxide bioavailability in a variety of disease states [13]. Therefore, the argument that EDHF is maximally active in the studied population becomes more pertinent in the presence of nitric oxide inhibition.

The EDHF mechanism may differ between vascular beds but the forearm arterial model employed here has been predictive of the behaviour of the coronary vasculature in prior studies of other aspects of endothelial function [30,51,52]. However, whilst the present study provides important data regarding rotigaptide in the peripheral resistance vasculature, caution should still be applied when considering extrapolation of the results to infer similar pharmacodynamic effects in other vascular beds.

In conclusion, we have demonstrated that intra-arterial rotigaptide does not augment vasomotion or endogenous fibrinolysis in healthy subjects. Whether enhancement of connexin 43-mediated intercellular communication influences vascular function in conditions associated with specific impairment of EDHF-mediated activity, such as type I diabetes mellitus, remains to be evaluated. Not only would this assessment provide important mechanistic insights to the pathophysiology of endothelial dysfunction but could also highlight an important novel therapeutic target.

Acknowledgments

We are grateful to Professors Stuart Cobbe and Godfrey Smith (University of Glasgow) for their expert advice with the electrophysiologic measurements. We thank the staff of the Wellcome Trust Clinical Research Facility in Edinburgh and Pamela Dawson for their help with these studies.

This work was funded by a project grant from the Chief Scientist's Office, Scottish Executive (CZB/4/520), a Bristol-Myers Squibb Cardiovascular Prize Fellowship, United Kingdom (NNL) and the British Heart Foundation (RCM and FLB).

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