

SPECIAL REPORT

The role of gap junctions in mediating endothelium-dependent responses to bradykinin in myometrial small arteries isolated from pregnant women

^{1,2}Louise C. Kenny, ²Philip N. Baker, ¹David A. Kendall, ¹Michael D. Randall & ^{*1}William R. Dunn

¹School of Biomedical Sciences, University of Nottingham Medical School, Queen's Medical Centre, Nottingham NG7 2UH and ²Maternal and Foetal Health Research Centre, University of Manchester, St Mary's Hospital, Whitworth Park, Manchester M13 0JH

Endothelium-dependent responses were assessed in myometrial small arteries isolated from pregnant women, using pressure myography. Responses to bradykinin were unaffected by the combined presence of the nitric oxide synthase (NOS) inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME, 100 μ M) and the cyclo-oxygenase inhibitor, indomethacin (10 μ M). The additional presence of clotrimazole (50 μ M) attenuated, but did not abolish, vasodilator responses to bradykinin. Raising extracellular [K⁺] (by between 1 and 15 mM) did not evoke a vasodilator response, nor did the cannabinoids, anandamide and methanandamide. Responses to bradykinin were attenuated in the presence of the gap junction inhibitors 18- α -glycyrrhetic acid (18- α GA, 100 μ M), carbenoxolone (100 μ M) and palmitoleic acid (50 μ M). SR141716A, the CB₁ receptor antagonist attenuated responses to bradykinin, but only at high concentrations (10 μ M). These results suggest that gap junctional communication is involved in the nitric oxide (NO)- and prostanoid-independent vasodilator responses to bradykinin in myometrial small arteries in normal pregnancy.

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Abbreviations: EDHF, endothelium-derived hyperpolarizing factor; NO, nitric oxide; NOS, nitric oxide synthase

Introduction Endothelium-dependent vasorelaxation responses in several human blood vessels, including subcutaneous (Buus *et al.*, 2000; Coats *et al.*, 2001; Coleman *et al.*, 2001), omental (Pascoal *et al.*, 1998) and myometrial small arteries (Kenny *et al.*, 2002) have been reported to be insensitive to combined inhibition of the cyclo-oxygenase and nitric oxide (NO) synthase enzymes. In some of these preparations the non-NO, non-prostanoid response was associated with vascular smooth muscle hyperpolarization consistent with the release of an endothelium-derived hyperpolarizing factor (EDHF) (Buus *et al.*, 2000; Coleman *et al.*, 2001).

A number of candidates have been proposed to be EDHFs including epoxyeicosatrienoic acids (EETs) (Campbell *et al.*, 1996), endocannabinoids (Randall *et al.*, 1996) and K⁺ (Edwards *et al.*, 1998). More recently, it has been suggested that EDHF responses may involve the transfer of a substance, or electrical current, directly between the endothelium and underlying vascular smooth muscle *via* heterocellular gap junctions (Chaytor *et al.*, 1998; Taylor *et al.*, 1998; Harris *et al.*, 2000; Coleman *et al.*, 2001).

The aim of the present study was to investigate the mechanisms involved in the vasodilator action of EDHF-type responses in human myometrial small arteries isolated from pregnant women.

Methods This investigation conformed to the principles outlined in the Declaration of Helsinki (1989). Ethical permission for the study was obtained from the Nottingham City Hospital Trust Ethical Committee and all subjects had given informed written consent to participation. Forty-nine healthy women had normotensive pregnancies with singleton fetuses subsequently shown to be above the tenth centile of birth weight for gestational age. They were undergoing elective Caesarean section at term for reasons such as previous cephalopelvic disproportion or malpresentation. Following delivery of the baby and placenta at Caesarean section, a full thickness biopsy of myometrium measuring approximately 2 cm by 1 cm was obtained from the upper margin of the transverse lower uterine incision. Specimens were placed in oxygenated physiological salt solution (PSS) at 4°C. With the use of a stereomicroscope, arteries between 100–400 μ m in diameter were identified, promptly dissected from the samples and utilized immediately.

Each artery was mounted on a pressure myograph as described in detail elsewhere (Wallis *et al.*, 1996). In brief, vessels were secured between two glass cannulae. One cannula was closed and the other connected to a system containing PSS, which in turn was linked to a pressure-servo unit. This allowed the intraluminal pressure to be precisely controlled. Vessels were circulated with PSS that was bubbled continuously with a 5% CO₂–95% O₂ gas mixture and circulated with a Masterflex pump. The vessel was imaged using a video camera and analysed with an appropriate dimension analyser (Living Systems Instrumentation, Burlington, VT, U.S.A.), which was linked to a MacLab data

*Author for correspondence;
E-mail: william.dunn@nottingham.ac.uk

acquisition system. The intraluminal pressure was set at 60 mmHg and the temperature of the organ bath was maintained at 37°C. The vessels were allowed to equilibrate under these conditions for 90 min. Over this time, vessels developed a variable degree of myogenic tone, which was supplemented by the addition of the thromboxane mimetic U46619 to reduce diameter by approximately 50% of the original diameter. Vessels were then exposed to increasing concentrations of the endothelium-dependent vasodilator, bradykinin in 3 fold increments. Following washing, and inducing tone, the concentration response curves to bradykinin were repeated in the presence and absence of various inhibitors. Initial experiments determined the effect of the cyclo-oxygenase inhibitor, indomethacin (10 μ M) and the NOS inhibitor, *N*-nitro-L-arginine methyl ester (L-NAME, 100 μ M) on responses to bradykinin. In subsequent experiments indomethacin (10 μ M) and L-NAME (100 μ M) were present throughout.

In a series of experiments, responses to bradykinin were obtained in the absence and presence of a variety of agents which have been reported to interfere with endothelium-dependent vasodilator responses, including: (1) clotrimazole (50 μ M), (McCulloch *et al.*, 1997), (2) 18- α -glycyrrhetic acid (18 α -GA, 100 μ M) (Taylor *et al.*, 1998), (3) carbenoxolone (100 μ M) (Edwards *et al.*, 1999), (4) palmitoleic acid (50 μ M) (Harris *et al.*, 2000), or (5) SR141716A (1 and 10 μ M) (Harris *et al.*, 2000; 2002). At the end of each experiment the vessels were washed for approximately 60 min in calcium free PSS in order to obtain the maximum passive diameter of each artery. In a separate series of experiments vessels were constricted (50% reduction in diameter) by myogenic tone supplemented with U46619, and then exposed to either (1) anandamide (10 nM–3 μ M in 3 fold increments), or (2) methanandamide (10 nM–3 μ M) or (3) raised extracellular [K⁺] (with additional increments of 2, 5, 10, 15 and 20 mM), to determine whether these agents caused vasodilatation.

Drugs and solutions 9,11-Dideoxy-11 α ,9 α -epoxy-methano-prostaglandin F_{2 α} (U46619), bradykinin acetate, indomethacin, L-NAME, clotrimazole, Ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), Ethylenediamine-tetraacetic acid (EDTA), 18- α -glycyrrhetic acid (18 α -GA), 3 β -Hydroxy-11-oxoolean-12-en-30-oic acid 3-hemisuccinate (carbenoxolone), cis-9-Hexadecenoic acid (palmitoleic acid) and R(+)-arachidonyl-1'-hydroxy-2'-propylamide (methanandamide) (Sigma, U.K.), and N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide hydrochloride (SR141716A), supplied by Sanofi-Winthrop. All drugs were made up fresh from stock solutions on the day of the experiment and dissolved in physiological salt solution with the exception of SR141716A and clotrimazole which were dissolved in ethanol and palmitoleic acid and 18 α -GA, which were dissolved in dimethyl sulphoxide (DMSO). Anandamide was synthesized by Dr P. DeBank (University of Nottingham) from arachidonoyl chloride and ethanolamine and dissolved in an inert oil/water emulsion. Extracellular [K⁺] was increased by equimolar exchange of NaCl with KCl. All drugs were added to the extraluminal side of the vessel. Control experiments confirmed that the vehicles had no significant effect on constrictor tone or bradykinin-induced relaxations at the final concentration employed.

The composition of the PSS was as follows (in mM): CaCl₂·2H₂O 1.6, NaCl 119, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.18, MgSO₄·7H₂O 1.17, Na₂EDTA 0.023, Glucose 5.5 (pH 7.4 when gassed with 5% CO₂-95% O₂). Calcium-free PSS was prepared by omitting calcium and adding 0.5 mM EGTA.

Drug analysis Vasodilator responses to bradykinin were calculated as percentages of the maximum possible vasodilator range, i.e. the diameter in calcium-free PSS minus the diameter in the presence of myogenic and U46619-induced tone. Results are shown graphically as means (\pm s.e.mean), with *n*=the number of patients. R_{max} refers to the maximum relaxation achieved in the presence of the highest concentration of bradykinin used (1 μ M). Statistical evaluation of concentration response curves within each group was performed using repeated measures analysis of variance (ANOVA) (with significance determined at *P*<0.05) with *post-hoc* testing (Bonferroni/Dunn).

Results Cumulative concentration response curves to bradykinin were reproducible over two successive runs (results not shown). Our preliminary experiments showed that responses to bradykinin were unaffected by combined inhibition of cyclo-oxygenase and NOS (see also Kenny *et al.*, 2002). Thereafter, all experiments were performed in the presence of indomethacin (10 μ M) and L-NAME (100 μ M).

In the presence of Clotrimazole (50 μ M), higher concentrations of U46619 were required to reduce diameter by 50%. Clotrimazole significantly attenuated but did not abolish response to bradykinin (R_{max} control 78.3 \pm 5.7% vs R_{max} clotrimazole 46.0 \pm 7.9%, *n*=5, *P*<0.05) (Figure 1).

The inhibitors of gap junction communication, 18 α -GA (100 μ M), carbenoxolone (100 μ M) and palmitoleic acid (50 μ M), virtually abolished responses to bradykinin (Figure 2).

SR141716A (1 μ M) had no effect on responses to bradykinin (R_{max} control 75.0 \pm 9.9% vs R_{max} SR141716A 72.4 \pm 10.6%, *n*=6, *P*>0.05). By contrast a higher concentration of SR141716A (10 μ M) significantly attenuated responses to bradykinin (Figure 3).

Anandamide and methanandamide did not evoke a relaxation response at concentrations up to 30 μ M (R_{max} anandamide 3.0 \pm 1.1%, *n*=4, R_{max} methanandamide 2.2 \pm 0.8%, *n*=5). Similarly incremental concentrations of K⁺ (additional to the 5.9 mM K⁺ already present in the PSS) did not evoke a relaxation and at concentrations of 15 mM

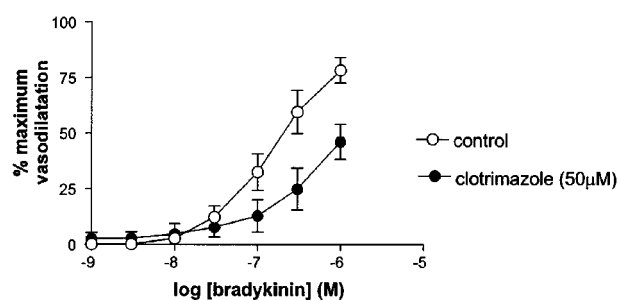


Figure 1 The effect of clotrimazole on responses to bradykinin (in the presence of L-NAME and indomethacin) in human isolated myometrial small arteries. Each point represents the mean \pm s.e.mean (*n*=5).

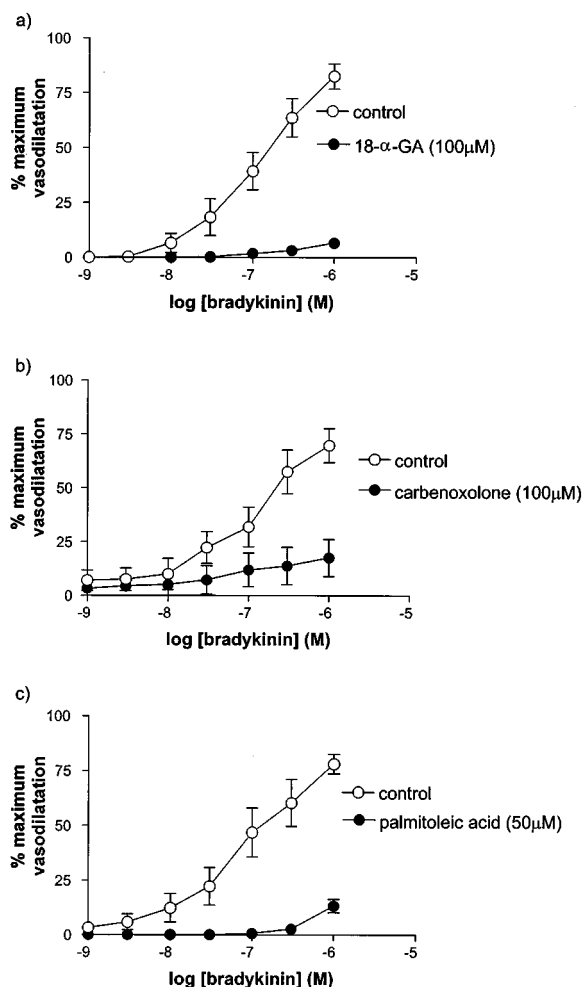


Figure 2 The effect of (a) 18- α -GA, (b) carbenoxolone and (c) palmitoleic acid on responses to bradykinin (in the presence of L-NAME and indomethacin) in human isolated myometrial small arteries. Each point represents the mean \pm s.e.mean ($n=6$).

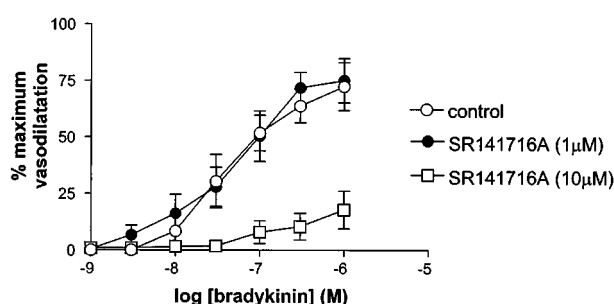


Figure 3 The effect of 1 μ M and 10 μ M SR 141716A on responses to bradykinin (in the presence of L-NAME and indomethacin) in human isolated myometrial small arteries. Each point represents the mean \pm s.e.mean ($n=6$).

and above caused a further increase in tone. In both sets of experiments, vasodilator responses to bradykinin were apparent.

Discussion A number of recent reports have indicated that endothelium-dependent responses are independent of NO and

prostanoid production in small arteries isolated from humans (Pascoal *et al.*, 1998; Buus *et al.*, 2000; Coats *et al.*, 2001; Coleman *et al.*, 2001; Kenny *et al.*, 2002). The non-NO, non-prostanoid responses in human subcutaneous arteries have been shown to be sensitive to depolarization, by raising extracellular KCl (Coats *et al.*, 2001; Kenny *et al.*, 2002) and to be associated with vascular smooth muscle hyperpolarization (Buus *et al.*, 2000; Coleman *et al.*, 2001). These observations are consistent with the release of an EDHF in these vessels. In the present study we investigated a number of potential mediators of the EDHF-type response in human myometrial small arteries.

In contrast to the observations of Edwards *et al.* (1998), who provided evidence to suggest that K^+ was an EDHF in rat arteries, no vasodilator response was seen in human myometrial, pressurized small arteries, in response to raising the concentration of extracellular K^+ over the range of 1–15 mM. Thus K^+ is unlikely to be an EDHF in these vessels. Coleman *et al.* (2001) also precluded a role for K^+ in mediating EDHF responses in human small arteries isolated from subcutaneous fat.

Endogenous cannabinoids have been suggested as possible candidates as EDHF. Evidence for this has been derived from studies which have demonstrated that anandamide evokes relaxant responses and that EDHF-type responses to a variety of endothelium-dependent agonists are inhibited by the cannabinoid receptor antagonist, SR141716A (Randall *et al.*, 1996). In the present study neither anandamide, nor its more metabolically stable analogue, methanandamide, evoked a vasodilator response in human small myometrial arteries. Similarly, SR141716A, at a concentration selective for CB_1 receptors (1 μ M), did not attenuate the response to bradykinin. These findings suggest that an endogenous cannabinoid is unlikely to be an EDHF in the myometrial vascular bed.

A number of reports have suggested that EDHF is an epoxide of arachidonic acid produced from the epoxygenase pathway by a cytochrome P-450 mono-oxygenase system (Campbell *et al.*, 1996; Coats *et al.*, 2001). This contention is largely based on the observation that inhibitors of cytochrome P₄₅₀ can block EDHF-type responses in vessels isolated from several vascular beds. In the present study, we have shown that the response to bradykinin was attenuated, although not abolished, by clotrimazole (50 μ M). This could be taken as evidence in support of a role for EETs in this preparation. However, this contention should be treated with caution, since it has been reported that clotrimazole can act as a K^+ channel blocker, and thus interfere with EDHF at its site of action, rather than preventing its synthesis (Zygmunt *et al.*, 1996). In addition, clotrimazole has been reported to block gap junction communication (Harris *et al.*, 2000).

Overall, our observations suggest the involvement of gap junction communication in mediating EDHF-type responses in human myometrial small arteries since 18 α -GA, carbenoxolone and palmitoleic acid virtually abolished non-NO, non-prostanoid responses to bradykinin. Furthermore, SR141716A at a high concentration (10 μ M), also abolished the response to bradykinin. Each of these agents have been shown to inhibit cell–cell coupling, mediated *via* functional gap junctions, in dye transfer studies (Chaytor *et al.*, 1999; Harris *et al.*, 2000). It should be noted that some of these agents have other actions. For example, it has recently been

reported that some glycyrrhetic derivatives (carbenoxelone and 18- β -glycyrrhetic acid (18- β -GA)) inhibit hyperpolarization in endothelial cells of guinea-pig and rat arteries (Tare *et al.*, 2002). It should be noted that the observation that carbenoxelone inhibited endothelial-dependent responses in the guinea-pig coronary artery (Tare *et al.*, 2002), contrast with observations in the guinea-pig carotid artery in which carbenoxelone was reported to have no effect on endothelial hyperpolarization (Edwards *et al.*, 1999). Interestingly, Tare *et al.* (2002) reported that 18- α -GA had only small, variable effects against endothelial and vascular smooth muscle hyperpolarizations in the guinea-pig coronary artery. This could be taken as evidence that endothelium-dependent responses in this preparation do not involve functional gap junctions and that 18- α -GA does not act as a non-selective inhibitor of endothelium-independent hyperpolarization. The observation that 18- α -GA abolished responses to bradykinin

in the present study thus, implicates the involvement of functional gap junctions. More importantly, the balance of evidence demonstrating that several chemically distinct agents that have been shown to interfere with gap junction communication, inhibited non-NO, non-prostanoid-mediated responses to bradykinin, suggest this is the mechanism of EDHF-type responses in human myometrial small arteries.

In conclusion, we have demonstrated that endothelium-dependent vasodilatation in a human small artery involves functional gap junctions.

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