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RESEARCH PAPER

Sex differences in endothelial function in porcine coronary arteries: a role for H_2O_2 and gap junctions?

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Keywords
endothelium-derived hyperpolarization (EDH); sex differences; gap junctions; hydrogen peroxide (H_2O_2); nitric oxide (NO); porcine coronary artery (PCA); intermediate-conductance calcium-activated K^+ channel (IK_{Ca}); small-conductance calcium-activated K^+ channel (SKc_a); bradykinin; connexin (Cx)

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BACKGROUND AND PURPOSE
Cardiovascular risk is higher in men and postmenopausal women compared with premenopausal women. This may be due to sex differences in endothelial function. Here, sex differences in endothelial function of porcine coronary arteries (PCAs) were investigated.

EXPERIMENTAL APPROACH
Distal PCAs were studied under myographic conditions and after precontraction with U46619. Concentration-response curves to bradykinin were constructed in the presence of a range of inhibitors.

KEY RESULTS
In male and female PCAs, bradykinin produced comparable vasorelaxant responses. Inhibition of NO and prostanoid synthesis produced greater inhibition in males compared with females. Removing H_2O_2 with PEG-catalase reduced the maximum relaxation in the absence, but not the presence of L-NAME and indomethacin in females, and had no effect in males. Blocking gap junctions with 100 μM carbenoxolone or 18α-glycyrrhetinic acid further inhibited the endothelium-derived hyperpolarization (EDH)-mediated response in females but not in males. In female PCAs, the maximum EDH-mediated response was reduced by inhibiting SK_{Ca} with apamin and by inhibiting IK_{Ca} with TRAM-34, or with both. In male PCAs, at maximum bradykinin concentration, the EDH-mediated response was reduced in the presence of apamin but not TRAM-34. Western blot did not detect any differences in connexins 40 or 43 or in IK_{Ca} expression between male and female PCAs.

CONCLUSIONS AND IMPLICATIONS
H_2O_2 mediated some part of endothelium-dependent vasorelaxation in female PCAs and EDH was more important in females, with differences in the contribution of gap junctions and IK_{Ca} channels. These findings may contribute to understanding vascular protection in premenopausal women.

Abbreviations
18α-GA, 18α-glycyrrhetinic acid; Cx, connexin; EDH, endothelium-derived hyperpolarization; IK_{Ca}, intermediate-conductance calcium-activated K^+ channel; MEGJs, myoendothelial gap junctions; PCA, porcine coronary arterie; PEG-catalase, polyethelene glycol-catalase; SK_{Ca}, small-conductance calcium-activated K^+ channel; SOD, superoxide dismutase; U46619, 9,11-dideoxy-9a,11a-epoxymethanoprostaglandin $F_{2\alpha}$

Introduction

Regulation of vascular tone is controlled by endothelium-derived relaxants including NO (Furchgott and Zawadzki, 1980), prostacyclin (Moncada *et al.*, 1976) and endothelium-dependent hyperpolarization (EDH)-type mechanisms (Taylor and Weston, 1988; Edwards *et al.*, 2010; Feletou and Vanhoutte, 2013). Many mediators have been proposed to be responsible for EDH over the past decade, yet none has appeared to be a 'universal EDH' (Griffith, 2004). However, in 2010, the EDH-mediated responses were classified into two categories (Edwards *et al.*, 2010): the first category is the 'classical' EDH pathway in which an increase in intracellular Ca^{2+} concentration hyperpolarizes the endothelial cells leading to activation of small (SK_{Ca}) and intermediate (IK_{Ca}) conductance Ca^{2+}-activated potassium channels on endothelial cells (Busse *et al.*, 2002; Gluais *et al.*, 2005a; Edwards *et al.*, 2010; channel nomenclature follows Alexander *et al.*, 2013). Activation of these potassium channels in turn hyperpolarizes the vascular smooth muscle either through the transfer of electrical signalling via myoendothelial gap junctions (MEGJs) (Chaytor *et al.*, 1998; Edwards *et al.*, 2000; Harris *et al.*, 2000; Kenny *et al.*, 2002; Sandow *et al.*, 2002; de Wit and Griffith, 2010; Chadha *et al.*, 2011; Kerr *et al.*, 2012) or through efflux of K^+ ions from endothelial SK_{Ca} and IK_{Ca} channels acting on barium-sensitive inwardly rectifying potassium channels and the ouabain-sensitive Na^+/K^+ ATPase pump respectively (Edwards *et al.*, 1998; 2010). Gap junctions are formed from two docking hemichannels with each hemichannel made up of six connexin (Cx) proteins (Griffith, 2004). In blood vessels, four subtypes of Cx proteins have been identified, Cx 37, 40, 43 and 45 (Chaytor *et al.*, 2003; Lang *et al.*, 2007; de Wit and Griffith, 2010). The Cx 43 subtype in particular has been demonstrated to play a functional role in the EDH-mediated vasorelaxation in subcutaneous resistance arteries from pregnant women (Lang *et al.*, 2007).

In the second category of the EDH-mediated pathway, smooth muscles cells are hyperpolarized by endothelium-derived mediators (Edwards *et al.*, 2010) such as H_2O_2 (Hayabuchi *et al.*, 1998; Yada *et al.*, 2003; Shimokawa, 2010) or arachidonic acid derivatives (epoxyeicosatrienoic acids) (Campbell *et al.*, 1996). H_2O_2 has been reported to act as a factor responsible for EDH in porcine coronary arteries (PCAs), human, murine and rat mesenteric arteries (Matoba *et al.*, 2000; 2002; 2003; Wheal *et al.*, 2012) (see Shimokawa, 2010). However, the responses to H_2O_2 may vary between species, vascular beds and experimental conditions (Chaytor *et al.*, 2003; Gluais *et al.*, 2005b; Lucchesi *et al.*, 2005).

Cardiovascular risk in men and postmenopausal women is higher than premenopausal women and sex differences in endothelial function have been suggested to contribute to this difference in risk (McCulloch and Randall, 1998; Villar *et al.*, 2008). To date, most studies on endothelial function have been conducted on either arteries from male animals only (Edwards *et al.*, 1998; Harris *et al.*, 2000; Matoba *et al.*, 2003; Leung *et al.*, 2006; Garry *et al.*, 2009) or from both sexes (Quignard *et al.*, 1999; Edwards *et al.*, 2000; Yang *et al.*, 2003; Chadha *et al.*, 2011; Huang *et al.*, 2011). However, previous studies have demonstrated clear sex differences in vascular function of the EDH-mediated pathways (see Feletou and Vanhoutte, 2006; Villar *et al.*, 2008). EDH-mediated responses have been reported to be up-regulated to compensate for the loss of NO (McCulloch *et al.*, 1997; Yada *et al.*, 2003; Wheal *et al.*, 2012) and this compensation was greater in females than males (McCulloch and Randall, 1998; White *et al.*, 2000; Garry *et al.*, 2009). Furthermore, in endothelial NO synthase (eNOS) and COX-1 double-knockout mice ('EDH mice'), the male mice were hypertensive while female mice were normotensive with greater endothelium-dependent vasorelaxation in female mice (Scotland *et al.*, 2005). However, in rat cerebral arteries, the EDH-mediated responses were greater in males compared with females (Sokoya *et al.*, 2007). In a previous study on mesenteric arteries from rats, EDH-mediated responses in females were partly dependent on increased expression of Cx 43, which was driven by oestrogen (Liu *et al.*, 2002).

The aim of the present study was to investigate the effects of sex on endothelium-dependent vasorelaxation of isolated coronary arteries from male or female pigs (PCAs), specifically the contributions of gap junction communication, endogenous H_2O_2 and Ca^{2+}-activated K^+ channels to the vasorelaxation induced by bradykinin.

Methods

Preparation of rings of distal PCAs

Hearts from male and female pigs (large white hybrid pigs, 4–6 months old, weighing ~50 kg) were collected from a local abattoir and transported to the laboratory in ice-cold modified Krebs'-Henseleit solution (118 mM NaCl, 4.8 mM KCl, 1.1 mM $MgSO_4$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 12 mM D-glucose, 1.25 mM $CaCl_2$) previously gassed with 5% CO_2 and 95% O_2. The distal part of the coronary artery was then dissected and placed in 2% w/v Ficoll in Krebs'-Henseleit solution for overnight storage at 4°C. The following day, tissues were finely dissected, cleaned of adherent connective and fatty tissues. PCAs were then cut into rings of about 2 mm in length and mounted in a multichannel wire myograph (Model 610 M, DMT, Aarhus N, Denmark) filled with 5 mL Krebs'-Henseleit solution gassed with 5% CO_2 and 95% O_2 and maintained at 37°C. Vessels were tensioned to 24.5 mN and left to equilibrate for approximately 30 min. Tension was measured and recorded using a PowerLab recording system (AD instruments, Oxfordshire, UK). The distal part of the PCAs was used because it has previously been reported that the EDH response is greater with decreasing vessel size (Shimokawa *et al.*, 1996) and the number of MEGJs appears to be greater in the distal part of the rat mesenteric arteries, compared with the proximal (Sandow and Hill, 2000). Here, the mean vessel size of PCAs from female pigs (0.86 ± 0.02 mm) did not differ significantly from the mean vessel size from male pigs (0.89 ± 0.02 mm) (two-tailed, unpaired Student's *t*-test). Seasonal variations in responses were not allowed for the present study design but each set of experiment has been carried out with an internal control.

Experimental protocol

Wire myography. After 30 min of equilibration, responses to 60 mM KCl were determined twice. The vascular tone was then raised to about 50–80% of the second KCl contraction tone by the addition of the thromboxane A_2 mimetic, 9,11-

dideoxy-9a,11a-epoxymethanoprostaglandin $F_{2\alpha}$ (U46619; 2 nM–50 μM). Once stable tone was achieved, concentration-response curves to bradykinin, an endothelium-dependent relaxant (0.01 nM–1 μM) or NS309, (6,7-dichloro-1*H*-indole-2,3-dione 3-oxime), a positive modulator of SK_{Ca} and IK_{Ca} channels (Leuranguer *et al.*, 2008; Dalsgaard *et al.*, 2009; Brondum *et al.*, 2010) (0.1 μM–0.1 mM) were constructed. To examine the selectivity of NS309, some experiments were precontracted with 60 mM KCl with their respective controls raised to the same tone with U46619. All inhibitors were incubated with the tissues for 1 h before precontraction with U46619. Vasorelaxation to bradykinin was studied in the absence or presence of N^G-nitro-L-arginine methyl ester (L-NAME) (300 μM) which is a NO synthase inhibitor to determine the NO-mediated component. Indomethacin (10 μM) was used to inhibit the synthesis of prostanoids. In some experiments, polyethelene glycol-catalase (PEG-catalase; 300 U mL^{-1}) (Hedegaard *et al.*, 2011) was added to remove intracellular H_2O_2. To study the role of gap junctions, a non-selective gap junction inhibitor carbenoxolone (100 μM) (Harris *et al.*, 2002; Tang and Vanhoutte, 2008) or 18α-glycyrrhetinic acid (18α-GA) (100 μM) (Kenny *et al.*, 2002; Matoba *et al.*, 2003) was used. Apamin (500 nM) and TRAM-34 (10 μM) (Gluais *et al.*, 2005a), SK_{Ca} and IK_{Ca} inhibitors, respectively, were used to study the role of K^+ channels in the bradykinin-induced vasorelaxation.

Western blotting. Western blot studies were carried out to determine the relative expression levels of Cx 37, 40, 43 and IK_{Ca} in PCAs from male and female pigs. PCAs from male and female pigs were finely dissected and cut into rings of about 1 cm in length. Vessels were then gassed with 5% CO_2 and 95% O_2 in Krebs'-Henseleit solution at 37°C for 1 h. Segments (designated F1–F5 for samples from females and M1–M5 for samples from males) were then homogenized on ice in lysis buffer (80 mM sodium β-glycerophosphate, 20 mM imidazole, 1 mM dithiothreitol, 1 mM sodium fluoride, pH 7.6) containing protease inhibitor cocktail (Calbiochem, VWR International Ltd, Lutterworth, Leicestershire, UK). Samples (F1–F5 and M1–M5) were diluted 1:1 in 2× Laemmli sample buffer and heated at 95°C for 5 min. After centrifugation at 13 000× *g* for 1 min, 5 μg of protein were loaded on a 4–20% Mini-PROTEAN TGX precast gel (Bio-Rad, Hemel Hempstead, Hertfordshire, UK) and transferred onto nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) using a Bio-Rad mini-transblot. The nitrocellulose membrane was then blocked with 5% w/v non-fat milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h before incubation with mouse monoclonal anti-Cx-43 antibody (C8093 Sigma-Aldrich) (1:1000) and mouse monoclonal anti-myosin light chain (MLC) antibody (M4401 Sigma-Aldrich) (1:500) overnight at 4°C. After washing in Tris-buffered saline containing 0.1% Tween 20, the blot was incubated with secondary antibody IRDye 800CW Goat anti-mouse IgG (1:10 000) (LI-COR Biosciences, Cambridge, UK) at 37°C for 1 h. The immunoblot was then visualized using a LI-COR Odyssey Infrared Imaging Scanner and the densities of bands determined using Odyssey (Application Software 3.0 LI-COR Biosciences).

Due to the high concentration of proteins required for all other antibodies, samples (designated F6–F10 and M6–M10) were prepared in a slightly modified protocol. Samples (F6–F10 and M6–M10) were homogenized on ice in lysis buffer (20 mM Tris, 1 mM EGTA, 320 mM sucrose, 0.1% Triton X100, 1 mM sodium fluoride, 10 mM sodium β-glycerophosphate, pH 7.6) containing protease inhibitor cocktail (Calbiochem) followed by centrifugation at 3000× *g* for 5 min at 4°C. Supernatant of the samples were then solubilized in 6× solubilization buffer and diluted to 1 mg mL^{-1} of protein with 1× solubilization buffer. Samples were then heated at 95°C for 5 min followed by centrifugation at 13 000× *g* for 1 min before loading to the precast gel. The amounts of protein concentration loaded with the respective dilution of antibody used were as followed: for Cx 40, 10 μg of PCAs samples with 20 μg of pig kidney lysate used as positive control were incubated with rabbit polyclonal anti-Cx 40 – aminoterminal end antibody (ab38580 Abcam®, Cambridge, UK) (1:100) and mouse monoclonal anti-GAPDH antibody (G8795 Sigma-Aldrich, Poole, Dorset, UK) (1:40 000). For Cx 37, 15 μg of PCAs samples with 20 μg of pig and rat kidney lysate used as positive controls were incubated with rabbit polyclonal anti-GJA4 antibody (C15878 Assay Biotech, Stratech Scientific Limited, Suffolk, UK) (1:500) and mouse monoclonal anti-β-Actin antibody (A2228 Sigma-Aldrich) (1:40 000). For IK_{Ca}, 15 μg of PCAs samples with 10 μg of pig kidney lysate used as positive control were incubated with mouse polyclonal anti-KCNN4 antibody (H00003783-B01P Abnova, Taipei, Taiwan) (1:500) and mouse monoclonal anti-GAPDH antibody (G8795 Sigma-Aldrich) (1:40 000). For all antibodies, the blocking and washing steps used were as described above and the same secondary antibody, IRDye® 800CW Goat anti-mouse IgG (1:10 000) (LI-COR Biosciences) were used for anti-mouse antibody and IRDye 680LT Goat anti-rabbit IgG (1:10 000) (LI-COR Biosciences) for anti-rabbit antibody.

Data analysis

Data are presented as mean percentage relaxation of U46619-induced tone with SEM and *n* being the number of separate animals. The concentration-response curves were fitted to a sigmoidal curve with a variable slope using four parameters logistic equation in GraphPad Prism (Version 6, GraphPad Software, La Jolla, CA, USA). The maximum percentage relaxation (R_{max}) and the negative log of concentration required to produce half the maximal relaxation of the induced tone (pEC_{50}) were calculated from the fitted curves. Data were analysed using two-tailed, paired or unpaired Student's *t*-test to compare differences between two groups. In three or more groups, one-way ANOVA was used and significant differences between groups were detected by Bonferroni's *post hoc* test. *P*-values of less than 0.05 were considered statistically significant.

Materials

All drugs were purchased from Sigma-Aldrich except for apamin and NS309 from Tocris Bioscience (Bristol, UK). Stock solutions of L-NAME, PEG-catalase, carbenoxolone and apamin were made in distilled water. Stock solution of indomethacin was made in absolute ethanol whereas TRAM-34, 18α-GA and NS309 were dissolved in DMSO. Stock solutions of bradykinin or the thromboxane A_2 mimetic U46619 (10 mM) were made in water and ethanol respectively. All further dilutions of the stock solutions were made using distilled water

except for NS309 which was further diluted with DMSO to 10 mM, 30% DMSO to 1 mM and distilled water to 0.1 mM.

Results

The effects of sex on EDH-type vasorelaxations

In PCAs from male and female animals, bradykinin produced comparable, concentration-dependent vasorelaxant effects, both in terms of the R_{max} (Figure 1A) and the pEC_{50} values (8.50 ± 0.08 in females; 8.38 ± 0.08 in males). In either set of PCAs, the addition of L-NAME and indomethacin reduced the maximum bradykinin-induced vasorelaxation compared with controls and this reduction was greater in male than in female PCAs. The concentration-response of arteries from both male and female pigs was also shifted significantly to the right in the presence of L-NAME and indomethacin, compared with their respective control responses (pEC_{50} values: 7.65 ± 0.16, females; 7.33 ± 0.27,

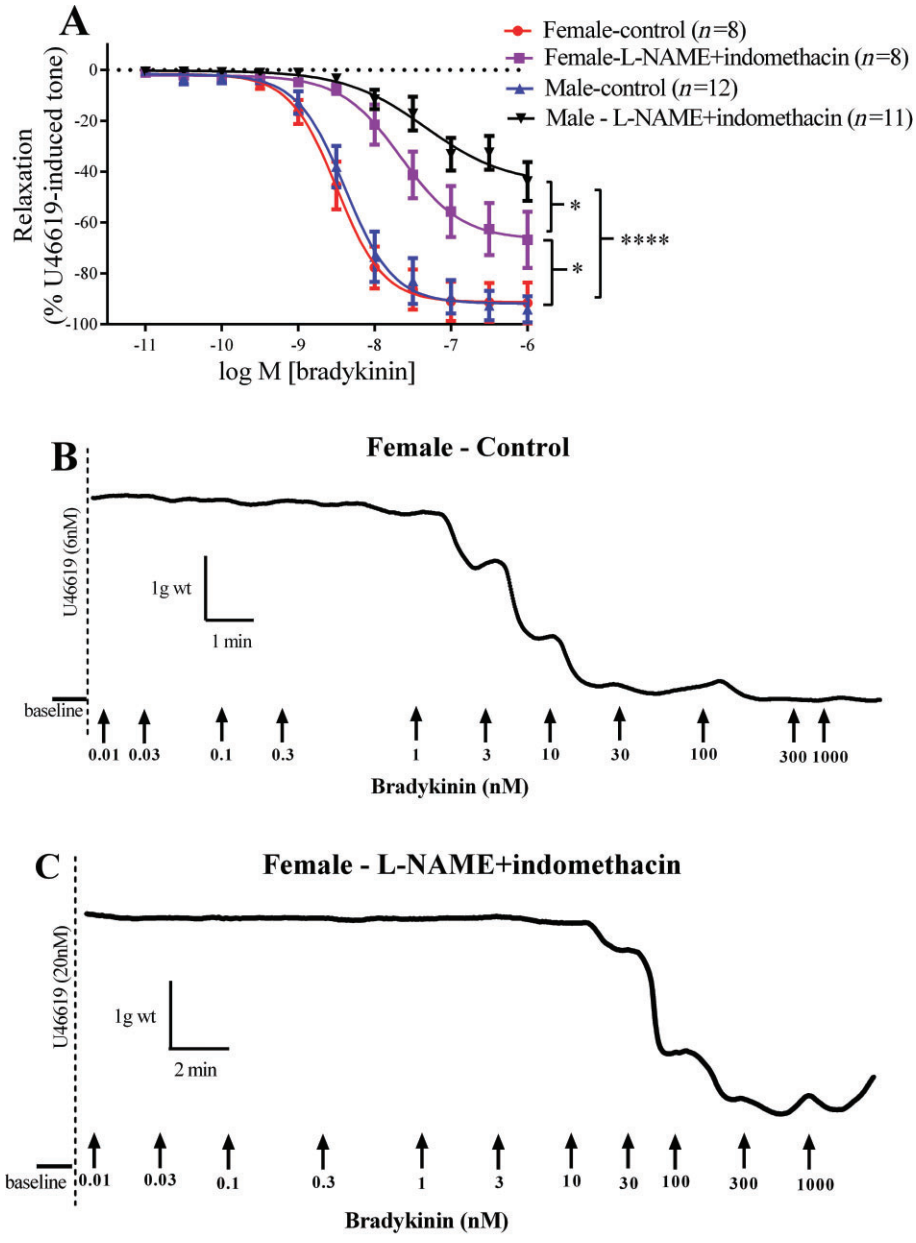

Figure 1

Log concentration-response curves for the vasorelaxant effects of bradykinin in the absence or presence of 300 μM L-NAME and 10 μM indomethacin in male and female PCAs (A). Data are expressed as a percentage change from U46619-induced tone and are mean ± SEM of 8–12 experiments. Original traces of recording showing the responses to increasing concentration of bradykinin (B,C). *P < 0.05, ****P < 0.0001; significantly different as indicated; one-way ANOVA followed by Bonferroni's *post hoc* test.

males). Original traces of recording showing the tissue responses with increasing concentrations of bradykinin are shown in Figure 1B and C.

The effects of L-NAME, indomethacin and PEG-catalase on bradykinin-induced vasorelaxation in PCAs from male and female pigs

In PCAs from females, treatment with PEG-catalase alone (Figure 2A) significantly reduced the maximum vasorelaxation to bradykinin (with pEC_{50} = 8.23 ± 0.13), compared with that under control conditions (with pEC_{50} = 8.46 ± 0.06). However, in the presence of L-NAME and indomethacin (pEC_{50} = 7.92 ± 0.2), no further inhibition of the R_{max} was observed when PEG-catalase was added (pEC_{50} = 7.63 ± 0.15).

Treatment of PCAs from males with PEG-catalase (Figure 2B) did not affect R_{max} to bradykinin (pEC_{50} = 8.05 ± 0.13) compared with control (pEC_{50} = 8.34 ± 0.09). The presence of L-NAME and indomethacin in male PCAs reduced the vasorelaxant responses to bradykinin (pEC_{50} = 7.26 ± 0.30) but the additional presence of PEG-catalase did not cause further reductions (pEC_{50} = 7.07 ± 0.50).

The effects of L-NAME, indomethacin and carbenoxolone on bradykinin-induced vasorelaxation in PCAs from male and female pigs

In PCAs from females, treatment with carbenoxolone alone did not affect vasorelaxation to bradykinin, either as the maximum response or as the pEC_{50} (Figure 3A). However, in the presence of L-NAME and indomethacin (pEC_{50} = 7.92 ± 0.20), addition of carbenoxolone further reduced the maximum relaxation to bradykinin (pEC_{50} = 7.62 ± 0.23).

In PCAs from males, the presence of carbenoxolone alone or in combination with L-NAME and indomethacin did not affect vasorelaxation to bradykinin (Figure 3B).

The effects of L-NAME, indomethacin and 18α-GA on bradykinin-induced vasorelaxation in PCAs from male and female pigs

The addition of 18α-GA to PCAs from female pigs did not affect bradykinin-induced vasorelaxation, compared with the controls (Figure 4A). In the presence of L-NAME and indomethacin, addition of 18α-GA did not affect the R_{max} but did shift the curve to the right by 2.2-fold.

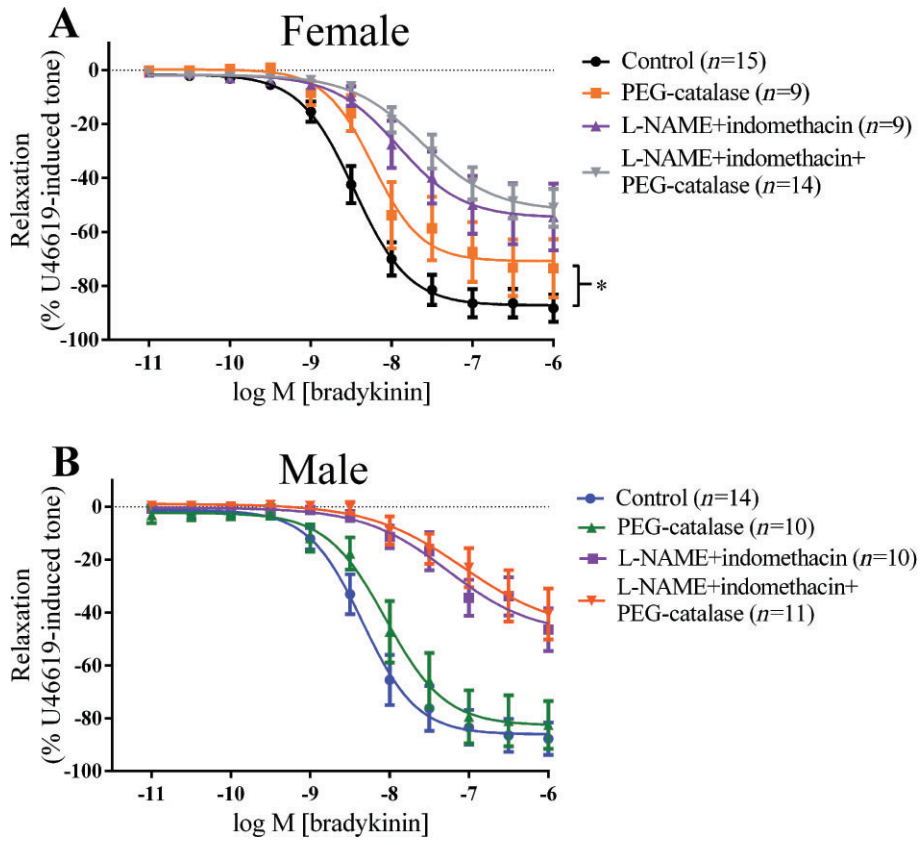

Figure 2

Log concentration-response curves for the vasorelaxant effects of bradykinin in the absence or presence of 300 μM L-NAME, 10 μM indomethacin or 300 U mL^{-1} PEG-catalase in female (A) and in male (B) PCAs. Data are expressed as a percentage change from U46619-induced tone and are mean ± SEM of 9–15 experiments. *P < 0.05; one-way ANOVA followed by Bonferroni's *post hoc* test.

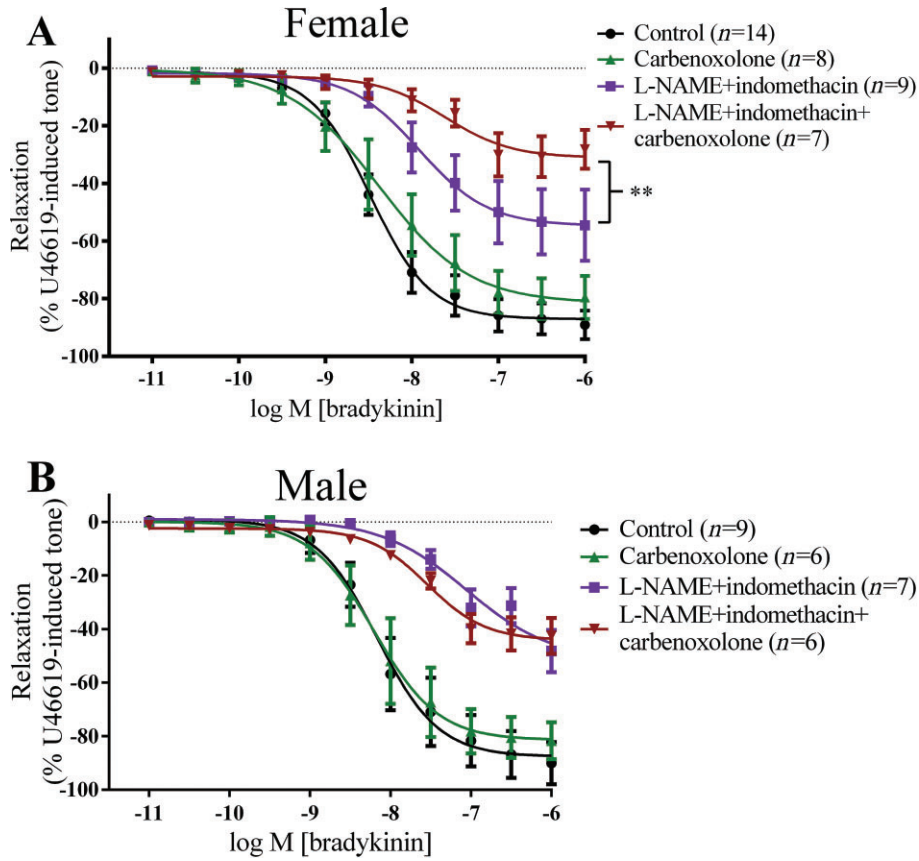

Figure 3

Log concentration-response curves for the vasorelaxant effects of bradykinin in the absence or presence of 300 μM L-NAME, 10 μM indomethacin or 100 μM carbenoxolone in female (A) and in male (B) PCAs. Data are expressed as a percentage change from U46619-induced tone and are mean ± SEM of 6–14 experiments. $^{**}P < 0.01$, one-way ANOVA followed by Bonferroni's *post hoc* test.

In PCAs from male pigs (Figure 4B), presence of 18α-GA alone had no effect on the bradykinin-induced vasorelaxation compared with the controls. Adding 18α-GA to L-NAME and indomethacin also did not affect the bradykinin-induced vasorelaxation.

The effects of L-NAME, indomethacin, TRAM-34 and/or apamin on bradykinin-induced vasorelaxation in PCAs from male and female pigs

In PCAs from females, treatment with TRAM-34 in the presence of L-NAME and indomethacin (Figure 5A), inhibited the maximum relaxation to bradykinin, without affecting pEC_{50} values (7.6 ± 0.05, without TRAM-34; 7.5 ± 0.2, with TRAM-34) Similarly, the maximum relaxation to bradykinin was inhibited by apamin in the presence of L-NAME and indomethacin (pEC_{50} = 7.6 ± 0.2) and the combination of channel blockers produced a greater inhibition of the maximum relaxation in the presence of L-NAME and indomethacin (pEC_{50} = 7.2 ± 0.2) (Figure 5A).

In PCAs from males, vasorelaxation produced at 1 μM of bradykinin was used for statistical analysis instead of R_{max} because the maximum relaxation was not fully defined in some of the groups. Treatment with TRAM-34 in the presence of L-NAME and indomethacin had no effect on the bradykinin-induced vasorelaxation at 1 μM bradykinin compared with L-NAME and indomethacin (Figure 5B). In contrast, addition of apamin alone significantly inhibited the vasorelaxation produced at 1 μM bradykinin, compared with L-NAME and indomethacin. Although adding both TRAM-34 and apamin, in the presence of L-NAME and indomethacin inhibited the vasorelaxation to 1 μM bradykinin, compared with that to L-NAME and indomethacin, this level of inhibition did not differ from that induced by apamin, L-NAME and indomethacin.

The effects of L-NAME, indomethacin and apamin and/or TRAM-34 on NS309-induced vasorelaxation in PCAs from male and female pigs

In PCAs from female (Figure 6A) and male (Figure 6B) pigs, the presence of L-NAME and indomethacin, with or without apamin, had no effect on the R_{max} or pEC_{50} values (5.81 ± 0.05, females; 5.82 ± 0.05, males) of the vasorelaxation induced by the K^+ channel opener, NS309. In PCAs from male pigs, in the presence of L-NAME and indomethacin, the addition of TRAM-34 did not affect responses to NS309 (Figure 6C), nor did the combination of TRAM-34 with apamin (Figure 6D).

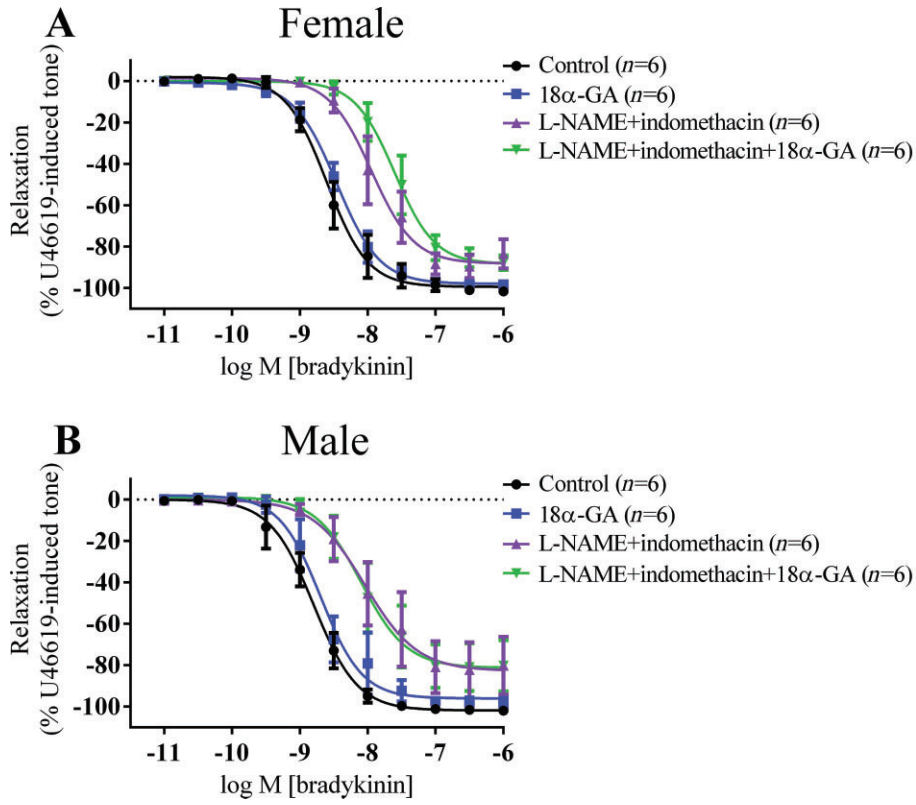

Figure 4

Log concentration-response curves for the vasorelaxant effects of bradykinin in the presence of 300 μM L-NAME, 10 μM indomethacin and 100 μM 18α-glycyrrhetinic acid (18α-GA) in female (A) and in male (B) PCAs. Data are expressed as a percentage change from U46619-induced tone and are mean ± S.E.M of six experiments. In female PCAs, presence of 18α-GA in L-NAME and indomethacin significantly shifted the bradykinin-induced vasorelaxation curve 2.2-fold to the right.

On the other hand, in PCAs from female pigs, the presence of L-NAME, indomethacin and both apamin and TRAM-34 significantly inhibited the NS309-induced vasorelaxation (Figure 6E) at 0.3 μM of NS309, with no differences in R_{max}.

Additional experiments to test the selectivity of NS309 were carried out by precontracting vessels with either 60 mM KCl or U46619 in the presence of L-NAME and indomethacin. As shown in Figure 6F, neither U46619 nor KCl affected the maximum relaxation of NS309, but 60 mM KCl did shift the concentration-response curve 8.1-fold to the right (experimental record in Figure 6G).

Expression of Cx 37, 40 and 43 and IK_{Ca} in PCAs from male and female pigs

Western blot analysis demonstrated the presence of Cx 43 (Figure 7A), Cx 40 (Figure 8A) and IK_{Ca} (Figure 10A), but not Cx 37 (Figure 9A), proteins in PCAs from male and female pigs. Further quantitative analysis based on the ratio of the protein band intensities to their respective loading control showed no significant differences between PCAs from male and female pigs in Cx 43:MLC (Figure 7B), Cx 40:GAPDH (Figure 8B) and IK_{Ca} : GAPDH (Figure 10B) (two-tailed, unpaired Student's *t*-test). In Figures 8A and 9, the red lower band below 37 kDa is a non-specific band produced by the secondary antibody, IRDye 680LT Goat anti-rabbit IgG (1:10 000) (LI-COR Biosciences). For Cx 40 (Figure 8A), no bands were observed at 40 kDa in the absence of the primary antibody (data not shown).

Discussion and conclusions

In the present study, bradykinin-induced vasorelaxation in PCAs from male and female pigs involved a substantial proportion of the response which was resistant to the effects of NO synthase inhibition and COX inhibition and this part of the relaxation was therefore attributed to EDH (McCulloch *et al.*, 1997; Edwards *et al.*, 2010). Here, we demonstrate a significant sex difference in this EDH-mediated response induced by bradykinin. Specifically, the EDH responses were more prominent in female PCAs than in those from males. Similar conclusions have been made in previous studies including isolated mesenteric arteries from rats (McCulloch and Randall, 1998; White *et al.*, 2000), mesenteric arteries from 'EDH mice' (eNOS/COX-1 double-knockout mouse) as well as an *in vivo* study with 'EDH mice' (Scotland *et al.*, 2005). However, another study in PCAs reported that the presence of L-NAME and indomethacin did not disclose any significant sex differences in the endothelium-dependent vasorelaxation to bradykinin (Barber and Miller, 1997). This

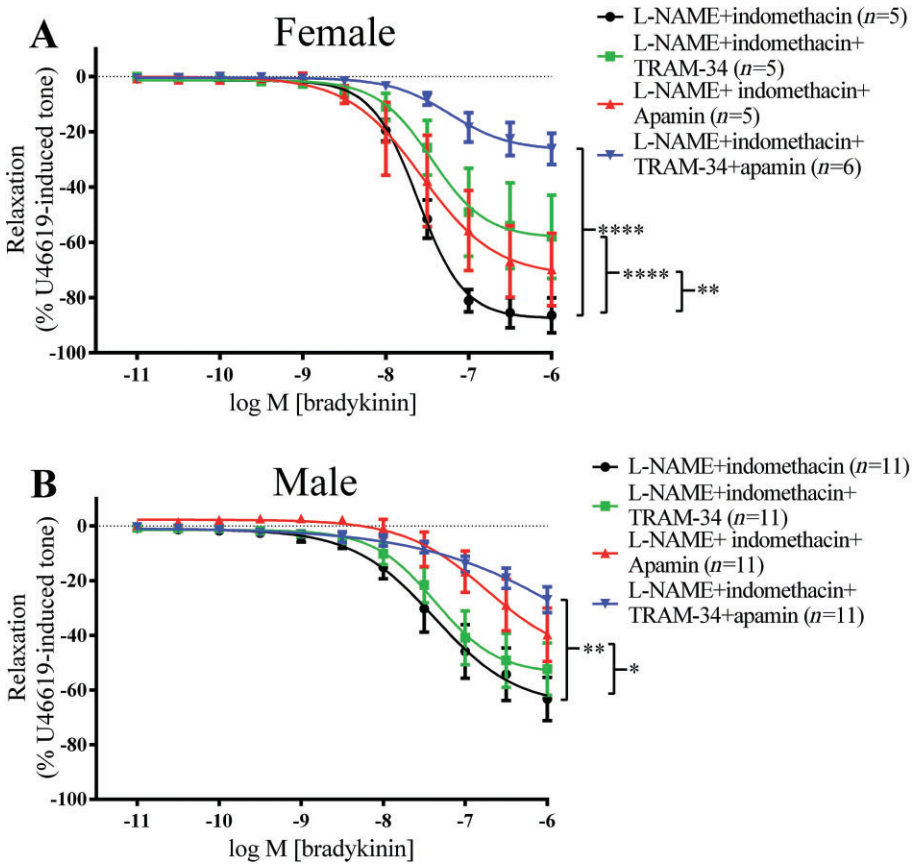

Figure 5

Log concentration-response curves for the vasorelaxant effects of bradykinin in the presence of 300 μM L-NAME, 10 μM indomethacin, 500 nM apamin and/or 10 μM TRAM-34 in female (A) and in male (B). Data are expressed as a percentage change from U46619-induced tone and are mean ± SEM of 5–11 experiments. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$; one-way ANOVA followed by Bonferroni's *post hoc* test.

difference could possibly be due to the size of the vessels used. In the present study, small distal PCAs where EDH is believed to be more prominent in endothelium-dependent relaxations (Shimokawa *et al.*, 1996) were used. However, in the study conducted by Barber and Miller (1997), the size of the vessels used was not reported.

The present study also demonstrated that NO played a greater role in PCAs from male pigs compared with female pigs. This might have been due to a higher expression of eNOS in males, compared with females, as a previous study in rat thoracic aorta reported that the gene expression levels of eNOS mRNA is higher in male rats compared with female rats (Kerr *et al.*, 1999). However, expression levels of eNOS alone do not necessarily translate to activity and function of NO because NO availability decreases rapidly as it reacts with superoxide anion (O_2^-) forming peroxynitrite (Figure 11B) (Kerr *et al.*, 1999).

Another relevant endogenous mediator is H_2O_2, formed by spontaneous or enzymic dismutation of O_2^- by superoxide dismutase (SOD) (Figure 11A; Shimokawa, 2010). On its own, H_2O_2 produced a concentration-dependent vasorelaxation in PCAs (Matoba *et al.*, 2003) and studies have reported that H_2O_2 is a mediator (Matoba *et al.*, 2000; 2002; 2003; Shimokawa and Morikawa, 2005; Edwards *et al.*, 2008; Hammond *et al.*, 2011) or modulator (Wheal *et al.*, 2012) of EDH. We therefore assessed the sex-related differences in endothelium-derived H_2O_2 in PCAs. We found that PEG-catalase alone significantly reduced vasorelaxation to bradykinin in PCAs from females but not males, suggesting that endogenous H_2O_2 played a significant role only in the responses of female PCAs. Conversely, in the presence of L-NAME and indomethacin, PEG-catalase did not affect the bradykinin-induced vasorelaxation in PCAs from both sexes, indicating that H_2O_2 does not play a significant role in the EDH-mediated pathway in distal PCAs. These findings differ from a previous study conducted with male PCAs, which concluded that H_2O_2 was an EDH mediator (Matoba *et al.*, 2003). In their study, porcine coronary microvessels (250–300 μm in diameter) were used and endothelium-dependent vasorelaxations to bradykinin were insensitive to indomethacin and L-NAME, which also differs from the findings in the present study. These differences, or at least that in the absence of NO and PGI_2, could be due to the difference in vessel size used. In the present study, experiments were repeated with a higher concentration of PEG-catalase (600 U mL^{-1}) in PCAs from males, but no further inhibition of the bradykinin-induced vasorelaxation was observed (data not shown). Thus a relative lack of catalase is not likely to explain this

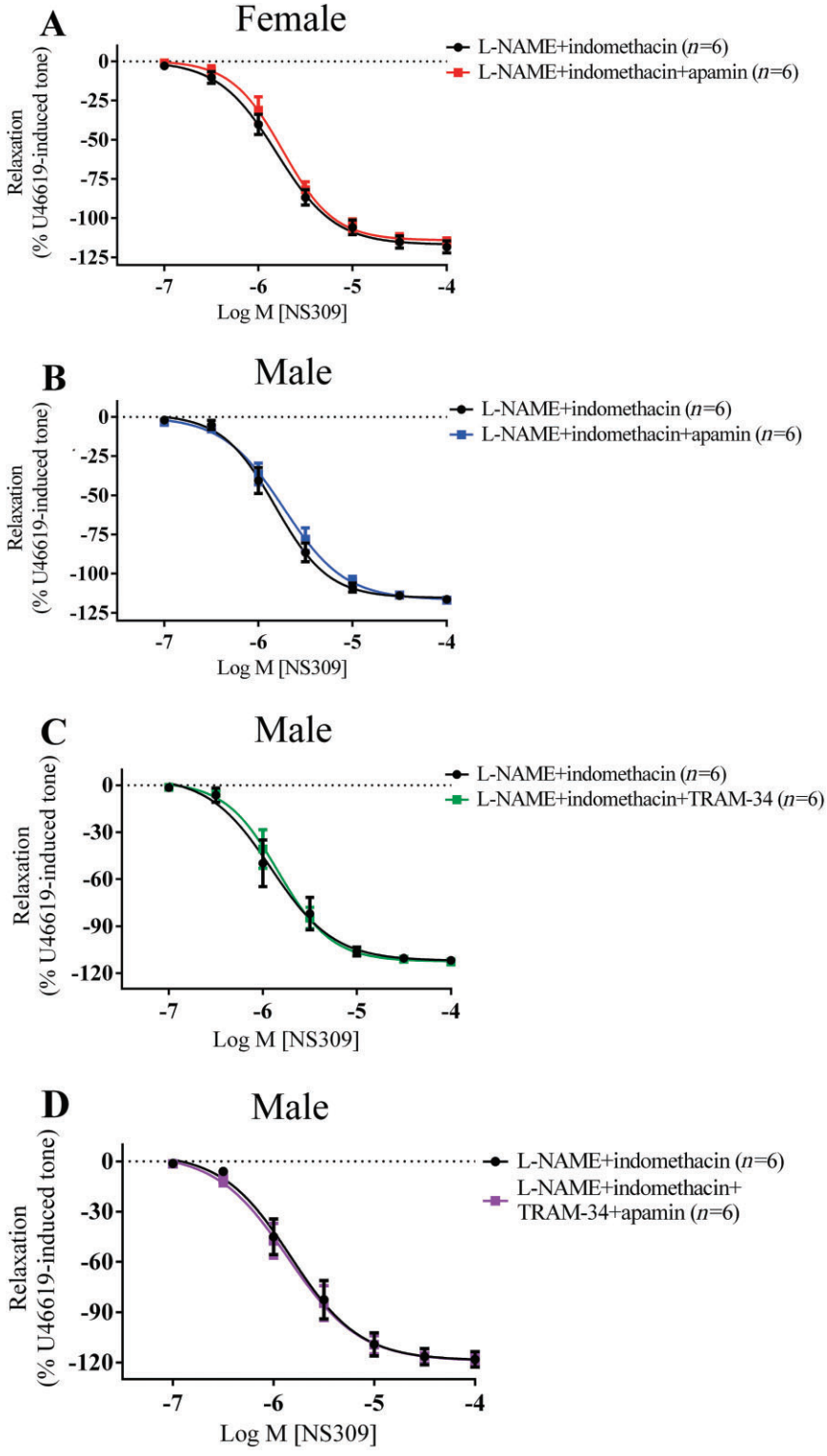

Figure 6

Log concentration-response curves for the vasorelaxant effects of NS309 in the presence of 300 μM L-NAME, 10 μM indomethacin and 500 nM apamin in female (A) and in male (B) PCAs or 10 μM TRAM-34 in male (C) or both apamin and TRAM-34 in male (D) and female (E) PCAs. *$P < 0.05$, two-tailed, paired Student's *t*-test. To examine the selectivity of NS309, log concentration-response curves for the vasorelaxant effects of NS309 in the presence of 300 μM L-NAME and 10 μM indomethacin precontracted with either U46619 or 60 mM KCl in female PCAs (F) was constructed where presence of 60 mM KCl significantly shifted the NS309-induced vasorelaxation 8.1-fold to the right ($P < 0.001$, two-tailed, paired Student's *t*-test). Data are expressed as a percentage change from U46619-induced tone and are mean ± SEM of five to ten experiments. Original traces of recording showing the responses to increasing concentration of NS309, a selective SK_{Ca} and IK_{Ca} channels activator (G).

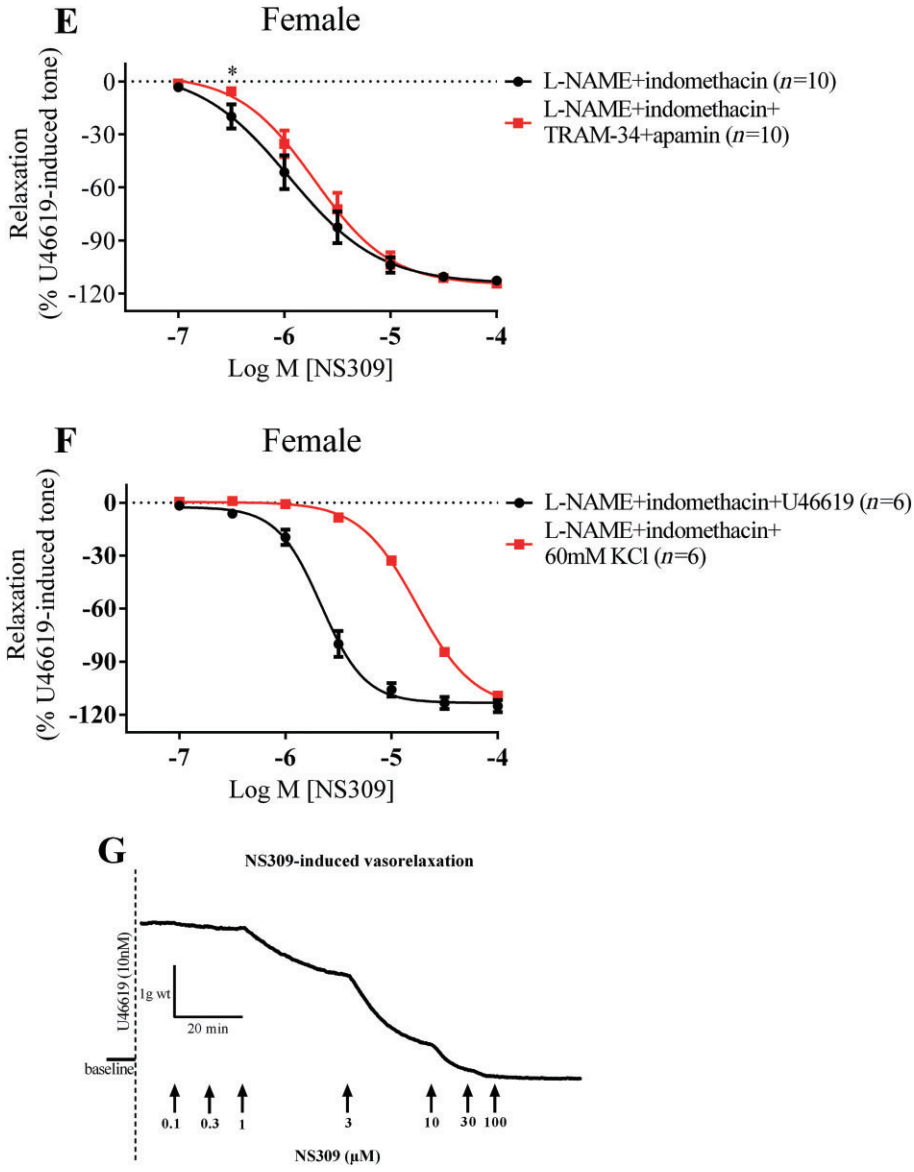

Figure 6
Continued

discrepancy. Another source of endothelium-derived H_2O_2 is the O_2^- generated as a by-product from the conversion of L-arginine to NO by eNOS (Figure 11A) (Heinzel *et al.*, 1992). Previous studies have reported that L-NAME caused a reduction in O_2^- generation (Kerr *et al.*, 1999) and inhibition of H_2O_2 formation (Heinzel *et al.*, 1992). Therefore, it is possible that formation of endogenous H_2O_2 in distal PCAs (as used in the present study) was dependent on the eNOS system where formation of H_2O_2 would also be inhibited in the presence of L-NAME. However, it should be noted that all our experiments were conducted in the presence of both L-NAME and indomethacin, and therefore the contribution of the COX-prostanoid pathway to the formation of H_2O_2 and its effects cannot be excluded.

On the other hand, in the NO-mediated response, a higher level of NO in PCAs from males could have reacted with O_2^-, forming peroxynitrite rather than H_2O_2, as the reaction of O_2^- with NO is three to four times faster than that with SOD (Wolin, 2009). This might explain why H_2O_2 did not play a role in the NO-mediated pathway in males. As for the response observed in PCAs from female pigs where endothelium-derived H_2O_2 was involved in the NO-mediated bradykinin-induced vasorelaxation, we propose that there may be a higher level of SOD present in the endothelial cells in female PCAs, leading to H_2O_2 formation. A previous study in mitochondria isolated from rat brain and liver showed higher expression and activities of manganese-SOD and glutathione peroxidase in females compared with males, thus providing a protective effect in females during oxidative stress (Borras *et al.*, 2003). However, further studies to determine the SOD levels and activity in PCAs are required. Also, as O_2^- in the blood vessels can be generated from several

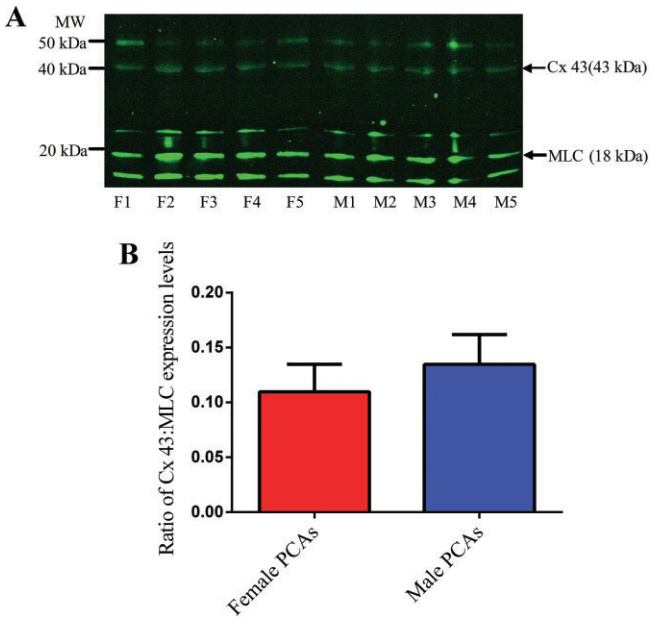

Figure 7

Connexin 43 (43 kDa) and MLC (18 kDa) expression levels in 5 μg of female (F1–F5) and male PCAs (M1–M5) (A). Ratio of the expression levels of connexin 43 to MLC in male and female PCAs based on the intensities of their bands (B). Data are expressed in the ratio of Cx43 to MLC intensities bands and are mean ± SEM of five experiments.

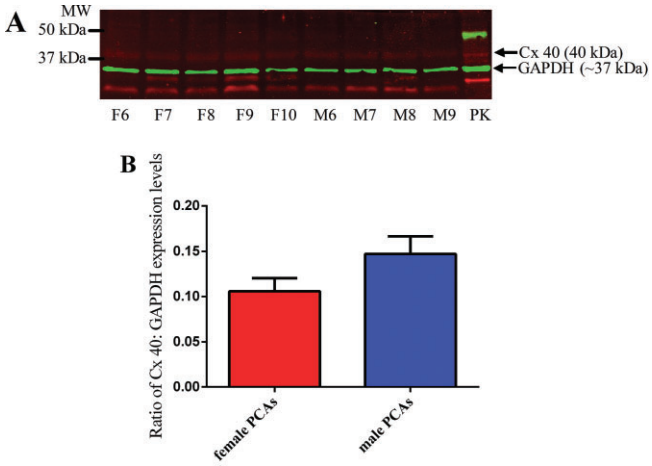

Figure 8

Connexin 40 (40 kDa) and GAPDH (~37 kDa) expression levels in 10 μg of female (F6–F10) and male PCAs (M6–M9) with 20 μg of pig kidney (PK) lysate as positive control (A). Ratio of the expression levels of connexin 40 to GAPDH in male and female PCAs based on the intensities of their bands (B). Data are expressed in the ratio of Cx40 to GAPDH intensities bands and are mean ± SEM of four to five experiments.

different pathways (Wolin, 2009) including NAPDH oxidase, it is possible that there are sex differences in the upstream reactions. Previous studies in age-matched rat aorta (Brandes and Mugge, 1997) and in young healthy human subjects (Ide

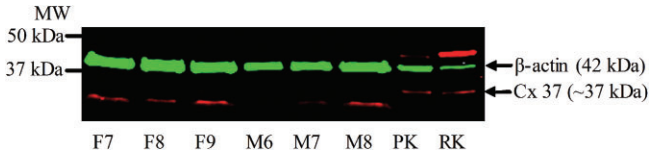

Figure 9

Connexin 37 (~37 kDa) and β-actin (42 kDa) expression levels in 15 μg of female (F7–F9) and male PCAs (M6–M8) with 20 μg of pig (PK) and rat kidney (RK) lysate as positive controls.

et al., 2002) concluded that males experience a greater oxidative stress compared with females and this was attributed to the increased production of O_2^- and reduced activity or availability of O_2^- scavengers.

Next, we investigated the role of gap junctional communication in male and female arteries as it has been reported that gap junctions play a role in the EDH-mediated response. We examined the effects of two different inhibitors (carbenoxolone and 18α-GA) alone or in combination with L-NAME and indomethacin against bradykinin-induced vasorelaxation. In PCAs from male and female pigs, the presence of carbenoxolone or 18α-GA alone had no effect on vasorelaxation to bradykinin indicating that gap junctional communication did not play a significant role in the NO-mediated response. Conversely, in the EDH pathway, carbenoxolone significantly reduced the maximum relaxation to bradykinin in PCAs from female but not male pigs. The inhibitory effects of both carbenoxolone and 18α-GA in the presence of L-NAME and indomethacin confirmed that gap junction communication played a significant role in the EDH responses induced by bradykinin in PCAs from female pigs but not male pigs. These findings concur with previous studies on isolated myometrial arteries and subcutaneous resistance arteries from pregnant women (Kenny *et al.*, 2002; Lang *et al.*, 2007) while the finding in PCAs from male pigs is in agreement with a previous study using 18α-GA in PCAs from male pigs (Matoba *et al.*, 2003). Gap junctions have been reported to play a role in both NO-dependent and EDH-mediated endothelium-dependent vasorelaxation in mesenteric arteries and thoracic aortae from male rabbits (Chaytor *et al.*, 1998), human mesenteric arteries (Chadha *et al.*, 2011) and PCAs of unspecified sex (Edwards *et al.*, 2000). However, another study conducted on PCAs of unspecified sex using three different types of gap junction inhibitors (18α-GA, 1-heptanol and Gap27) had no effect on the EDH response (Yang *et al.*, 2003). In the NO-dependent responses of the present study, the difference in findings could be due to the difference in the size and/or type of vascular tissue used or species differences (Feletou and Vanhoutte, 2006). The inconsistency of findings for the role of gap junctions in all previous studies could possibly be due to the unspecified sex used (Yang *et al.*, 2003; Chadha *et al.*, 2011).

We have now demonstrated clear sex differences in the role of gap junctional communication in EDH responses. As Cx 43 has previously been implicated in EDH-mediated relaxations (Lang *et al.*, 2007), we investigated whether differential expression of this protein could explain the difference in the sensitivity to gap junction inhibition. However,

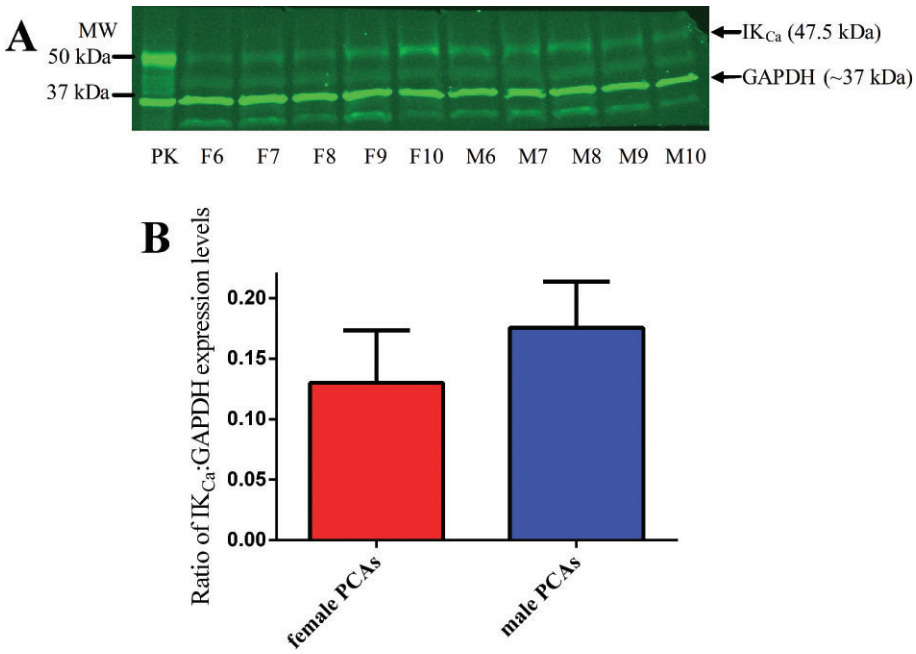

Figure 10

IK_{Ca} (47.5 kDa) and GAPDH (~37 kDa) expression levels in 15 μg of female (F6–F10) and male PCAs (M6–M10) with 10 μg of pig kidney (PK) lysate as positive control (A). Ratio of the expression levels of IK_{Ca} to GAPDH in male and female PCAs based on the intensities of their bands (B). Data are expressed in the ratio of IK_{Ca} to GAPDH intensities bands and are mean ± SEM of five experiments.

in the present study, our Western blot analysis for Cx 43 protein in PCAs from male and female pigs did not detect any differences, indicating that expression *per se* did not contribute to the sex differences observed. Further, Western blot analysis on other subtypes of Cx proteins that have been previously identified in blood vessels (Chaytor *et al.*, 2003; Lang *et al.*, 2007; de Wit and Griffith, 2010) showed no differences in the expression level of Cx 40 proteins and we could not detect Cx 37 proteins in PCAs from male and female pigs. Therefore, the differences in EDH-mediated responses between PCAs from male and female pigs may be due to differences in the operation of the gap junctions, rather than the expression of gap junction proteins. One possibility is that calcium-activated potassium channels (K_{Ca}) (Gluais *et al.*, 2005a; Chadha *et al.*, 2011) which are located near the MEGJs differentially influence gap junctional communication, in males and females.

Given the potential differences in K_{Ca}-channel activity between sexes, we then examined the effects of apamin, the SK_{Ca} blocker and the TRAM-34, an inhibitor of IK_{Ca} channels on EDH-mediated responses (Yang *et al.*, 2003; Gluais *et al.*, 2005a; Edwards *et al.*, 2010; Chadha *et al.*, 2011). Here, we demonstrated that in the EDH-mediated response, IK_{Ca} channels played a role only in PCAs from female pigs, while the SK_{Ca} channels play a role in both sexes. This is in agreement with a previous study conducted on carotid arteries from male guinea pigs where TRAM-34 alone had no effect on endothelium-dependent hyperpolarization, but apamin significantly inhibited acetylcholine-evoked hyperpolarization (Gluais *et al.*, 2005a). Furthermore, another study examining sex differences in EDH-mediated responses reported that apamin had the same effect on the maximum relaxation to acetylcholine in male and female rat mesenteric arteries (White *et al.*, 2000), which is comparable with the present study. Taken together, the present study demonstrates that gap junctional communication and IK_{Ca} channels play a role in the EDH-mediated response in PCAs from females, but not males. Interestingly, previous studies using immunohistochemistry with specific antibodies showed that IK_{Ca} channels are colocalized with myoendothelial Cx proteins with their function being related to the EDH-mediated activity (Sandow *et al.*, 2006; Chadha *et al.*, 2011). A previous study in male obese rats demonstrated an up-regulation in IK_{Ca} and MEGJ expression and activity to compensate for the loss of NO-mediated responses as observed in male age-matched control rats (Chadha *et al.*, 2010). However, Western blot analysis in the present study for IK_{Ca} expression level showed no sex-related differences between PCAs.

In order to determine whether there is a difference in the function of IK_{Ca} channels between males and females, NS309 was used as a potent and selective SK_{Ca} and IK_{Ca} channel activator (Leuranguer *et al.*, 2008). NS309 produced a comparable concentration-dependent vasorelaxation in male and female PCAs. Blocking SK_{Ca} channels specifically with apamin had no effect on the NS309-induced vasorelaxation in either male or female pigs. Further experiments blocking IK_{Ca} channels specifically with TRAM-34 also had no effect on the NS309-induced vasorelaxation. This finding is in line with a previous study measuring the membrane potential of guinea pig carotid arteries where NS309 (10 μM)-induced hyperpolarization was not significantly affected by TRAM-34 or apamin alone in the presence of L-NAME and indomethacin. It is therefore possible that when either SK_{Ca} or IK_{Ca} channels are blocked separately, there is a compensatory response

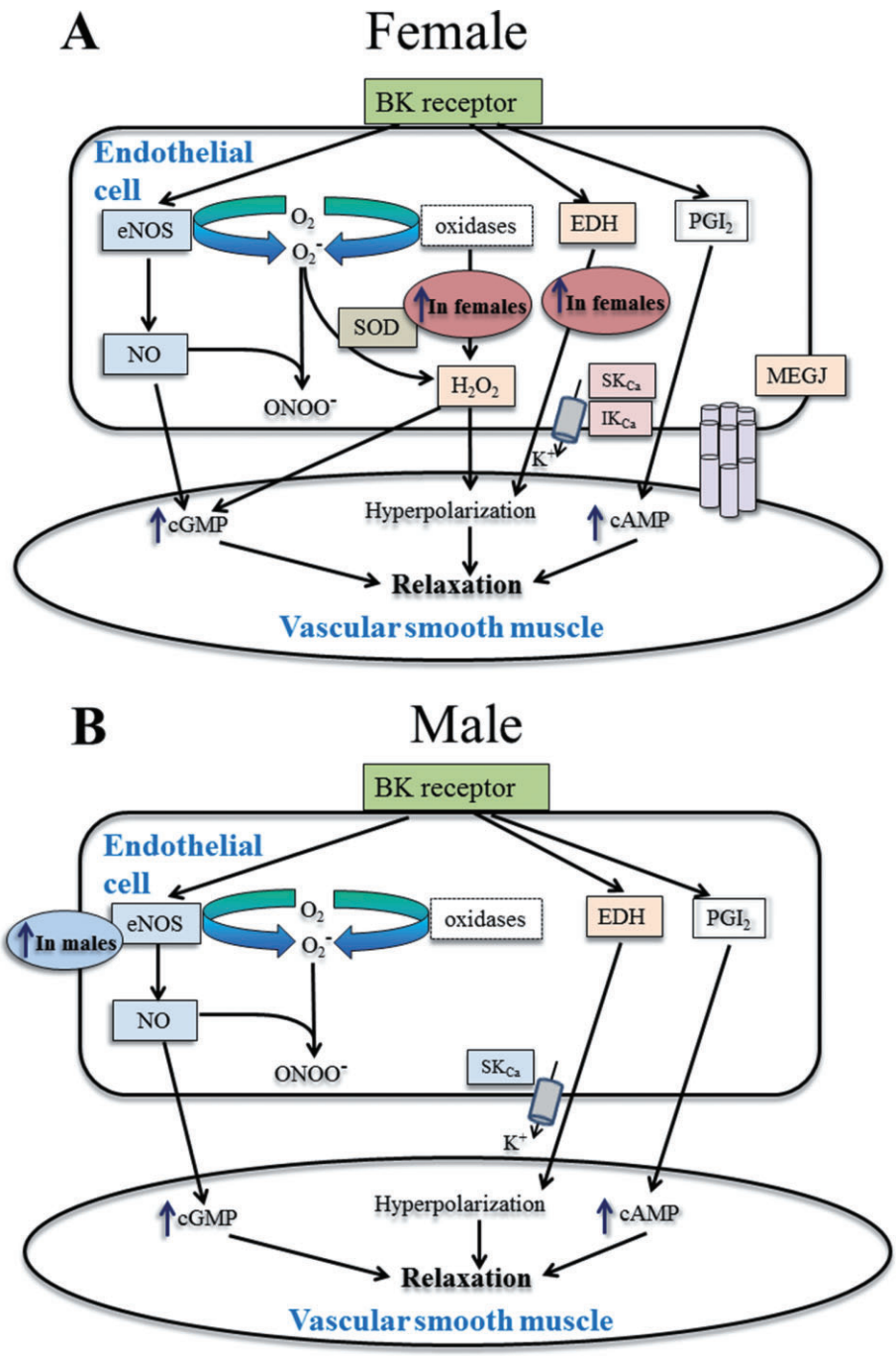

Figure 11

Hypothesized mechanism of action of sex differences in endothelial function underlying bradykinin-induced vasorelaxation in isolated PCAs from female (A) and male (B) pigs. Present study demonstrated a clear sex differences in endothelial function where only in PCAs from female pigs have greater EDH-mediated responses specifically the gap junction communication whereas endogenous H_2O_2 plays a role in the NO-mediated pathway in female pigs. Figure adapted from Shimokawa (2010).

involving the other K_{Ca} channels activated by NS309. However, blocking both SK_{Ca} and IK_{Ca} channels had little effect on the NS309-induced vasorelaxation, although there was slight inhibition at 0.3 μM in PCAs from female pigs. These data suggest that NS309 is not mediating relaxation through SK_{Ca} or IK_{Ca} channels in the PCA and therefore cannot be used to determine if there is a difference in the activity of these channels between males and females. Additional experiments to investigate the selectivity of NS309 using high potassium demonstrated that at higher concentrations of NS309 (>3 μM), other vasorelaxation pathways may be involved. This observation is in line with previous

studies where loss of selectivity for IK_{Ca} and SK_{Ca} has been reported at higher concentrations of NS309 (Dalsgaard *et al.*, 2009; Kroigaard *et al.*, 2012). Furthermore, NS309 inhibited voltage-dependent Ca^{2+} channels (Morimura *et al.*, 2006). Therefore, in the present study, use of NS309 as a selective activator of SK_{Ca} and IK_{Ca} is questionable and it may not be appropriate to draw any conclusions to support the bradykinin-induced vasorelaxation findings. To our knowledge, NS309 is a more potent and selective SK_{Ca} and IK_{Ca} activator compared with other activators such as 1-ethyl-2-benzimidazolinone (Leuranguer *et al.*, 2008) or 5,6-dichloro-1-ethyl-1,3-dihydro-2H-benzimidazole-2-one (Morimura *et al.*, 2006).

In conclusion, the present study demonstrates that both NO and EDH-mediated responses contribute significantly towards the endothelium-dependent vasorelaxation induced by bradykinin in male and female isolated distal PCAs. Clear sex differences in endothelial function have been demonstrated where the EDH-mediated responses play a greater role in PCAs from female compared with male pigs. In PCAs from females, endogenous H_2O_2 played a role in the bradykinin-induced vasorelaxation. Furthermore, gap junctional communication and the IK_{Ca} channels appear to be more important in the EDH-mediated pathway in PCAs from females and this could be compensation for the diminished response of a NO-mediated pathway in these PCAs. The sex differences in endothelial function demonstrated in the present study may contribute to a better understanding of the cardiovascular protective effects observed in premenopausal women.

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Conflicts of interest

The authors declare no conflicts of interest.

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