

RESEARCH PAPER

Enhanced endothelin receptor type B-mediated vasodilation and underlying $[Ca^{2+}]_i$ in mesenteric microvessels of pregnant rats

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BACKGROUND AND PURPOSE

Normal pregnancy is associated with decreased vascular resistance and increased release of vasodilators. Endothelin-1 (ET-1) causes vasoconstriction via endothelin receptor type A (ET_A R), but could activate ET_B R in the endothelium and release vasodilator substances. However, the roles of ET_B R in the regulation of vascular function during pregnancy and the vascular mediators involved are unclear.

EXPERIMENTAL APPROACH

Pressurized mesenteric microvessels from pregnant and virgin Sprague–Dawley rats were loaded with fura-2/AM for simultaneous measurement of diameter and $[Ca^{2+}]_i$.

KEY RESULTS

High KCl (51 mM) and phenylephrine (PHE) caused increases in vasoconstriction and $[Ca^{2+}]_i$ that were similar in pregnant and virgin rats. ET-1 caused vasoconstriction that was less in pregnant than virgin rats, with small increases in $[Ca^{2+}]_i$. Pretreatment with the ET_B R antagonist BQ-788 caused greater enhancement of ET-1-induced vasoconstriction in pregnant rats. ACh caused endothelium-dependent relaxation and decreased $[Ca^{2+}]_i$, and was more potent in pregnant than in virgin rats. ET-1 + ET_A R antagonist BQ-123, and the ET_B R agonists sarafotoxin 6c (S6c) and IRL-1620 caused greater vasodilation in pregnant than in virgin rats with no changes in $[Ca^{2+}]_i$, suggesting up-regulated ET_B R-mediated relaxation pathways. ACh-, S6c- and IRL-1620-induced relaxation was reduced by the NO synthase inhibitor N_{ω} -nitro-L-arginine methyl ester, and abolished by tetraethylammonium or endothelium removal. Western blots revealed greater amount of ET_B R in intact microvessels of pregnant than virgin rats, but reduced levels in endothelium-denuded microvessels, supporting a role of endothelial ET_B R.

CONCLUSIONS AND IMPLICATIONS

The enhanced ET_B R-mediated microvascular relaxation may contribute to the decreased vasoconstriction and vascular resistance during pregnancy.

Abbreviations

BQ-123, cyclo[D-Trp-D-Asp-Pro-D-Val-Leu]; BQ-788, N-[(cis-2,6-dimethyl-1-piperidiny)carbonyl]-4-methyl-L-leucyl-1-(methoxycarbonyl)-D-tryptophyl-D-norleucine sodium salt; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial NOS; ET-1, endothelin-1; ET_A R, endothelin receptor type A; ET_B R, endothelin receptor type B; INDO, indomethacin; IRL-1620, Suc-Asp-Glu-Glu-Ala-Val-Tyr-Phe-Ala-His-Leu-Asp-Ile-Ile-Trp; L-NAME, N_{ω} -nitro-L-arginine methyl ester; pD_2 ($-\log EC_{50}$), concentration of drug required to evoke a half-maximal response; PGI_2 , prostacyclin; PHE, phenylephrine; S6c, sarafotoxin 6c; SNP, sodium nitroprusside; TEA, tetraethylammonium chloride; VSM, vascular smooth muscle

Introduction

The maternal cardiovascular system undergoes marked adaptive changes during pregnancy in order to meet the metabolic demands of the growing fetus. During pregnancy, there is an increase in maternal blood volume and cardiac output, decreased systemic vascular resistance, and increased blood flow to the uteroplacental circulation (Poston *et al.*, 1995; Thornburg *et al.*, 2000). To accommodate for the increased blood volume during pregnancy, vascular tone is shifted towards vasodilation, and BP may remain unchanged or is slightly decreased (Thornburg *et al.*, 2000). Studies have shown that the vasoconstriction responses to vasopressors are attenuated in some vascular beds during pregnancy (Crandall *et al.*, 1990; Davidge and McLaughlin, 1992; Mazzuca *et al.*, 2012). Furthermore, the responses to vasodilator stimuli are enhanced during pregnancy via both endothelium-dependent and endothelium-independent mechanisms (Davidge and McLaughlin, 1992; Gerber *et al.*, 1998; Cooke and Davidge, 2003; Mazzuca *et al.*, 2012). The endothelium is known to release endothelium-derived vasodilators such as NO, prostacyclin (PGI₂) and endothelium-derived hyperpolarizing factor (EDHF) (Vanhoutte *et al.*, 2009; Flammer and Luscher, 2010). These endothelium-derived vasodilators act on vascular smooth muscle (VSM) causing a decrease in VSM intracellular free Ca²⁺ concentration ([Ca²⁺]_i) and vascular relaxation.

In addition to endothelium-derived vasodilators, the endothelium releases contracting factors such as thromboxane A₂, angiotensin II (ANG II) and endothelin-1 (ET-1). ET-1 is a major vasoconstrictor and regulator of BP (Yanagisawa *et al.*, 1988; Schiffrin, 2005). The vascular effects of ET-1 are mediated by at least two receptor subtypes: endothelin receptor type A (ET_AR) and endothelin receptor type B (ET_BR) (Arai *et al.*, 1990; Sakurai *et al.*, 1990). ET_ARs are mainly expressed in VSM to induce vasoconstriction (Davenport and Maguire, 2011). On the other hand, ET_BRs are largely expressed in endothelial cells and mediate the release of endothelium-derived vasodilator substances such as NO, PGI₂, and EDHF, and could also play a role in ET-1 clearance (Nakashima and Vanhoutte, 1993; Tirapelli *et al.*, 2005). Although a role of ET-1 and ET_AR in modulation of cardiovascular and renal function during normal and hypertensive pregnancy has been suggested (Conrad *et al.*, 1999; Thaete and Neerhof, 2000; Alexander *et al.*, 2001; Gandley *et al.*, 2001; Roberts *et al.*, 2006; George and Granger, 2011; Tam Tam *et al.*, 2011; George *et al.*, 2012), the role of ET_BR in mediating the enhanced vasodilator response during pregnancy, the vascular mediators involved, and the underlying changes in vascular [Ca²⁺]_i are unclear.

The present study was designed to test the hypothesis that ET_BR-mediated signalling plays a role in the regulation of microvascular function during pregnancy. We used mesenteric microvessels from late pregnant and virgin rats to determine whether (i) normal pregnancy is associated with attenuated vasoconstriction, enhanced microvascular relaxation and parallel changes in underlying microvascular [Ca²⁺]_i; (ii) the pregnancy-associated changes in microvascular function involve enhanced endothelial ET_BR activity; (iii) the pregnancy-associated changes in ET_BR activity involve changes in endothelium-derived vasodilators; and (iv) the

pregnancy-associated changes in ET_BR activity reflect changes in microvascular ET_BR levels.

Methods

Animals and tissue preparation

Time-pregnant (day 11 of gestation) and age-matched virgin female Sprague–Dawley rats (12 weeks of age) were purchased from Charles River Laboratories (Wilmington, MA, USA). The rats were housed in the animal facility for at least 1 week and had access to *ad libitum* standard rat chow and tap water in 12 h light/dark cycle. Virgin rats were studied during oestrus in order to control for reproductive and endocrine confounders. Oestrus cycle was determined by taking a vaginal smear with a pasteur pipette (Mazzuca *et al.*, 2010). An oestrus smear primarily contained a nucleated cornified squamous cell and was confirmed prior to all experiments (Yener *et al.*, 2007). Late pregnant rats (day 19 of gestation) and age-matched virgin rats were killed by inhalation of CO₂. The abdominal cavity was opened and the small intestine, adjacent mesentery and mesenteric arterial arcade were rapidly excised and placed in ice-cold oxygenated Krebs physiological solution. With the aid of a dissection microscope, small second- and third-order mesenteric arteries were carefully isolated and cleaned of surrounding fat and adipose tissue and used for microvascular functional studies. The remainder of the vascular tissue was stored at –80°C for Western blot experiments. All experimental procedures followed the guidelines of the Institutional Animal Care and Use Committee at Harvard Medical School.

Microvessel cannulation and pressurization

Segments of small mesenteric microvessels [outside diameter (OD) ~300 µm], ~4–5 mm in length, were transferred to a temperature-controlled perfusion chamber and mounted between two glass micropipettes (cannulas), and secured with 10–0 ophthalmic nylon monofilament (Living Systems Instrumentation, Burlington, VT, USA), as previously described (Chen and Khalil, 2008). One end of the microvessel was mounted on a glass micropipette and the lumen was gently rinsed with Krebs solution to ensure removal of any remaining blood, then the other end of the microvessel was mounted on a second cannula and tied in place. The microvessel segment in the perfusion chamber was placed on an inverted microscope (TE300; Nikon, Melville, NY, USA). A stopcock located distal to the vessel was closed, the proximal end was connected to a pressure transducer pressure-servo control system (Living Systems Instrumentation), and the vessel was gradually pressurized to 60 mmHg and then maintained at constant pressure with the pressure-servo control unit. The microvessel was bathed in 5 mL of Krebs bubbled with 95% O₂ and 5% CO₂ at 37°C and was continuously superfused with fresh Krebs at a rate of 1 mL·min^{–1} using a peristaltic mini-pump (Master-Flex; Cole-Parmer, Vernon Hills, IL, USA). Drugs were added abluminally to the bath solution. The vessel was allowed to equilibrate for 60 min before testing its functional viability using high potassium chloride (KCl) depolarizing solution (51 mM), phenylephrine (PHE, 10^{–5} M) and ACh (10^{–5} M). Microvessels were unaccep-

table if they showed leaks or if they failed to produce maintained constriction to KCl and PHE or dilation to ACh.

The mesenteric microvessels were continuously monitored using a video camera connected to a monitor, and the microvessel diameter was measured using automatic edge-detection system (Crescent Electronics, Sandy, UT, USA) and digitized at 1 Hz using a personal computer as previously described (Chen and Khalil, 2008). Snap pictures of the microvessel were taken at rest and following steady-state constriction to different vasoconstrictor stimuli using a digital camera (Cool-Snap; Photometrics, Tucson, AZ, USA).

Fura-2 loading and $[Ca^{2+}]_i$ measurement

For measurement of $[Ca^{2+}]_i$, microvessels were incubated in Krebs solution containing the cell permeable Ca^{2+} indicator fura-2/AM (5 μ M) and the mild detergent cremophor EL (0.25%) for 1 h, as previously described (Chen and Khalil, 2008). The microvessel was washed three times in Krebs solution to remove extracellular fura-2/AM, and incubated in normal Krebs for an additional 30 min to allow for deesterification of the trapped intracellular fura-2/AM into the Ca^{2+} -sensitive fura-2. The fura-2-loaded microvessel was excited alternately at 340 and 380 nm, the emitted light was collected at 510 nm every 1 s, and the fluorescence signal was measured using Felix fluorescence data acquisition and analysis software (Photon Technology International, Birmingham, NJ, USA). The 340/380 ratio was calculated and represented the changes in $[Ca^{2+}]_i$. The signal-to-noise ratio was improved by averaging 10 consecutive 340/380 fluorescence ratio readings.

Simultaneous measurement of microvessel diameter and $[Ca^{2+}]_i$

Our initial experiments demonstrated that KCl- and PHE-induced changes in $[Ca^{2+}]_i$ were rather small. Furthermore, the ET-1 response was relatively slow in onset, particularly at low concentrations, and a cumulative-constriction response curve would require prolonged exposure to excitation light, which would cause significant photobleaching of fura-2 and affect the accuracy of $[Ca^{2+}]_i$ measurements. Therefore, to accurately compare the $[Ca^{2+}]_i$ -dependent constriction induced by various agonists, we used maximal concentrations and an 8 min exposure time. The maximal concentrations of KCl, PHE and ET-1 used were based on previous reports from our laboratory (Chen and Khalil, 2008).

In all experiments, the microvessel from pregnant or virgin rats was first stimulated with 51 mM KCl and the simultaneous changes in constriction and 340/380 ratio (indicative of $[Ca^{2+}]_i$) were recorded. The microvessel was washed with Krebs solution and allowed to recover for 20 min. The microvessel was then stimulated with PHE (10^{-5} M) or ET-1 (10^{-7} M) and the simultaneous changes in constriction and 340/380 ratio were recorded. In a separate set of microvessels, the role of ET_BR in modulating ET-1 responses was tested by pretreating the microvessel with the selective ET_BR antagonist BQ-788 (10^{-6} M) for 30 min, then ET-1 (10^{-7} M) was added and the microvessel constriction and 340/380 ratio were recorded.

To test for endothelial function, microvessels were sub-maximally precontracted (~70% of maximum) with PHE (6×10^{-6} M), then ACh (10^{-5} M) was added and the simultaneous changes in microvessel diameter and 340/380 ratio were recorded over an 8 min exposure time. PHE (6×10^{-6} M) produced similar precontraction in microvessels from pregnant and virgin rats. To further test endothelium-dependent vasodilation, experiments were conducted using increasing concentrations of ACh (10^{-9} – 10^{-5} M), added cumulatively 2 min for each concentration.

To test ET_BR-mediated relaxation, microvessels were pretreated with the ET_AR antagonist BQ-123 (10^{-6} M) for 30 min to block ET_AR-mediated vasoconstriction, precontracted with PHE (6×10^{-6} M), then stimulated with increasing concentrations of ET-1 (10^{-11} – 10^{-7} M), and the microvascular relaxation and 340/380 ratio were recorded. To further test ET_BR-mediated relaxation, mesenteric microvessels were precontracted with PHE (6×10^{-6} M) then stimulated with increasing concentrations of the ET_BR agonist sarafotoxin 6c (S6c, 10^{-11} – 10^{-7} M) or IRL-1620 (10^{-11} – 10^{-7} M), and the percentage relaxation and underlying $[Ca^{2+}]_i$ were measured. Each agonist dose was applied cumulatively, allowing each relaxation to plateau between successive doses.

To elucidate the vasodilator mediator released during stimulation by ACh, S6c and IRL-1620, concentration-relaxation curves were repeated in the presence of N^ω-nitro-L-arginine methyl ester (L-NAME, 3×10^{-4} M) and indomethacin (INDO, 10^{-6} M) to block NOS and COX respectively. Previous studies have shown minimal contribution of PGI₂ to vasodilation in rat mesenteric arteries (Wigg *et al.*, 2001). Therefore, in all experiments, both NOS and COX activities were blocked simultaneously. To test for a hyperpolarization pathway, experiments were repeated in the presence of tetraethylammonium chloride (TEA, 30 mM), a non-selective K⁺ channel blocker.

To confirm the role of endothelium, the endothelium was removed by gently injecting air bubbles through the microvessels while still mounted in the arterial chamber (total volume of injected air bubbles ~0.3 mL). Endothelium removal was determined by the absence of vasodilator responses (<10%) to ACh (10^{-5} M), and the integrity of VSM function was confirmed by the maintenance of the constrictor response to PHE (10^{-5} M).

To directly test the ability of VSM to relax, microvessels were precontracted with PHE (6×10^{-6} M), then stimulated with the exogenous NO donor sodium nitroprusside (SNP, 10^{-5} M), and the simultaneous changes in microvessel diameter and 340/380 ratio were recorded over an 8 min exposure time. To further test VSM function, experiments were repeated using increasing SNP concentrations (10^{-9} – 10^{-5} M) applied cumulatively, allowing each relaxation to plateau between successive doses.

Western blot analysis

Protein was extracted by homogenizing mesenteric arteries from three samples, each sample containing arteries pooled from 4 rats per group. In some samples, the endothelium was removed by passing bubbles through the lumen of the artery while mounted in the arterial chamber then pinning down the excised mesenteric artery in a Petri dish and inserting a thin wire (OD 150 μ m) into the vessel lumen and carefully rubbing the microvessel interior three times forwards and backwards. Arteries were then homogenized in a

homogenization buffer containing 20 mM 3-[N-morpholino]propane sulfonic acid, 4% SDS, 10% glycerol, 2.3 mg dithiothreitol, 1.2 mM EDTA, 0.02% BSA, 5.5 M leupeptin, 5.5 M pepstatin, 2.15 M aprotinin and 20 M 4-(2-aminoethyl)-benzenesulfonyl fluoride, using a 2 mL tight-fitting homogenizer (Kontes Glass Co., Vineland, NJ, USA). The homogenate was centrifuged at $10\,000\times g$ for 5 min. The supernatant was collected, and protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA, USA). Protein extracts (20 μ g) were combined with an equal volume of 2X Laemmli loading buffer, boiled for 5 min, and size fractionated by electrophoresis on 8% SDS-polyacrylamide gels. Proteins were transferred from the gel to a nitrocellulose membrane by electroblotting. Membranes were incubated in 5% non-fat dry milk in TBS-Tween for 1 h and then overnight at 4°C with polyclonal rabbit anti-ET_AR antibody or anti-ET_BR antibody (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were washed five times for 15 min each in TBS-Tween then incubated with HRP-conjugated secondary antibody (1:1000) for 1.5 h, and the immunoreactive bands were detected using enhanced chemiluminescence Western blotting detection reagent (GE Healthcare Bio-Sciences, Piscataway, NJ, USA). The blots were subsequently reprobed for β -actin (1:2000). Data were analysed by optical densitometry and ImageJ software (National Institutes of Health, Bethesda, MD, USA). The densitometry values represented the pixel intensity normalized to β -actin to correct for loading as previously described (Yin *et al.*, 2012).

Solutions and drugs

Krebs solution contained 120 mM NaCl, 5.9 mM KCl, 25 mM NaHCO₃, 1.2 mM NaH₂PO₄, 11.5 mM dextrose, 2.5 mM CaCl₂, 1.2 mM MgCl₂, at pH 7.4, and bubbled with 95% O₂ and 5% CO₂. The 51 mM KCl was prepared as Krebs solution with equimolar substitution of NaCl with KCl. Stock solutions of PHE, ACh, SNP, ET-1 and L-NAME (10^{-1} M; Sigma-Aldrich, St. Louis, MO, USA), S6c and IRL-1620 (10^{-3} M; Tocris Bioscience, Hallsville, MO, USA) were prepared in distilled water. TEA (30 mM, Sigma-Aldrich) was prepared as Krebs solution with equimolar substitution of NaCl with TEA. INDO (10^{-2} M; Sigma-Aldrich), the ET_AR antagonist BQ-123, the ET_BR antagonist BQ-788 (10^{-3} M; Tocris Bioscience) and the Ca²⁺ indicator fura-2/AM (10^{-3} M; Invitrogen, Carlsbad, CA, USA) were prepared in dimethylsulfoxide (DMSO). The final concentration of DMSO in the experimental solution was <0.1%. All other chemicals were of reagent grade or better. All drugs and molecular targets nomenclature used in this manuscript conform to BJP's Guide on Receptors and Channels (Alexander *et al.*, 2011).

Statistical analysis

Experiments were conducted on mesenteric microvessels isolated from 4 to 12 different rats per group (virgin and pregnant), and cumulative data were presented as means $\pm$ SEM, with the 'n' value representing the number of rats per group. Time-dependent constriction was measured as [(resting diameter – constriction diameter)/resting diameter] $\times$ 100. Time-dependent relaxation was measured as [(relaxation diameter – PHE preconstriction diameter)/(resting diameter – PHE

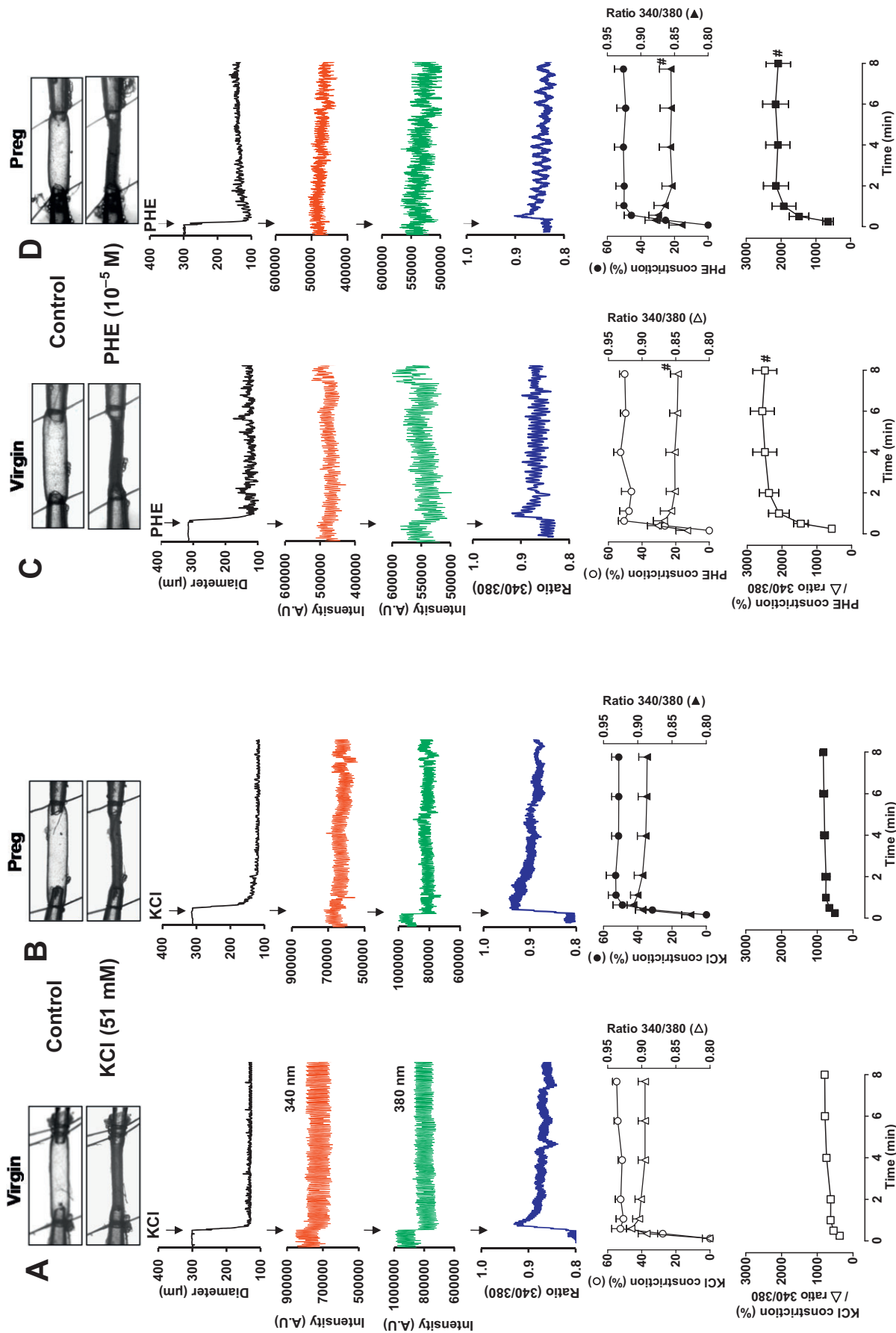
preconstriction diameter)] $\times$ 100. Concentration-dependent relaxations were expressed as percentage of maximum relaxation to the specific vasodilator, concentration–relaxation curves were constructed, sigmoidal curves were fitted to the data using the least squares method, and the pD₂ values (–log EC₅₀) were measured using Prism (v.5.01; GraphPad Software, San Diego, CA, USA). For vascular function experiments, data were first analysed using repeated measures ANOVA (response to drug in a specific group vs. time or concentration). When a statistical difference was observed, data were further analysed using Bonferroni's *post hoc* correction for multiple comparisons. Student's unpaired *t*-test was used for comparison of two means. ET_AR and ET_BR protein levels were analysed and compared between groups using Student's unpaired *t*-test. Differences were statistically significant when $P < 0.05$.

Results

Effect of KCl and PHE

In mesenteric microvessels of both virgin (Figure 1A) and pregnant rats (Figure 1B), membrane depolarization by KCl (51 mM) caused a rapid initial decrease in diameter (time-to-maximum vasoconstriction: virgin = 18.7 ± 2.4 s, pregnant = 22.3 ± 1.8 s) that reached steady state within 33.6 ± 5.4 s in virgin rats and 40.4 ± 4.2 s in pregnant rats (Table 1), and was maintained for the 8 min duration. Furthermore, in microvessels of both virgin and pregnant rats, KCl caused a slight change in the 340 nm fura-2 fluorescence signal, a significant decrease in the 380 nm fluorescence signal, and an increase in the 340/380 fluorescence ratio, indicating a simultaneous increase in [Ca²⁺]_i. The initial KCl-induced increase in [Ca²⁺]_i was rapid (time to peak: virgin = 11.2 ± 0.7 s, pregnant = 11.3 ± 1.0 s), reached steady state within 36.7 ± 2.9 s in virgin rats and 34.4 ± 5.4 s in pregnant rats (Table 1), and was maintained for the 8 min duration. Cumulative data indicated that the initial and maintained KCl-induced vasoconstriction and [Ca²⁺]_i were not significantly different in mesenteric microvessels of virgin and pregnant rats. To further test the Ca²⁺ dependency of the KCl response, the Δ vasoconstriction per Δ [Ca²⁺]_i (340/380 ratio) was calculated. The KCl-induced Δ vasoconstriction per Δ 340/380 ratio was not significantly different in microvessels of virgin (Figure 1A, bottom panel) and pregnant rats (Figure 1B, bottom panel).

In mesenteric microvessels of both virgin (Figure 1C) and pregnant rats (Figure 1D), stimulation with the α -adrenergic receptor agonist PHE (10^{-5} M) caused a rapid initial decrease in diameter (time-to-maximum vasoconstriction: virgin = 16.6 ± 2.5 s, pregnant = 19.8 ± 2.8 s) that reached steady state within 31.9 ± 4.6 s in virgin rats and 37.3 ± 2.7 s in pregnant rats (Table 1), and was maintained for the 8 min duration. The PHE-induced vasoconstriction was preceded by a rapid initial spike in [Ca²⁺]_i (time to peak: virgin = 10.9 ± 1.4 s, pregnant = 12.9 ± 1.7 s), followed by a smaller increase in [Ca²⁺]_i that reached steady state within 42.6 ± 8.6 s in virgin rats and 36.7 ± 3.1 s in pregnant rats (Table 1), and was maintained for the 8 min duration. Cumulative data demonstrated no significant differences in PHE-induced initial or maintained vasoconstriction or [Ca²⁺]_i in mesenteric

**Figure 1**

Effect of KCl (51 mM) (A, B) and PHE (10⁻⁵ M) (C, D) on vasoconstriction and [Ca²⁺]_i in mesenteric microvessels of virgin and pregnant (Preg) rats. Images of fura-2 loaded microvessels from virgin and pregnant rats were taken at rest and after stimulation with KCl or PHE. Simultaneous measurements of microvessel diameter, fura-2340 and 380 nm fluorescence signal, and 340/380 ratio were recorded. Cumulative KCl or PHE-induced vasoconstriction, 340/380 ratio, and vasoconstriction over Δ 340/380 ratio in mesenteric microvessels of virgin and pregnant rats were presented as means ± SEM, *n* = 8–12 different rats per group. # Measurements during stimulation with PHE (C and D) are significantly different (*P* < 0.05) from corresponding KCl measurements (A and B).

Table 1

Time-to-peak and time-to-steady-state vasoconstriction and $[Ca^{2+}]_i$ in response to KCl (51 mM), PHE (10^{-5} M) and ET-1 (10^{-7} M) in mesenteric microvessels of virgin and pregnant rats

	Virgin			Pregnant		
	KCl	PHE	ET-1	KCl	PHE	ET-1
Time-to-maximum constriction (s)	18.7 ± 2.4	16.6 ± 2.5	69.4 ± 10.9 [#]	22.3 ± 1.8	19.8 ± 2.8	39.7 ± 6.7 ^{**}
Time-to-peak $[Ca^{2+}]_i$ (s)	11.2 ± 0.7	10.9 ± 1.4	24.3 ± 3.6 [#]	11.3 ± 1.0	12.9 ± 1.7	18.9 ± 2.7
Time-to-steady-state constriction (s)	33.6 ± 5.4	31.9 ± 4.6	88.7 ± 14.8 [#]	40.4 ± 4.2	37.3 ± 2.7	76.3 ± 10.7 [#]
Time-to-steady-state $[Ca^{2+}]_i$ (s)	36.7 ± 2.9	42.6 ± 8.6	95.3 ± 9.4 [#]	34.4 ± 5.4	36.7 ± 3.1	102.0 ± 14.3 [#]

s, sec.

n = 8–12 different rats per group.

**P* < 0.05 pregnant versus virgin.

[#]*P* < 0.05, measurements during stimulation with ET-1 are significantly different from corresponding measurements during stimulation with KCl or PHE.

microvessels of virgin (Figure 1C) and pregnant rats (Figure 1D). Furthermore, the PHE-induced Δ vasoconstriction per Δ 340/380 ratio was not significantly different between virgin (Figure 1C, bottom panel) and pregnant rats (Figure 1D, bottom panel). However, the PHE-induced Δ vasoconstriction per Δ 340/380 ratio (Figure 1C, bottom panel) was significantly greater (*P* < 0.05) than that induced by KCl (Figure 1A, bottom panel) in microvessels of virgin rats. Similarly, the PHE-induced Δ vasoconstriction per Δ 340/380 ratio (Figure 1D, bottom panel) was significantly greater (*P* < 0.05) than that induced by KCl (Figure 1B, bottom panel) in microvessels of pregnant rats.

Effect of ET-1

In mesenteric microvessels of both virgin (Figure 2A) and pregnant rats (Figure 2B), stimulation with ET-1 (10^{-7} M) caused an initial decrease in diameter that was relatively slower (*P* < 0.05) than that induced by KCl or PHE (time-to-maximum vasoconstriction: virgin = 69.4 ± 10.9 s, pregnant = 39.7 ± 6.7 s; time to steady state: virgin = 88.7 ± 14.8 s, pregnant = 76.3 ± 10.7 s) (Table 1), and was maintained for the 8 min duration. The ET-1-induced time-to-maximum constriction was faster (*P* < 0.05) in microvessels of pregnant than virgin rats (Table 1). The ET-1-induced vasoconstriction was associated with an initial peak in $[Ca^{2+}]_i$ that was slower (*P* < 0.05) than that induced by KCl or PHE (time to peak: virgin = 24.3 ± 3.6 s, pregnant = 18.9 ± 2.7 s), followed by a smaller increase in $[Ca^{2+}]_i$ that was slower in reaching steady state within 95.3 ± 9.4 s in virgin rats (Figure 2C) and 102.0 ± 14.3 s in pregnant rats (Figure 2D) (Table 1), and was maintained for the 8 min duration. Cumulative data showed that ET-1-induced vasoconstriction was significantly reduced (*P* < 0.05) in microvessels of pregnant (Figure 2F) compared with virgin rats (Figure 2E). On the other hand, the ET-1-induced initial and maintained $[Ca^{2+}]_i$ was not significantly different in microvessels of pregnant (Figure 2F) compared with virgin rats (Figure 2E). The ET-1-induced Δ vasoconstriction per Δ 340/380 ratio (Figure 2G, bottom panel) was significantly greater (*P* < 0.05) than that induced by KCl in microvessels of virgin rats (Figure 1A, bottom panel). Similarly, the ET-1-

induced Δ vasoconstriction per Δ 340/380 ratio (Figure 2H, bottom panel) was significantly greater (*P* < 0.05) than that induced by KCl in microvessels of pregnant rats (Figure 1B, bottom panel). Also, the ET-1-induced Δ vasoconstriction per Δ 340/380 ratio was significantly reduced (*P* < 0.05) in microvessels of pregnant (Figure 2H) compared with virgin rats (Figure 2G).

Effect of ET_BR blockade

When compared with control ET-1 experiments in the absence of blockers, pretreatment with the selective ET_BR antagonist BQ-788 (10^{-6} M) significantly enhanced (*P* < 0.05) ET-1-induced vasoconstriction in microvessels of both virgin (Figure 3A) and pregnant rats (Figure 3B). In the presence of BQ-788, the maximal ET-1-induced vasoconstriction was not significantly different in pregnant (Figure 3B) versus virgin rats (Figure 3A). However, when compared with control experiments in the absence of blockers, the ET-1-induced vasoconstriction in the presence of BQ-788 was ~1.7-fold greater in pregnant (Figure 3B) compared with ~1.2-fold greater in virgin rats (Figure 3A). The enhanced ET-1-induced vasoconstriction in the presence of BQ-788 was not associated with significant changes in $[Ca^{2+}]_i$ in microvessels of either virgin (Figure 3C) or pregnant rats (Figure 3D).

Effect of ACh and SNP

In microvessels of both virgin (Figure 4A) and pregnant rats (Figure 4B) preconstricted with PHE (6×10^{-6} M), ACh (10^{-5} M) caused rapid relaxation (time-to-maximum vasodilation: virgin = 59.1 ± 5.0 s, pregnant = 54.6 ± 4.8 s) that reached steady state within 75.8 ± 4.8 s in virgin rats and 68.2 ± 5.2 s in pregnant rats, and was maintained for the 8 min duration. ACh-induced vasodilation was associated with a rapid initial decrease in $[Ca^{2+}]_i$ within 24.7 ± 1.9 s in virgin rats and 26.5 ± 3.9 s in pregnant rats that reached steady state in 59.7 ± 5.0 s in virgin rats and 51.0 ± 12.2 s in pregnant rats, and was maintained for the 8 min duration. Cumulative data demonstrated no significant differences in ACh-induced initial or maintained vasodilation or $[Ca^{2+}]_i$ in mesenteric microvessels of virgin (Figure 4A) and pregnant

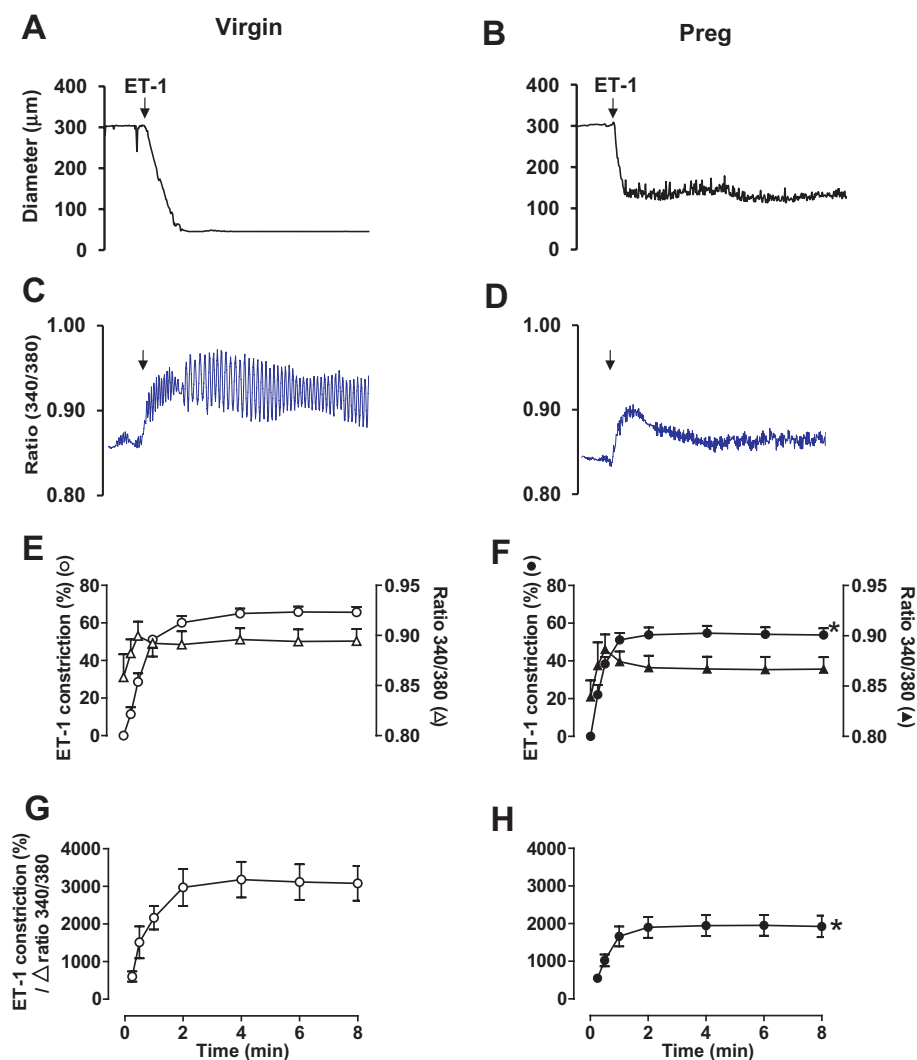


Figure 2

Effect of ET-1 (10^{-7} M) on vasoconstriction and $[Ca^{2+}]_i$ in mesenteric microvessels of virgin and pregnant rats. Simultaneous measurements of ET-1-induced changes in diameter (A, B) and fura-2 340/380 fluorescence ratio (C, D) were recorded in isolated microvessels from virgin (A, C) and pregnant rats (B, D). Cumulative ET-1-induced vasoconstriction, $[Ca^{2+}]_i$, and vasoconstriction per Δ 340/340 ratio in mesenteric microvessels of virgin (E, G) and pregnant rats (F, H) were presented as means $\pm$ SEM, $n = 8$ –12 different rats per group. * Measurements in pregnant rats (F and H) are significantly different ($P < 0.05$) from corresponding measurements in virgin rats (E and G).

rats (Figure 4B). ACh also evoked concentration-dependent relaxation in mesenteric microvessels of both virgin and pregnant rats (Figure 4E). Although the maximal ACh-induced relaxation was not different, ACh was significantly more potent ($P < 0.05$) in inducing vasodilation in microvessels of pregnant ($pD_2 = 6.69 \pm 0.14$) compared with virgin rats ($pD_2 = 6.14 \pm 0.16$) (Figure 4E).

In microvessels of both virgin (Figure 4C) and pregnant rats (Figure 4D), the exogenous NO donor SNP (10^{-5} M) caused rapid relaxation (time-to-maximum vasodilation: virgin = 66.8 ± 6.9 s, pregnant = 60.4 ± 8.6 s) that reached steady state within 77.5 ± 8.1 s in virgin rats and 73.0 ± 7.1 s in pregnant rats, and was maintained for the 8 min duration. SNP-induced vasodilation was associated with a rapid initial decrease in $[Ca^{2+}]_i$ within 30.0 ± 3.2 s in virgin rats and 25.3 ± 4.8 s in pregnant rats, reached steady state within 77.5 ± 8.1 s

in virgin rats and 73.0 ± 7.1 s in pregnant rats, and was maintained for the 8 min duration. Cumulative data demonstrated no significant differences in SNP-induced initial or maintained vasodilation or $[Ca^{2+}]_i$ in mesenteric microvessels of virgin (Figure 4C) and pregnant rats (Figure 4D). SNP also evoked concentration-dependent relaxation, and the maximal SNP-induced relaxation was not different in microvessels of virgin and pregnant rats (Figure 4F). Furthermore, SNP was equally potent in inducing relaxation in microvessels of virgin ($pD_2 = 6.51 \pm 0.14$) and pregnant rats ($pD_2 = 6.49 \pm 0.23$) (Figure 4F).

Effect of ET_BR activation

In microvessels pretreated with the ET_AR antagonist BQ-123 (10^{-6} M) and preconstricted with PHE (6×10^{-6} M), ET-1 induced concentration-dependent relaxation that was signifi-

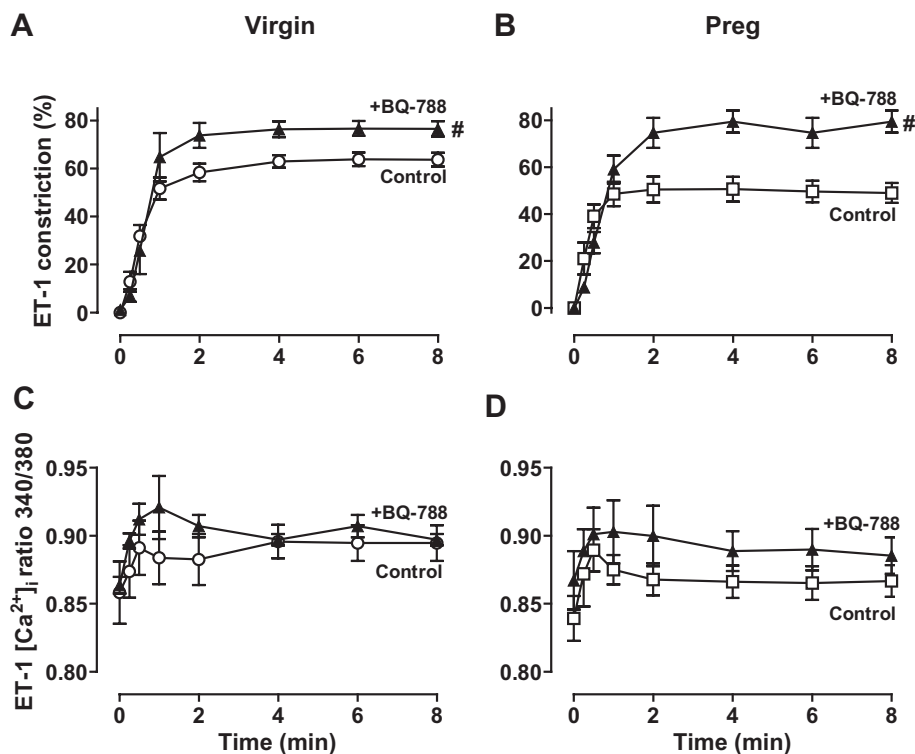


Figure 3

Effect of ET_BR blockade on ET-1-induced constriction and [Ca²⁺]_i. Mesenteric microvessels of virgin and pregnant rats were either non-treated or pretreated with BQ-788 (10⁻⁶ M), then stimulated with ET-1 (10⁻⁷ M), and vasoconstriction and [Ca²⁺]_i measurements were recorded in virgin (A, C) and pregnant rats (B, D). Line graphs represent means ± SEM, *n* = 6 different rats per group. # Measurements in the presence of BQ-788 are significantly different (*P* < 0.05) from corresponding control measurements in the absence of BQ-788.

cantly greater (*P* < 0.05) in pregnant than in virgin rats (Figure 5A). The BQ-123 + ET-1-induced relaxation was not associated with significant changes in [Ca²⁺]_i in microvessels of virgin or pregnant rats (Figure 5B). Furthermore, the selective ET_BR agonists S6c (Figure 5C) and IRL-1620 (Figure 5E) caused concentration-dependent relaxation that was significantly greater (*P* < 0.05) in microvessels of pregnant compared with virgin rats. The differences in S6c and IRL-1620-induced relaxation in microvessels of pregnant versus virgin rats were not associated with significant differences in the underlying [Ca²⁺]_i between virgin (Figure 5D) and pregnant rats (Figure 5F).

Role of endothelium in microvascular relaxation

To test the role of the endothelium, microvessel relaxation was compared in endothelium-denuded and intact microvessels. ACh-induced relaxation was abolished in endothelium-denuded compared with intact microvessels of virgin (Figure 6A) and pregnant rats (Figure 6B). Furthermore, microvascular relaxation in response to BQ123 + ET-1 (data not shown), S6c (Figure 6C) and IRL-1620 (Figure 6D) was abolished in endothelium-denuded compared with intact microvessels of pregnant rats.

Effect of blocking NO, PGI₂ and EDHF

To investigate the vascular mediator released during microvessel relaxation, we tested the effects of blockade of

NOS and COX with L-NAME (3 × 10⁻⁴ M) and INDO (10⁻⁶ M) respectively. Pretreatment with L-NAME + INDO did not cause significant changes in maximal ACh-induced relaxation, but caused a significant shift to the right (*P* < 0.05) in the concentration–relaxation curves in microvessels of virgin (Figure 6A) and pregnant rats (Figure 6B). The L-NAME + INDO-resistant component of ACh relaxation was attributed to EDHF, and ACh was more potent (*P* < 0.05) in stimulating this EDHF-mediated component in microvessels of pregnant (pD₂ = 6.24 ± 0.05) (Figure 6B) compared with virgin rats (pD₂ = 5.58 ± 0.56) (Figure 6A). The remaining L-NAME + INDO-resistant and EDHF-mediated component of ACh relaxation was almost abolished by the non-selective K⁺ channel blocker TEA (30 mM) in microvessels of both virgin (Figure 6A) and pregnant rats (Figure 6B).

To test the vascular mediators involved in ET_BR-mediated relaxation, microvessels were treated with blockers of NO, PGI₂ and EDHF pathway. In mesenteric arteries of virgin rats, L-NAME and INDO insignificantly reduced the small S6c and IRL-1620-induced maximal relaxation (S6c without blockers = 8.39 ± 6.79%, with L-NAME + INDO = 4.01 ± 3.70%, *P* = 0.58; IRL-1620 without blockers = 7.50 ± 4.49%, with L-NAME + INDO = 1.28 ± 0.97, *P* = 0.21), and any remaining S6c or IRL-1620-induced relaxation in microvessels treated with L-NAME + INDO was almost negligible (<4%), suggesting a little role of EDHF. In contrast, in microvessels of pregnant rats, pretreatment with L-NAME + INDO caused significant reduction in S6c-induced maximal relaxation

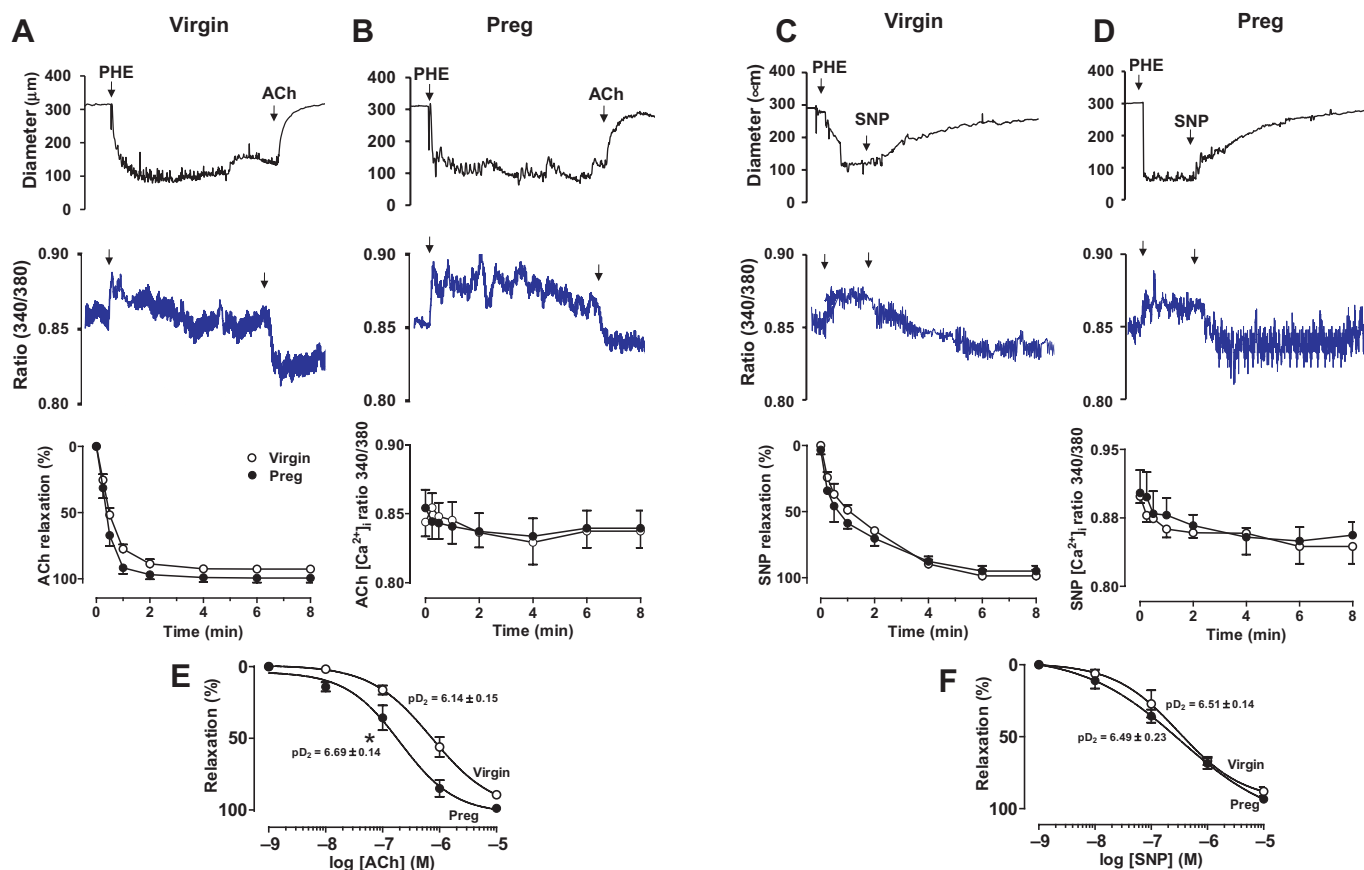


Figure 4

Effect of ACh (10⁻⁵ M) on endothelium-dependent vasodilation, and SNP (10⁻⁵ M) on endothelium-independent VSM relaxation and [Ca²⁺]_i in mesenteric microvessels of virgin (A, C) and pregnant rats (B, D). Simultaneous measurements of microvessel diameter and fura-2 340/380 fluorescence ratio were recorded, and cumulative ACh- and SNP-induced time-dependent changes in microvascular relaxation and [Ca²⁺]_i were presented. Concentration–relaxation curves to ACh (10⁻⁹–10⁻⁵ M) (E) and SNP (10⁻⁹–10⁻⁵ M) (F) were also constructed in mesenteric microvessels of virgin and pregnant rats, and the pD₂ value was measured. Line graphs represent means ± SEM of vasodilation, *n* = 8–12 different rats per group. **P* < 0.05, pregnant versus virgin.

(23.42 ± 7.39%) when compared with control relaxation in the absence of blockers (42.45 ± 7.85%, *P* < 0.05, Figure 6C). Pretreatment with L-NAME + INDO caused insignificant reduction in IRL-1620-induced maximal relaxation in microvessels of pregnant rats (20.08 ± 6.21%) when compared with that in the absence of blockers (33.46 ± 6.46, *P* = 0.15, Figure 6D). Importantly, the remaining S6c or IRL-1620-induced relaxation in mesenteric arteries of pregnant rats treated with L-NAME + INDO was still significant (>20%), suggesting a greater role of EDHF-dependent component of ET_BR-mediated relaxation in microvessels of pregnant compared with virgin rats. Additional pretreatment with the non-selective K⁺ channel blocker TEA abolished the remaining relaxation to S6c (Figure 6C) and IRL-1620 (Figure 6D) in microvessels of pregnant rats.

ET_AR and ET_BR protein level

Western blot analysis revealed ET_AR (Figure 7A) and ET_BR proteins (Figure 7B) in mesenteric microvessels of both virgin and pregnant rats. ET_AR protein level was not significantly different in endothelium-intact or endothelium-denuded

microvessels of pregnant compared with virgin rats, or between endothelium-denuded and intact microvessels of either virgin or pregnant rats (Figure 7A). In contrast, ET_BR protein level was significantly greater (*P* < 0.05) in intact microvessels of pregnant compared with virgin rats (Figure 7B). Furthermore, ET_BR protein level was significantly reduced (*P* < 0.05) in endothelium-denuded microvessels of pregnant compared with virgin rats, and in endothelium-denuded compared with intact microvessels of pregnant but not virgin rats (Figure 7B).

Discussion and conclusions

The main findings of the present study are: (i) ET-1-induced vasoconstriction is reduced in mesenteric microvessels of pregnant compared with virgin rats, with small increases in [Ca²⁺]_i; (ii) the pregnancy-associated changes in microvascular function partly involve enhanced endothelial ET_BR-mediated relaxation; and (iii) the pregnancy-associated increase in ET_BR-mediated relaxation involves changes in endothelium-

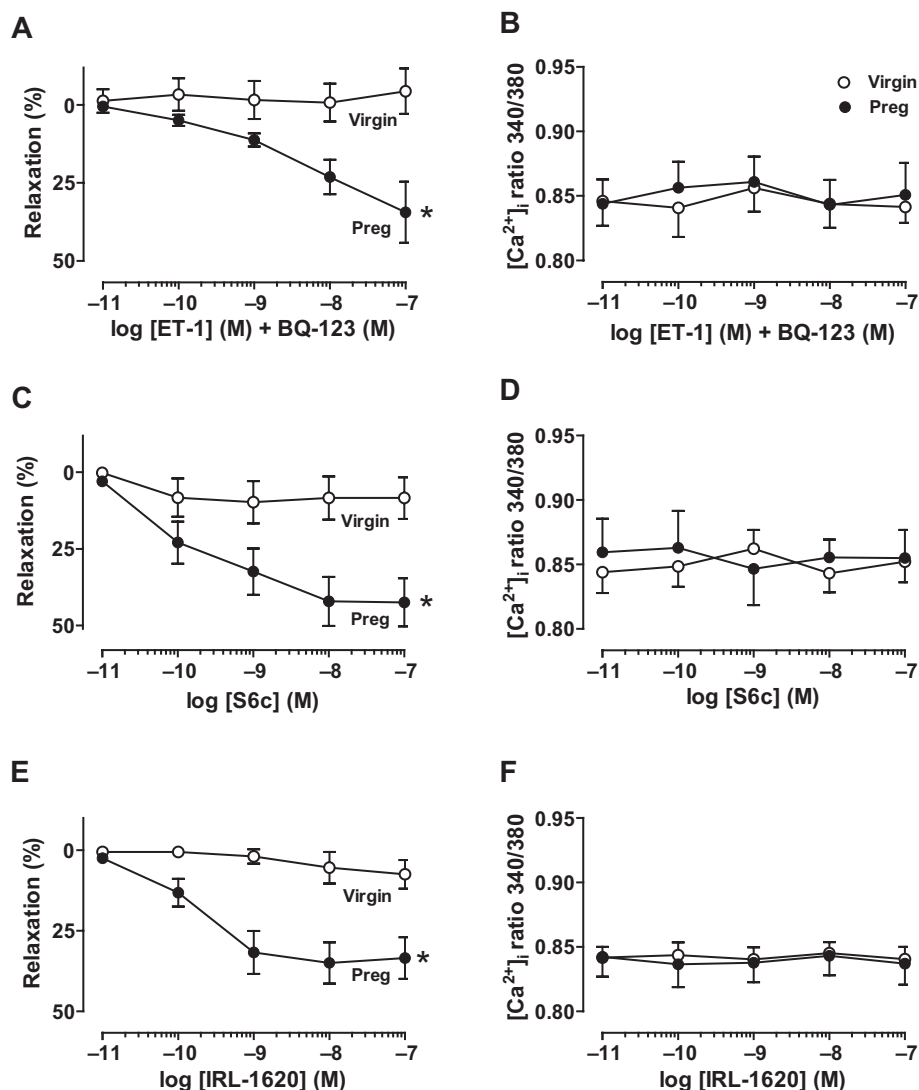


Figure 5

Effect of ET_BR-mediated activation. Microvessels of virgin and pregnant rats were pretreated with the ET_AR antagonist BQ-123 (10^{-6} M) to block ET_AR-mediated vasoconstriction. Microvessels were then precontracted with PHE (6×10^{-6} M), then stimulated with increasing concentrations of ET-1 (10^{-11} – 10^{-7} M), and the % relaxation of PHE contraction (A) and underlying $[Ca^{2+}]_i$ (B) was recorded. Concentration–relaxation curves (C, E) and underlying $[Ca^{2+}]_i$ (D, F) in response to the ET_BR agonist S6c (10^{-11} – 10^{-7} M) (C, D) and IRL-1620 (10^{-11} – 10^{-7} M) (E, F) were compared in microvessels of virgin and pregnant rats. Line graphs represent means $\pm$ SEM of vasodilation and $[Ca^{2+}]_i$, $n = 8$ –12 different rats per group. * $P < 0.05$, pregnant versus virgin.

dependent NO- and EDHF-mediated pathways, and reflects increases in microvascular endothelial ET_BR protein levels.

Normal pregnancy is associated with increased maternal blood volume and cardiac output and decreased systemic vascular resistance (Poston *et al.*, 1995; Thornburg *et al.*, 2000), but BP may remain unchanged or slightly decreased (Khalil *et al.*, 1998; Thornburg *et al.*, 2000). BP regulation is a multifactorial process involving neuronal, renal and vascular control mechanisms (Hall *et al.*, 1986; Lohmeier, 2001; Cain and Khalil, 2002), and the lack of detectable changes in BP during pregnancy does not rule out possible changes in blood vessels. Pregnancy is associated with increased flow-mediated vasodilation, arterial compliance, and G_q-endothelial cell and G_s-VSM receptor-coupled vasodilation pathways, as well as

decreased G_q-VSM-coupled receptor-mediated vasoconstriction (van Drongelen *et al.*, 2012). Furthermore, *in vivo* studies have shown that the pressor response to PHE and ANG II is reduced in the systemic and uterine circulations of pregnant sheep and rats (Naden and Rosenfeld, 1981; Paller, 1984; Magness and Rosenfeld, 1986; Rosenfeld and Naden, 1989). The blunted vasoconstrictor responses *in vivo* may be compensated by cardiac baroreceptor reflexes (D'Angelo and Osol, 1993), thereby normalizing any changes in BP during pregnancy. In light of this, we directly assessed vascular function in isolated mesenteric vessels because they contribute to vascular resistance in conscious rats (Christensen and Mulvany, 1993), and previous studies have reported pregnancy-associated alterations in these vessels (Anderson *et al.*, 2006a;

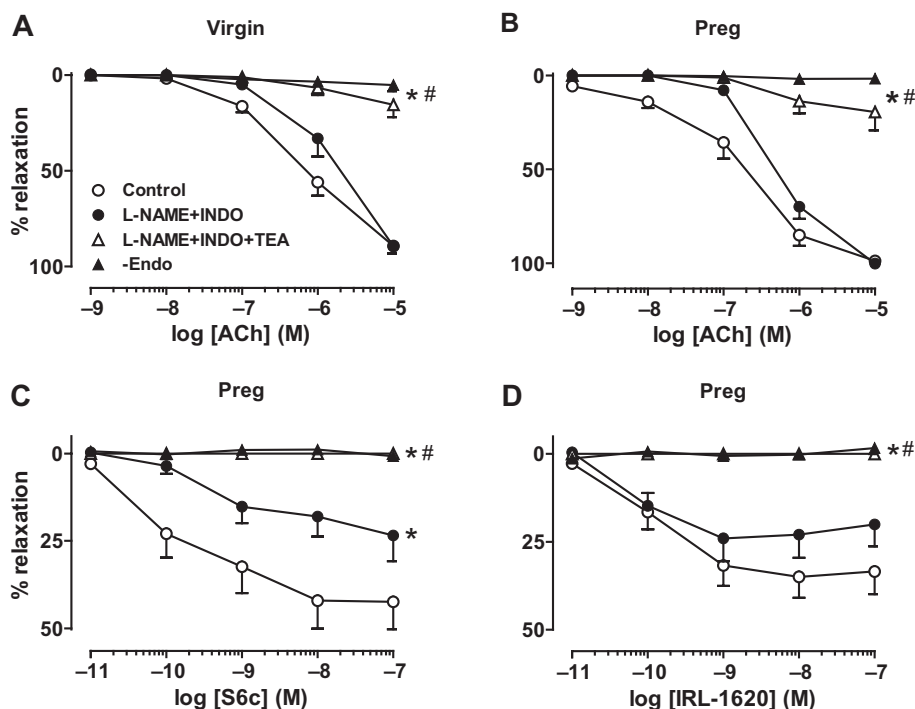


Figure 6

Effect of blocking NO, PGI₂ and EDHF in mesenteric microvessels of virgin and pregnant rats. Mesenteric microvessels from virgin (A) and pregnant rats (B, C, D) were precontracted with PHE (6×10^{-6} M) and concentration–relaxation curves in response to ACh (10^{-9} – 10^{-5} M) (A, B), S6c (10^{-11} – 10^{-7} M) (C), and IRL-1620 (10^{-11} – 10^{-7} M) (D), and in the absence and presence of L-NAME (3×10^{-4} M) and INDO (10^{-6} M) and TEA (30 mM) or in endothelium-denuded microvessels (–Endo) were recorded. Line graphs represent means $\pm$ SEM of vasodilation, $n = 8$ –12 different rats per group. –Endo experiments were $n = 4$ rats per group. * $P < 0.05$ versus control measurements in the absence of blockers. # $P < 0.05$ versus measurements in the presence of L-NAME + INDO.

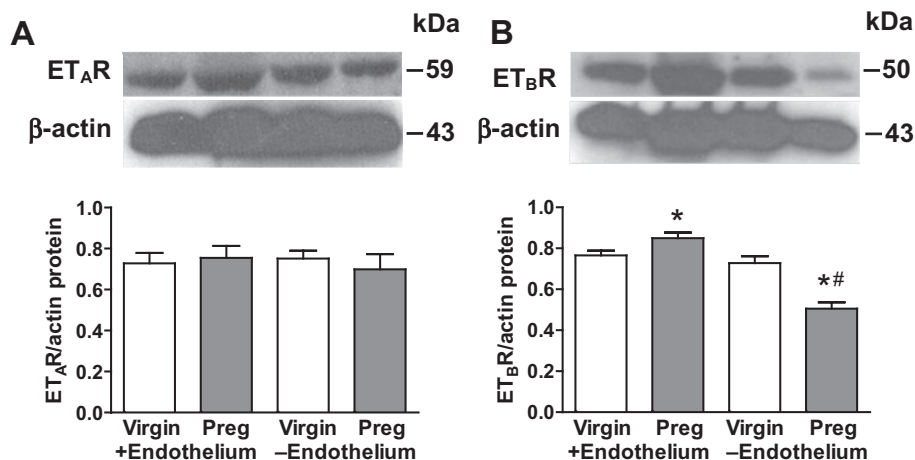


Figure 7

Western blot analysis of ET_AR and ET_BR. Mesenteric microvessels with (+) and without (–) endothelium of both virgin and pregnant rats were homogenized and prepared for Western blot analysis of protein levels of ET_AR (A) and ET_BR (B). Bar graphs represent means $\pm$ SEM. $n = 3$ samples from pooled mesenteric arteries from 4 different rats per group. * $P < 0.05$ pregnant versus virgin. # $P < 0.05$ –Endothelium versus +Endothelium.

Chen and Khalil, 2008). Furthermore, studies have measured $[Ca^{2+}]_i$ in blood vessels loaded with fura-2, and suggested that the measured $[Ca^{2+}]_i$ reflected mainly changes in VSM (Himpens *et al.*, 1990; Khalil and van Breemen, 1990; Bolz

et al., 1999; Chen and Khalil, 2008). In the present study, the increases in vasoconstriction and $[Ca^{2+}]_i$ during microvessel activation by KCl, PHE and ET-1, and the parallel vasodilation and decreased $[Ca^{2+}]_i$ during stimulation by ACh and SNP,

support that the detected changes in microvessel $[Ca^{2+}]_i$ originated mainly from VSM.

We found that KCl, which stimulates Ca^{2+} entry through voltage-gated Ca^{2+} channels (Khalil and van Breemen, 1988), caused microvascular constriction and increased $[Ca^{2+}]_i$ that were not different in pregnant and virgin rats. Our data agree with other reports (D'Angelo and Osol, 1993; Mazzuca *et al.*, 2012), and support that in the mesenteric vascular bed Ca^{2+} influx through voltage-gated Ca^{2+} channels is not altered during late pregnancy. Furthermore, consistent with other studies (Parent *et al.*, 1990; D'Angelo and Osol, 1993; Pascoal *et al.*, 1995), maximal doses of the α -adrenergic receptor agonist PHE (10^{-5} M) caused vasoconstriction that was not different in pregnant and virgin rats. In order to accurately measure $[Ca^{2+}]_i$, we used the maximal PHE concentration, and found that PHE-induced $[Ca^{2+}]_i$ was not different in pregnant versus virgin rats. Although we did not test a dose-dependent response to PHE, other studies have shown that the sensitivity to PHE either increased (D'Angelo and Osol, 1993), decreased (Parent *et al.*, 1990; Davidge and McLaughlin, 1992) or unchanged (Pascoal *et al.*, 1995; Mazzuca *et al.*, 2012) in mesenteric arteries of late pregnant compared with virgin rats. The cause of these discrepancies is unclear but may be related to differences in methodology, rat strain, gestational time or the vessel order along the vascular tree (van Drongelen *et al.*, 2012).

The KCl- and PHE-induced constriction in microvessels of pregnant and virgin rats was associated with increases in $[Ca^{2+}]_i$, suggesting activation of Ca^{2+} -dependent mechanisms of VSM contraction. While in both virgin and pregnant rats the maximal PHE-induced constriction was similar to that of KCl, $[Ca^{2+}]_i$ was smaller during activation by PHE compared with KCl. Assuming that KCl mainly stimulates Ca^{2+} entry, Ca^{2+} -calmodulin, and myosin light chain phosphorylation, the similar magnitude of PHE contraction despite the significantly less $[Ca^{2+}]_i$ and greater Δ constriction to $\Delta [Ca^{2+}]_i$ ratio suggests activation of additional mechanisms that increase the myofilament force sensitivity to $[Ca^{2+}]_i$ such as rho kinase, MAPK (Somlyo and Somlyo, 2003; Hilgers and Webb, 2005; Puetz *et al.*, 2009) and PKC (Khalil *et al.*, 1992; Khalil, 2010).

ET-1 is a major regulator of vascular function, acting via both ET_A R and ET_B R (Yanagisawa *et al.*, 1988; Arai *et al.*, 1990; Kohan *et al.*, 2011). ET-1 activates ET_A R in VSM cells causing vasoconstriction and increases in $[Ca^{2+}]_i$, PKC, MAPK, and other pathways of VSM contraction and cell growth (Schiffrin and Touyz, 1998; Cain *et al.*, 2002; Dallas and Khalil, 2003; Touyz *et al.*, 2004). ET_B R in endothelial cells may promote vascular relaxation (Giardina *et al.*, 2001; Tirapelli *et al.*, 2005; Tykocki *et al.*, 2009) and has been identified in the systemic, renal, pulmonary, coronary and cerebral circulation (Mazzuca and Khalil, 2012). We found that ET-1 induced vasoconstriction in mesenteric microvessels of virgin and pregnant rats. Similar to PHE, ET-1-induced constriction was similar to KCl, but associated with smaller increases in $[Ca^{2+}]_i$, and greater Δ vasoconstriction per $\Delta [Ca^{2+}]_i$, supporting that ET-1 causes VSM contraction not only by increasing $[Ca^{2+}]_i$ but also by activating Ca^{2+} sensitization pathways such as PKC and rho kinase (Dallas and Khalil, 2003; Tykocki and Watts, 2010; Lima *et al.*, 2011). ET-1-induced constriction was reduced in microvessels of pregnant versus virgin rats and associated with small increases in $[Ca^{2+}]_i$ that were not differ-

ent in virgin and pregnant rats. Furthermore, the ET-1-induced Δ vasoconstriction per $\Delta [Ca^{2+}]_i$ was reduced in microvessels of pregnant compared with virgin rats. These findings suggest that the reduced ET-1-induced vasoconstriction in pregnant rats may not be exclusively due to changes in VSM $[Ca^{2+}]_i$ but could involve reduced $[Ca^{2+}]_i$ sensitization pathways such as PKC or rho kinase, and further analysis of these pathways should be examined in future studies.

Because endothelial ET_B R could have opposing effects to VSM ET_A R, we investigated whether the reduced ET-1 vasoconstriction in pregnant rats was due to up-regulation of endothelial ET_B R. We first tested the effects of blocking ET_B R using BQ-788 on ET-1-induced vasoconstriction. We reasoned that if BQ-788 blocks endothelial ET_B R-mediated vasodilation, then it will allow ET-1 to mainly activate ET_A R and cause greater vasoconstriction. BQ-788 enhanced ET-1-induced vasoconstriction. While maximal ET-1-induced constriction was not different in virgin and pregnant rats, the fold change in ET-1 constriction with ET_B R blockade was greater in pregnant than in virgin rats, suggesting enhanced ET_B R-mediated vasodilation during pregnancy. Interestingly, $[Ca^{2+}]_i$ was not altered by BQ-788, suggesting that the increase in ET-1 vasoconstriction may be largely due to activation of Ca^{2+} -sensitization pathways.

We then examined the effects of activation of ET_B R on microvascular function. Microvascular ET_B R activity was enhanced during pregnancy because pretreatment with the ET_A R antagonist BQ-123 then adding ET-1, or treatment with the ET_B R agonists S6c and IRL-1620 caused greater relaxation in microvessels of pregnant than virgin rats. The enhanced ET_B R activity could be partly due to increased endothelial ET_B R expression because (i) removal of the endothelium abolished BQ-123 + ET-1-, S6c- and IRL-1620-induced relaxation in pregnant rats; (ii) Western blots showed greater ET_B R levels in endothelium-intact microvessels of pregnant than virgin rats; and (iii) removal of the endothelium did not show a difference in ET_A R levels, but reduced ET_B R expression in pregnant versus virgin rats, supporting that endothelial ET_B R plays a role in promoting vasodilation during pregnancy. The enhanced ET_B R dilation is unlikely due to increased VSM sensitivity to vasodilators because SNP-induced relaxation was not different in pregnant and virgin rats, which is in accordance with other studies (Gerber *et al.*, 1998; Cooke and Davidge, 2003; Mazzuca *et al.*, 2012).

In search for the factors involved in the enhanced ET_B R-mediated relaxation in pregnant rats, we examined the various endothelium-dependent relaxation factors released by known vasodilators such as ACh. Although the maximal ACh-induced relaxation was not different, ACh was more potent in inducing vasodilation in microvessels of pregnant than virgin rats, which is consistent with other studies (Davidge and McLaughlin, 1992; Kim *et al.*, 1994; Gerber *et al.*, 1998; Mazzuca *et al.*, 2012). ACh-induced relaxation was abolished in endothelium-denuded microvessels of virgin and pregnant rats, supporting endothelium-dependent release of vasodilator substances. In many blood vessels, NO is a major endothelium-derived vasodilator (Fleming and Busse, 2003; Félétou and Vanhoutte, 2006; Flammer and Luscher, 2010), while PGI_2 may be important in specialized blood vessels such as the pulmonary artery (Majed and Khalil, 2012). The NOS inhibitor L-NAME did not inhibit maximal

ACh relaxation in microvessels of virgin or pregnant rats, but reduced the sensitivity to ACh, suggesting that the increased sensitivity to ACh-induced relaxation in microvessels of pregnant rats may be partly due to changes in NO release. This is supported by reports that endothelial NOS (eNOS) is up-regulated in the systemic circulation during pregnancy (Magness *et al.*, 2001). The pregnancy-associated increase in NO synthesis could be partly due to up-regulation of oestrogen, which is known to increase eNOS expression/activity (Magness *et al.*, 2001; Rupnow *et al.*, 2001). Activation of endothelial cells is known to increase $[Ca^{2+}]_i$, which in turn activates eNOS and increases NO production. In addition to increases in endothelial $[Ca^{2+}]_i$, phosphorylation by MAPK and Akt could lead to further activation of eNOS. NO is known to diffuse into VSM and increase cGMP production, which in turn decreases VSM $[Ca^{2+}]_i$ by inhibiting Ca^{2+} influx and promoting Ca^{2+} extrusion (Félétou and Vanhoutte, 2006). In the present study, ACh-stimulated increase in endothelial $[Ca^{2+}]_i$ could not be detected either because any increase in endothelial Ca^{2+} is masked by the concomitant decrease in VSM $[Ca^{2+}]_i$ with the net observation of vasodilation and simultaneous decrease in $[Ca^{2+}]_i$, or because eNOS activation is less dependent on Ca^{2+} and more dependent on other pathways such as MAPK and Akt, and these possibilities need to be further examined. The increased sensitivity to ACh in mesenteric arteries of pregnant rats may also be due to increased cGMP production in the systemic circulation during pregnancy (Conrad *et al.*, 1993), although this is less likely because the microvascular relaxation to SNP, an exogenous NO donor and a stimulator of cGMP production, was not different in virgin and pregnant rats.

In addition to NO and possibly PGI₂, endothelium-dependent relaxation may involve EDHF, an important vasodilator in small resistance arteries that contributes to the regulation of vascular resistance and blood flow (Shimokawa *et al.*, 1996; Busse *et al.*, 2002; Féletou and Vanhoutte, 2009). EDHF produces relaxation by membrane hyperpolarization, inactivation of voltage-gated Ca^{2+} channels (Nelson *et al.*, 1990) and decreasing the activity of PLC (Itoh *et al.*, 1992). In the presence of L-NAME and INDO to block NOS and COX, respectively, ACh still caused microvascular relaxation and the sensitivity to ACh was enhanced in pregnant compared with virgin rats, supporting that EDHF-mediated pathways are up-regulated in the mesenteric circulation during pregnancy (Gerber *et al.*, 1998; Dalle Lucca *et al.*, 2000; Mazzuca *et al.*, 2012). The observations that blockade with L-NAME + INDO did not inhibit maximal ACh-induced relaxation but reduced the sensitivity to ACh, while ACh relaxation was abolished by blocking EDHF with TEA and the sensitivity to EDHF-mediated relaxation was greater in pregnant than virgin rats, suggest that ACh-induced mesenteric microvascular relaxation was only partly due to changes in NO synthesis/release and, to a large extent, due to release of EDHF.

Similar to ACh, the microvessel relaxation in response to ET_BR activation by BQ-123 + ET-1 or the ET_BR agonists S6c and IRL-1620 was abolished by endothelium removal, supporting an endothelium-dependent ET_BR-mediated vasodilation. In comparison with ACh, the enhanced endothelium-dependent ET_BR-mediated relaxation in microvessels of pregnant rats was partially inhibited by L-NAME supporting a role

of NO. S6c and IRL-1620 did not cause detectable change in $[Ca^{2+}]_i$ likely because the increase in ET_BR-mediated $[Ca^{2+}]_i$ in endothelial cells is cancelled by the concomitant decrease in $[Ca^{2+}]_i$ in VSM, or perhaps due to eNOS activation by other pathways such as MAPK and Akt. In microvessels of pregnant rats, a significant S6c- and IRL-1620-induced relaxation remained in the presence of L-NAME and INDO, supporting a role of EDHF. The remaining EDHF-mediated S6c- and IRL-1620-induced relaxation was abolished by TEA, a non-selective K⁺ channel blocker. Although the present study did not identify which ET_BR-mediated EDHF(s) were up-regulated in the mesenteric arteries during pregnancy, studies have shown that the up-regulation of EDHF is due to enhanced myoendothelial gap junction communication in subcutaneous and myometrial arteries of pregnant women (Kenny *et al.*, 2002; Luksha *et al.*, 2004; Lang *et al.*, 2007) and aortic rings from pregnant rats (Dantas *et al.*, 1999). Other studies have shown that 4-aminopyridine, but not apamin, charybdotoxin or glibenclamide reduces IRL-1620-induced relaxation, indicating that voltage-dependent K⁺ channel (K_v) but not small-conductance Ca^{2+} -activated K⁺ channel (K_{Ca2.3}), intermediate-conductance Ca^{2+} -activated K⁺ channel (K_{Ca3.1}) or ATP-sensitive K⁺ channel (K_{ATP}), respectively, contributes to the ET_BR/EDHF-mediated response (Tirapelli *et al.*, 2005). These channels could be involved in the prominent EDHF-dependent ET_BR-mediated relaxation in mesenteric microvessels of pregnant rats. Other proposed EDHF mechanisms including cytochrome p450 pathway of arachidonic acid such as epoxyeicosatrienoic acids, inward rectifier K⁺ channel (K_{IR}), Na⁺/K⁺-ATPase, hydrogen peroxide (H₂O₂), and C-type natriuretic peptide may be involved, and warrant further investigation.

An important question is what causes the enhanced endothelial ET_BR activity during pregnancy. The pregnancy-associated increase in microvascular relaxation may take place at the ET_BR level, and several factors such as oestrogen and relaxin may affect the expression and activity of ET_BR during pregnancy (Nuedling *et al.*, 2003; Conrad, 2011). Furthermore, post-translation modification and O-GlcNAcylation may modulate the vascular effects of ET-1 (Lima *et al.*, 2011), and may affect ET_BR activity during pregnancy.

We should caution that ET_BRs have been identified not only in the endothelium, but have also been proposed to be expressed in VSM, and to promote vasoconstriction by mechanisms similar to those activated by ET_AR (Touyz *et al.*, 1995; Tostes *et al.*, 2008), and therefore the net physiological impact of ET_BR stimulation may depend on the balance between the opposing effects of ET_BR in endothelial cells and VSM. Importantly, in freshly isolated male Wistar-Kyoto rat mesenteric arteries mounted on wire or perfusion myographs, stimulation with the ET_BR agonists S6c and IRL-1620 produced negligible vasoconstrictor effects (Adner *et al.*, 1998a,b), suggesting little role of VSM ET_BR in mesenteric artery vasoconstriction or that any VSM ET_BR-mediated contraction mechanisms are counterbalanced by endothelial ET_BR-mediated vasodilation. Different effects of ET_BR activation have also been observed in various vascular beds and in health and disease (Schneider *et al.*, 2007; Davenport and Maguire, 2011; Mazzuca and Khalil, 2012). However, the observation that ET_BR agonists caused vasodilation in mesen-

teric microvessels of pregnant rats supports greater amount of endothelial than VSM ET_BR. This is further supported by the Western blot data that ET_BR levels were greater in endothelium-intact than endothelium-denuded microvessels of pregnant rats. Conversely, in virgin rats, ET_BR levels were not different in endothelium-denuded and intact mesenteric arteries, suggesting that most of the detected ET_BR represents VSM ET_BR as opposed to endothelial ET_BR. This may also explain the minimal ET_BR-mediated vasodilator response to BQ-123 + ET-1, S6c and IRL-1620 in mesenteric microvessels of virgin rats.

The present findings could have important physiological and pathological implications. Up-regulation of endothelial ET_BR could lead to enhanced microvascular relaxation, attenuated vasoconstriction, and may play a protective role in modulating BP during normal pregnancy. Conversely, decreased expression/activity of ET_BR may play a role in the endothelial dysfunction and vasoconstriction in hypertension in pregnancy and pre-eclampsia. Mesenteric microvascular constriction is enhanced in rat models of hypertension in pregnancy (Anderson *et al.*, 2006b). Because endothelial dysfunction is a major factor in the pathogenesis of pre-eclampsia (Maynard *et al.*, 2003; Reslan and Khalil, 2010), it could cause down-regulation of endothelial ET_BR leading to decreased vasodilation and increased BP. Interestingly, some studies have examined the effects of ET_BR modulation *in vivo* and demonstrated that chronic subcutaneous infusion of the ET_BR antagonist A-192621 caused transient increases in BP in pregnant rats (Thaete and Neerhof, 2000; Madsen *et al.*, 2001), which could be well explained by blockade of endothelial ET_BR-mediated vasodilation. However, *in vivo* changes in BP should be interpreted with caution as BP regulation involves multiple renal, hormonal, neuronal and vascular control mechanisms. For instance, ET_BR in the renal medulla may play a role in salt and water excretion, and blockade of renal ET_BR *in vivo* could cause salt and water retention and hypertension (Kohan *et al.*, 2011). Furthermore, ET_BR may mediate ET-1 clearance, and blockade of these receptors *in vivo* would allow more ET-1 to activate ET_AR to promote vasoconstriction and increase BP. One approach to circumvent the long-term renal effects on BP is to study the acute effects of intravenous infusion of an ET_BR receptor antagonist over the course of minutes in pregnant rats. Over such a short time course, it is unlikely that the renal haemodynamics would play a significant role in modulating the BP response. However, during the acute infusion of an ET_BR modulator *in vivo*, any changes in vascular reactivity may be compensated by baroreceptor reflexes, which could mask any vascular effects on BP (D'Angelo and Osol, 1993). The present study was pivotal in that it addressed the 'direct effects' of ET_BR agonists and antagonists in the mesenteric vascular bed, and highlighted the important role of endothelial ET_BR activation in enhancing the vasodilatory response commonly observed during pregnancy.

ET-1 has been suggested to play a role in pre-eclampsia (Maynard *et al.*, 2003). ET-1 levels are increased in pre-eclampsia, particularly when accompanied with intrauterine growth restriction (McQueen *et al.*, 1993; Furuhashi *et al.*, 1995), and modulation of the ET-1 system may be an important target in the management of pre-eclampsia. Studies have shown that infusion of ET_AR antagonist reduces BP in the

L-NAME-infused reduced uterine perfusion pressure and sFlt-1-infused rat models of hypertension in pregnancy (Olson *et al.*, 1999; Alexander *et al.*, 2001; Murphy *et al.*, 2010; 2012; Tam Tam *et al.*, 2011). While the decrease in BP could be due to blockade of ET_AR in VSM and reduction of vasoconstriction, it could also be due to the possibility that most ET-1 would be directed towards endothelial ET_BR and promote vasodilation. Endothelial ET_BR agonists or conditional genetic activation of endothelial ET_BR may represent novel approaches in the management of hypertension in pregnancy and pre-eclampsia.

In summary, the present study provided evidence that ET_BR expression and activity are enhanced during pregnancy, and may explain the blunted vasoconstrictor response to ET-1 during pregnancy. The role of endothelial ET_BR needs to be further evaluated in the pathogenesis and management of hypertension in pregnancy and pre-eclampsia.

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Conflict of interest

The authors declare no conflict of interest.

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