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Adaptive Regulation of Endothelin Receptor Type A and Type B in Vascular Smooth Muscle Cells during Pregnancy in Rats

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Abstract

Normal pregnancy is associated with systemic vasodilation and decreased vascular contraction, partly due to increased release of endothelium-derived vasodilator substances. Endothelin-1 (ET-1) is an endothelium-derived vasoconstrictor acting via endothelin receptor type A (ET_AR) and possibly type B (ET_BR) in vascular smooth muscle cells (VSMCs), with additional vasodilator effects via endothelial ET_BR. However, the role of ET-1 receptor subtypes in the regulation of vascular function during pregnancy is unclear. We investigated whether the decreased vascular contraction during pregnancy reflects changes in the expression/activity of ET_AR and ET_BR. Contraction was measured in single aortic VSMCs isolated from virgin, mid-pregnant (mid-Preg, day 12) and late-Preg (day 19) Sprague-Dawley rats, and the mRNA expression, protein amount, tissue and cellular distribution of ET_AR and ET_BR were examined using RT-PCR, Western blots, immunohistochemistry and immunofluorescence. Phenylephrine (Phe, 10^{-5} M), KCl (51 mM) and ET-1 (10^{-6} M) caused VSMC contraction that was in late-Preg < mid-Preg and virgin rats. In VSMCs treated with ET_BR antagonist BQ788, ET-1 caused significant contraction that was still in late-Preg < mid-Preg and virgin rats. In VSMCs treated with the ET_AR antagonist BQ123, ET-1 caused a small contraction; and the ET_BR agonists IRL-1620 and sarafotoxin 6c (S6c) caused similar contraction that was in late-Preg < mid-Preg and virgin rats. RT-PCR revealed similar ET_AR, but greater ET_BR mRNA expression in pregnant vs. virgin rats. Western blots revealed similar ET_AR, and greater protein amount of ET_BR in endothelium-intact vessels, but reduced ET_BR in endothelium-denuded vessels of pregnant vs. virgin rats. Immunohistochemistry revealed prominent ET_BR staining in the intima, but reduced ET_AR and ET_BR in the aortic media of pregnant rats. Immunofluorescence signal for ET_AR and ET_BR was less in VSMCs of pregnant vs. virgin rats. The pregnancy-associated decrease in ET_AR- and ET_BR-mediated VSMC contraction appears to involve downregulation of ET_AR and ET_BR expression/activity in VSM, and may play a role in the adaptive vasodilation during pregnancy.

Keywords

vascular smooth muscle; contraction; pregnancy; preeclampsia

INTRODUCTION

Normal pregnancy is associated with significant hemodynamic and cardiovascular changes to meet the metabolic needs of mother and fetus. During pregnancy the cardiac output increases while the systemic vascular resistance, arterial pressure and pressor response to circulating angiotensin II (ANG II) are decreased (Khalil and Granger, 2002; Stennett et al.,

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2009). The pregnancy-associated reduction in blood pressure has been largely explained by increased release of endothelium-derived vasodilators such as nitric oxide (NO) and activation of the NO-cGMP relaxation pathway. This is supported by reports that the expression of NO synthase (NOS) and NO production are increased during pregnancy (Conrad and Whittemore, 1992; Crews et al., 1999; Goetz et al., 1994; Molnar and Hertelendy, 1992; Nathan et al., 1995; Sladek et al., 1997), and that the plasma levels of cGMP are elevated during pregnancy (Conrad et al., 1999; Conrad and Vernier, 1989). Other endothelium-derived vasodilators including prostacyclin (PGI_2) (Ylikorkala et al., 1986), endothelium-derived hyperpolarizing factor (EDHF) (Fulep et al., 2001; Gerber et al., 1998), and hemeoxygenase products (Bainbridge and Smith, 2005; McLaughlin et al., 2000) are also elevated during pregnancy.

In addition to endothelium-derived vasodilators, the endothelium releases vasoconstrictor factors such as ANG II and endothelin-1 (ET-1). Normal pregnancy is associated with upregulation of the renin-angiotensin-system and elevated plasma levels of ANG II, but reduced vascular reactivity to vasoconstrictors (Davidge and McLaughlin, 1992; Nathan et al., 1995; Stennett et al., 2009). Although ET-1 is a major endothelium-derived vasoconstrictor and an important modulator of vascular tone and blood pressure (Hynynen and Khalil, 2006; Schiffrin, 2001; Schiffrin and Touyz, 1998), its role and vascular effects during pregnancy have not been fully investigated.

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) (Mazzuca and Khalil, 2012; Sakurai et al., 1990; Touyz et al., 1995b). Although the role of ET_AR in vascular contraction is well-characterized (Luscher and Barton, 2000; Schiffrin and Touyz, 1998), the role of ET_BR is less defined. ET-1 interacts with ET_AR, and possibly ET_BR, in vascular smooth muscle cells (VSMCs), initiating a series of biochemical events that lead to vascular contraction (Fellner and Arendshorst, 2004; Kanashiro et al., 2000; Ko et al., 2005; McNair et al., 2004; Pollock et al., 2005; Schroeder et al., 2000; Sirous et al., 2001; Touyz et al., 1995a). ET-1 could also activate ET_BR in endothelial cells to induce vascular relaxation (D'Orleans-Juste et al., 2002; Hynynen and Khalil, 2006; McMurdo et al., 1994) by promoting the release of NO, PGI_2 and EDHF (Nakashima and Vanhoutte, 1993; Novak et al., 2004; Tirapelli et al., 2005).

We have previously shown that vascular contraction is reduced during pregnancy (Crews et al., 1999; Khalil et al., 1998). While the vascular changes during pregnancy have been partly explained by increased release of NO (Conrad and Whittemore, 1992; Crews et al., 1999; Goetz et al., 1994; Molnar and Hertelendy, 1992; Nathan et al., 1995; Sladek et al., 1997), PGI_2 (Ylikorkala et al., 1986), and EDHF (Fulep et al., 2001; Gerber et al., 1998), whether the pregnancy-associated decrease in vascular contraction involves changes in ET receptor expression or activity is less clear. We have recently shown that ET-1 induced vaoconstriction is reduced in mesenteric microvessels of pregnant rats compared with virgin rats (Mazzuca et al., 2013). However, because different ET receptor subtypes are expressed in various vascular cells, studying the pregnancy-associated changes in ET receptors in a multicellular vascular preparation could be difficult. Therefore, the present study was primarily performed on single VSMCs freshly isolated from the aorta of female rats to investigate whether: 1) ET_BR is expressed and functional in VSMCs of female rats, 2) ET_BR-mediated VSMC contraction is modified during the course of pregnancy, and 3) the pregnancy-associated changes in ET_BR-mediated VSMC contraction reflect changes in the expression, cellular distribution and activity ET_BR. For comparison, the pregnancy associated changes in vascular ET_BR expression, distribution and activity were compared in parallel with ET_AR.

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METHODS

Animals and Tissue Preparation

Age-matched virgin, mid-pregnant (mid-Preg, day 12) and late-Preg (day 19) Sprague-Dawley rats (12 weeks old, Charles River laboratory, Wilmington, MA) were housed in the animal facility and maintained on *ad libitum* standard rat chow and tap water in 12 hr/12 hr light/dark cycle. Because vascular reactivity can be influenced by sex hormones during the estrous cycle, all experiments on virgin rats were conducted during estrus, in order to control for endocrine confounders. The estrous cycle was determined by taking a vaginal smear with a pasteur pipette (Mazzuca et al., 2010). An estrus smear primarily contained anucleated cornified squamous cells (Yener et al., 2007) and was confirmed prior to all experiments. After measuring body weight (virgin 251.0±8.61, mid-Preg 289.5±4.2, late-Preg 362.5±10.4 g), blood pressure was measured via a carotid artery catheter connected to pressure transducer (virgin 105±7, late-Preg 96±7 mmHg). Rats were euthanized by inhalation of CO_2, and the thoracic aorta was rapidly excised, placed in oxygenated Krebs solution, and cleaned of connective and adipose tissue under microscopic visualization. All procedures followed the NIH guide for the Care of Laboratory Animal Welfare Act, and approved protocols by the Animal Care and Use Committee at Harvard Medical School.

Isolation of Single VSMCs

Single aortic VSMCs were freshly isolated as previously described with a few modifications, specifically avoiding aspiration through a pipette or centrifugation (Ma et al., 2010; Murphy and Khalil, 2000). The aorta was opened by cutting along its longitudinal axis, the endothelium was removed by scraping the vessel interior five times with curved forceps, the adventitia was carefully peeled off using sharp-tipped forceps, and the remaining aortic media was sectioned into 2x2 mm strips. Aortic strips (50 mg) were placed in a siliconized flask containing a tissue digestion mixture of collagenase type II (236 U/mg protein activity, Worthington, Freehold, NJ), elastase grade II (3.25 U/mg protein activity, Boehringer Mannheim, Indianapolis, IN), and trypsin inhibitor type II-soybean (10,000 U/ml, Sigma, St. Louis, MO) in 7.5 ml of Ca^{2+}- and Mg^{2+}-free Hank's solution supplemented with 30% bovine serum albumin (Sigma). The tissue was incubated 3 consecutive times in the tissue digestion mixture to yield 3 batches of cells. For the first batch, the digestion medium contained 6 mg collagenase, 4 mg elastase and 147 μl trypsin inhibitor, and the incubation period was 75 min. For batches 2 and 3, the collagenase was reduced to 3 mg, the trypsin inhibitor was reduced to 122 μl and the incubation period was reduced to 30 min. The aortic tissue preparation was placed in a shaking water bath at 34°C in an atmosphere of 95% O_2 and 5% CO_2. At the end of each incubation period, the preparation was rinsed with 12.5 ml Ca^{2+}- and Mg^{2+}-free Hank's solution, and the isolated cells were poured over either ice-cooled wells with glass bottom (Biotechs, Butler, PA) (for cell contraction studies), or glass cover slips placed in 6-well plates (for immunofluorescence studies). By using the gravitational force, single aortic VSMCs were allowed to settle and adhere to the glass cover slips. Ca^{2+} was added gradually back to the preparation in order to avoid the "Ca^{2+} paradox" (Nayler et al., 1984). The cell isolation procedure consistently yielded spindle-shaped and viable VSMCs that showed significant contraction in response to various contractile stimuli.

Cell Contraction

Freshly isolated aortic VSMCs in the glass-bottom wells were placed on a slide warmer at 37°C (Biotechs) and on the stage of an inverted Nikon Eclipse-300 microscope. The cells were viewed using a 20X or 40X objective, and cell images were acquired using a Nikon digital camera and image acquisition software. After measuring the resting cell length, the cells were stimulated with phenylephrine (Phe, 10^{-5} M), high KCl depolarizing solution (51 mM), or ET-1 (10^{-6} M) for 10 min and the changes in cell length were recorded. The

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magnitude of cell contraction was expressed as $((L_i-L)/L_i) \times 100$, where L_i is the initial cell length and L is the final cell length. We also tested the effects of ET receptor modulators whose specificity has previously been established including selective ET_AR antagonist BQ123 (Sakamoto et al., 1993), ET_BR antagonist BQ788 (Ishikawa et al., 1994; Okada and Nishikibe, 2002) and the ET_BR agonists IRL-1620 (Ekelund et al., 1994; Miasiro et al., 1998) and sarafotoxin 6c (S6c) (Williams et al., 1991). To test the role of ET_AR, ET-1 induced VSMC contraction was measured in the presence of the ET_BR antagonist BQ788 (10^{-6} M). To test the role of ET_BR, VSMC contraction to ET-1 in the presence of the ET_AR antagonist BQ123, or in response to the ET_BR agonists IRL-1620 and S6c (10^{-6} M) was measured. The concentrations selected were determined from preliminary concentration-response curves in isolated vessels which indicated that these concentrations produced the maximal responses (Mazzuca et al., 2013).

Real-Time RT-PCR Analysis

RNA was isolated from aortic strips of virgin and pregnant rats using RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA). 1 μg of total RNA was used for reverse transcription to synthesize single-strand cDNA in a 20 μl-reaction mixture according to the protocol of First-Strand cDNA Synthesis Kit (Amersham Biosciences, Pittsburgh, PA). 2 μl of cDNA dilution (1:5 for ET_AR and ET_BR, and 1:25 for α-actin) of reverse transcription (RT) product was applied to 20 μl RT-PCR reaction. Quantification of gene expression was performed using real-time quantitative RT-PCR machine (Mx4000 Multiplex Quantitative PCR System, Stratagene, La Jolla, CA) and employing published oligonucleotide primers specific for ET_AR and ET_BR (Integrated DNA Technologies (IDT), Coralville, IA), and the Bio-Rad iQ SYBR Green Supermix for amplicon detection (Bio-Rad, Hercules, CA). α-Actin primer was included in the RT-PCR reaction as internal standard to normalize the results.

Primer	Sequence	
ET_AR	Forward	5′- CAGCCTGGCCCTTGGAGACCTTAT -3′
	Reverse	5′- TTCTGTGCTGCTCGCCCTTGTATT -3′
ET_BR	Forward	5′- GATACGACAACTTCCGCTCCA- 3′
	Reverse	5′- GTCCACGATGAGGACAATGAG- 3′
α-actin	Forward	5′- GACACCAGGGAGTGATGGTT -3′
	Reverse	5′- GTTAGCAAGGTCGGATGCTC -3′

PCR was carried out with 1 cycle for 10 min at 95°C then 40–45 cycles of 30 sec denaturation at 95°C, 45 sec of annealing at 56°C, and 30 sec of extension at 72°C, followed by 1 min of final extension step at 95°C. The number of PCR cycles varies according to the expression level of the target gene. An appropriate primer concentration and number of cycles was determined to ensure that the PCR is taking place in the linear range and thereby guarantees a proportional relationship between input RNA and the cycles readout. The gene expression was calculated relative to the housekeeping gene α-actin (Ma et al., 2010).

Western Blot Analysis

Endothelium-intact aortic segments from virgin, mid-Preg and late-Preg rats were homogenized in a buffer containing 20 mM 3-[N-morpholino]propane sulfonic acid (MOPS), 4% SDS, 10% glycerol, 10 mM dithiothreitol, 1.2 mM EDTA, 0.02% BSA, 5.5 μM leupeptin, 5.5 μM pepstatin, 2.15 μM aprotinin and 20 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, using a 2 ml tight-fitting homogenizer (Kontes Glass, Vineland,

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NJ, USA). The homogenate was centrifuged at 10,000g for 2 min, the supernatant was collected, and the protein concentration was determined using a protein assay kit (Bio-Rad). In some experiments, tissue homogenate was prepared from endothelium-denuded aorta of virgin and pregnant rats. In these experiments, the aorta was opened by cutting along its longitudinal axis and the endothelium was removed by scraping the vessel interior five times with curved forceps.

Protein extracts (20 μg) 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. The membrane was incubated in 5% dried non-fat milk for 1 hr, then treated with polyclonal antibody to ET_AR or ET_BR (1:1000) (Santa Cruz Biotechnology) for 24 hr. Negative control experiments were performed with the omission of primary antibody, and exhibited no detectable immunoreactive bands. The nitrocellulose membranes were washed 5 times for 15 min each in TBS-Tween then incubated in horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:1000) for 1.5 hr, and the immunoreactive bands were detected using Enhanced Chemi-Luminescence (ECL) Western blotting detection reagent (GE Healthcare Bio-Sciences, Piscataway, NJ). The blots were subsequently reprobed for β-actin using monoclonal anti-β-actiin antibody (1:2000, Sigma). The reactive bands were analyzed quantitatively by optical densitometry and ImageJ software (National Institutes of Health, Bethesda, MD, USA). The densitometry values represented the pixel intensity, and were normalized to β-actin to correct for loading (Ma et al., 2010).

Histology and Quantitative Morphometry

To assess whether pregnancy is associated with adaptive vascular tissue changes in the relative thickness of the intima, media and adventitia, cryosections (6 μm) of aortic rings from virgin, mid-Preg and late-Preg rats were placed on glass slides and prepared for staining with hematoxylin and eosin. Stained sections were coded and labeled in a blinded fashion. Images were acquired on a Nikon microscope with digital camera mount and analyzed using ImageJ software (NIH). Outlines of the vessel lumen, internal elastic lamina and external vascular wall were defined and the total wall thickness and relative thickness of the intima, media and adventitia were measured (Stennett et al., 2009).

Immunohistochemistry

To determine the tissue distribution of ET receptor subtypes in the aorta of virgin, mid-Preg and late-Preg rats, cryosections of the aorta (6 μm thick) were thawed and fixed in ice-cold acetone for 10 min. Endogenous peroxidase was quenched in 1.5% H_2O_2 solution for 10 min, and nonspecific binding was blocked in 10% horse serum. Tissue sections were incubated with polyclonal ET_AR and ET_BR antibodies (1:500, Santa Cruz Biotechnology). After being rinsed with phosphate-buffered saline (PBS), tissue sections were incubated with biotinylated anti-rabbit secondary antibody, rinsed with PBS, and then incubated with avidin-labeled peroxidase (VectaStain Elite ABC Kit, Vector Laboratories, Burlingame, CA). Positive labeling was visualized using diaminobenzadine and appeared as brown spots. Negative control slides were run in the absence of primary antibody, and showed no detectable immunostaining. Specimens were counterstained with hematoxylin for 30 seconds, rinsed with PBS, topped with cytoseal 60, then covered with slide coverslips.

Images of aortic tissue sections were acquired on a Nikon microscope with digital camera mount using the same magnification, light intensity, exposure time and camera gain, and the images were analyzed using ImageJ software. The total number of pixels in the tissue section image was first defined, then the number of brown spots (pixels) was counted and presented as % of total pixels. The number of pixels in the specific vascular layer (intima,

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media and adventitia) was also defined and transformed into the area in μm^2 using a calibration bar. The number of brown spots (pixels) representing ET_AR and ET_BR in each vascular layer was then counted and presented as number of pixels/μm^2 (Stennett et al., 2009).

Immunofluorescence

Isolated aortic VSMCs on cover-slips were fixed for 10 min in 2% paraformaldehyde in Hank's solution (pH 7.4), and the excess fixative was quenched two times 5 min each with 0.1 mM glycine in Hank's. The cells were permeabilized with 0.1% Triton X-100 in 1% bovine serum albumin (BSA) Hank's for 10 min then washed 3 times 5 min each with 0.05% Triton X-100 in 1% BSA Hank's. Cells were blocked in 1% BSA Hank's supplemented with 2% goat serum for 45 min then reacted with polyclonal antibody to ET_AR or ET_BR (1:500) (Santa Cruz Biotechnology). Cells were washed 3 times 10 min each in 1% BSA Hank's, then incubated in fluorescein isothiocyanate (FITC)-labeled anti-rabbit immunoglobulin G (Sigma) for 30 min. For double staining of the nucleus, the DNA marker ethidium dimer (1:1000, Invitrogen, Carlsbad, CA) was used, and excess label was removed by washing in 1% BSA Hank's. All procedures were performed at 22°C.

Fluorescently-labeled cells were viewed on a Nikon microscope using a 40X objective. For FITC, an excitation filter at 485 nm, a dichroic filter at 500 nm, and a longpass filter at 530 nm were used. For ethidium dimer, a Texas Red excitation filter at 488 nm, a dichroic filter at 500 nm, and a long-pass filter at 615 nm were used. An excitation light shutter was opened only when taking measurements to minimize photobleaching. Fluorescent images were acquired by a Quantix cooled intensified charge-coupled device (CCD) camera (Photometric, Tucson, AZ) using the same magnification, light intensity, exposure time and camera gain, and the images were analyzed using ImageJ software. Images were background subtracted. Only cells with intact nuclei as determined by ethidium dimer staining were analyzed. Average FITC cell fluorescence intensity was measured by tracing the area along the borders of the cell image, integrating the total fluorescence intensity in the cell image, and dividing by the number of pixels in the cell image. To test for subcellular localization of ET_AR and ET_BR at the cell surface, a line was traced along the cell border and the average pixel intensity was determined. Nuclear staining was determined by tracing the area of ethidium dimer fluorescence. We have used a similar fluorescence microscopy approach to determine the subcellular distribution of protein kinase C (PKC) in aortic VSMCs and found that PKC activation with Phe caused translocation of PKC from the cytosol to the vicinity of the surface membrane (Khalil & Morgan, 1991: Khalil et al., 1992). In these VSMCs, the surface membrane area was identified using the cell membrane marker 7-decylBODIPY-1-propionic acid, and the nuclear area was identified using ethidium dimmer. The difference between the total fluorescence and the combined fluorescence in the surface membrane and the nucleus represented the cytosolic fluorescence. The subcellular distribution was determined as fluorescence intensity in the cell surface, cytosol and nuclear region relative to total cell fluorescence.

Solutions, Drugs and Chemicals

Krebs solution was used for dissecting the tissue and contained (in mM): 120 NaCl, 5.9 KCl, 25 $NaHCO_3$, 1.2 NaH_2PO_4, 11.5 dextrose, 2.5 $CaCl_2$, and 1.2 $MgCl_2$. Krebs solution was bubbled with 95% O_2 and 5% CO_2 for 30 min, at an adjusted pH 7.4. Hank's solution was used for cell isolation and for performing the experiments and contained (in mM): 137 NaCl, 5.4 KCl, 0.44 KH_2PO4, 0.42 Na_2HPO_4, 4.17 $NaHCO_3$, 5.55 dextrose and 10 HEPES. Hank's solution was bubbled for 30 min with a 95% O_2 5% CO_2 mixture and the pH was adjusted to 7.4. High KCl (51 mM) depolarizing solution had the same composition as Hank's solution with equimolar substitution of NaCl with KCl. PBS was used to rinse the

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slides in the immunohistochemistry experiments and contained (in mM): 137 NaCl, 2.7 KCl, 8 Na_2HPO_4, 2 KH_2PO_4, at pH 7.4. Stock solutions of Phe (10^{-1} M), ET-1 (10^{-2} M) (Sigma), IRL-1620, and sarafotoxin 6c (S6c) (10^{-2} M) (Tocris, Ellisville, MO) were prepared in distilled water. Stock solutions of the ET_AR antagonist BQ-123 and the ET_BR antagonist BQ-788 (10^{-3} M; Tocris) were prepared in DMSO. The final concentration of DMSO in the experimental solution was <0.1%. All other chemicals were of reagent grade or better.

Statistical Analysis

Cell contraction data were collected from 6 to 12 aortic VSMCs from each rat for each experiment, the average data from different cells of each rat was measured, and the cumulative data from 6 to 12 different rats were used for statistical analysis. For analysis of the RT-PCR data and determination of mRNA levels, the $\Delta\Delta C_T$ method was used as previously described (Livak and Schmittgen, 2001; Pojoga et al., 2011). The averages and SD were determined for all the technical replicates C_T. With the use of the average technical replicate C_T, ΔC_T was calculated for each group (C_T of target gene − C_T of control gene actin). After the average C_T was determined for each group, $\Delta\Delta C_T$ values (C_T of pregnant group − C_T of control virgin group) were calculated and presented as fold increase relative to the measurements in virgin rats $2^{-(\Delta\Delta C_T)}$. RT-PCR, Western blots, histology and immunohistochemistry were performed on specimens from 4 to 7 rats. Data were first analyzed using one way ANOVA with Scheffe's F test, where F=(variance between groups/ variance within groups). When a statistical difference was observed, the data were further analyzed using Student-Newman-Keuls *post-hoc* test for multiple comparisons. Student's unpaired *t*-test was used for comparison of two means. Data were presented as means±SD with n = number of rats, and differences were considered statistically significant if $p<0.05$.

RESULTS

The cell isolation procedure produced aortic VSMCs of variable lengths. Only spindle-shaped cells ≥ 50 μm in length were selected for this study. The resting cell length was not significantly different between virgin (68.55±4.71 μm), mid-Preg (67.94±7.44 μm) and late-Preg rats (67.17±5.01 μm). Also, the resting cell width was not significantly different between virgin (10.41±1.30 μm), mid-Preg (10.34±1.35 μm) and late-Preg rats (10.61±1.34 μm). VSMCs of virgin and pregnant rats were responsive to contractile stimuli. The α-adrenergic receptor agonist Phe (10^{-5} M) (Fig. 1A) and membrane depolarization by 51 mM KCl (Fig. 1B) caused significant contraction that was reduced in VSMCs of late-Preg compared with virgin and mid-Preg rats (Fig. 1).

ET-1 (10^{-6} M) also caused significant contraction in aortic VSMCs that was reduced in late-Preg compared with virgin and mid-Preg rats (Fig. 2A). In VSMCs of virgin and pregnant rats pretreated with the ET_AR antagonist BQ123 (10^{-6} M) for 10 min, ET-1 caused a small contraction that was still reduced in late-Preg compared with virgin and mid-Preg rats (Fig. 2B). In VSMCs of virgin and pregnant rats pretreated with the ET_BR antagonist BQ788 (10^{-6} M) for 10 min, ET-1 caused significant contraction that was less in late-Preg than virgin and mid-Preg rats (Fig. 2C). In VSMCs of virgin and pregnant rats pretreated with both BQ123 (10^{-6} M) and BQ788 (10^{-6} M), ET-1 contraction was almost abolished (Fig. 2D).

In aortic VSMCs of virgin rats, the ET_BR agonist IRL-1620 (10^{-6} M) caused a small but significant contraction. IRL-1620 induced contraction was significantly reduced in late-Preg compared with virgin and mid-Preg rats (Fig. 3A). Similarly, the ET_BR agonist S6c (10^{-6} M) caused a small contraction that was significantly reduced in late-Preg compared with virgin and mid-Preg rats (Fig. 3B).

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RT-PCR analysis of aortic tissue homogenate revealed little change in ET_AR, but enhanced ET_BR mRNA expression in late-Preg compared with virgin rats (Fig. 4). Western blot analysis of aortic tissue homogenate from virgin and pregnant rats revealed immunoreactive bands corresponding to ET_AR at 59 kDa and ET_BR at 50 kDa. In tissue homogenates of endothelium-intact aorta, optical density analysis revealed little change in the protein amount of ET_AR, but increased amount of ET_BR in late-Preg compared with virgin rats (Fig. 5A). In tissue homogenates from endothelium-denuded aorta, optical density analysis revealed similar amount of ET_AR, but reduced amount of ET_BR in late-Preg compared with virgin rats (Fig. 5B).

Histological analysis of tissue sections indicated that the aortic wall thickness was thinner in late-Preg compared with virgin and mid-Preg rats (Fig. 6A, 6B). Additional morphometric analysis indicated that the tunica intima and tunica media thickness as % of total thickness was significantly greater, while the % tunica adventitia thickness was significantly reduced in aortic tissue sections of late-Preg compared with virgin and mid-Preg rats (Fig. 6C).

Immunohistochemical analysis revealed detectable ET_AR and ET_BR staining in aortic tissue sections of virgin and pregnant rats (Fig. 7A, 7B). Quantitative image analysis of tissue sections demonstrated that the total brown staining for ET_AR was reduced in late-Preg compared with virgin and mid-Preg rats (Fig. 7C). In contrast, the total ET_BR brown staining was significantly greater in late-Preg compared with virgin and mid-Preg rats (Fig. 7C). Analysis of the distribution of ET_AR in the intima, media and adventitia by counting the number of brown spots in the specific tissue layer demonstrated that ET_AR staining was evenly distributed in the tunica intima and adventitia, but was significantly reduced in the tunica media of late-Preg compared with virgin and mid-Preg rats (Fig. 7D). In contrast, ET_BR demonstrated intense staining in the intima and reduced staining in the media in aortic tissue sections of late-Preg compared with virgin and mid-Preg rats (Fig. 7E).

To further test for pregnancy-associated changes in cellular ET_AR and ET_BR expression, we examined single aortic VSMCs fluorescently labeled with ET_AR and ET_BR antibodies and double-stained with the nuclear DNA marker ethidium dimer (Fig. 8A, 8B). The average fluorescence signal for ET_AR was reduced in VSMCs of late-Preg compared with virgin and mid-Preg rats (Fig. 8C). Similarly, the average ET_BR fluorescence was significantly reduced in late-Preg compared with virgin and mid-Preg rats (Fig. 8C). Optical dissection of the subcellular distribution of ET_AR revealed a decrease in the fluorescence signal in the cell periphery in mid-Preg and late-Preg compared with virgin rats (Fig. 8D). On the other hand, the average ET_AR fluorescence in the cytosol and in the center of the cell coinciding with the nucleus was enhanced in mid-Preg and late-Preg compared with virgin rats (Fig. 8D). Analysis of the subcellular distribution of the ET_BR fluorescence signal in VSMCs revealed a decrease in the cell periphery and an increase in the cytosol and nuclear region in late-Preg compared with mid-Preg and virgin rats (Fig. 8E).

DISCUSSION

The main findings of the present study are: 1) In addition to ET_AR, ET_BR is expressed and functional, and mediates measurable contraction in aortic VSMCs of female rats, 2) A major component of ET-1 induced VSMC contraction is mediated by ET_AR and a smaller component is mediated via ET_BR, and both components are reduced in late-Preg compared with mid-Preg and virgin rats, 3) The pregnancy-related decrease in ET_AR- and ET_BR-mediated VSMC contraction was associated with decreased ET_BR mRNA expression and protein amount and decreased ET_AR and ET_BR immunostaining in the aortic media and VSMCs.

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Normal pregnancy is associated with reduced vascular resistance, blood pressure and vascular reactivity to vasoconstrictors such as Phe and ANG II (Crews et al., 1999; Davidge and McLaughlin, 1992; Khalil et al., 1998; Nathan et al., 1995). Although ET-1 is a major vasoconstrictor, its role in modulating vascular function during pregnancy is less clear. We found that aortic VSMC contraction to ET-1 was reduced in late-Preg compared with mid-Preg and virgin rats. This is consistent with our previous report that ET-1 induced vasoconstriction is reduced in mesenteric vessels of pregnant compared with virgin rats (Mazzuca et al., 2013). The pregnancy-related changes in ET-1 induced VSMC contraction could be due to changes in the relative expression, distribution, or activity of ET receptor subtypes.

ET_AR is widely expressed in various blood vessels and the kidney and mediates most of the effects of ET-1 (Khalil, 2011; LaDouceur et al., 1993; Schiffrin and Touyz, 1998; Seo et al., 1994). In addition to ET_AR, ET-1 could activate ET_BR. In the adult rat, ET_BR is expressed in the kidney, heart, and the aorta, mesenteric, coronary and renal vessels (Hynynen and Khalil, 2006; Mazzuca and Khalil, 2012; Ohuchi et al., 2000). ET_BR may counteract the effects of ET_AR. For instance, ET_AR stimulates VSMC proliferation, whereas ET_BR inhibits proliferation and promotes cell differentiation (Ohlstein et al., 1992; Schiffrin and Touyz, 1998). ET_AR mediates anti-natriuretic and renal vasoconstrictor effects, while ET_BR promotes natriuresis and renal vasodilation (Nakano et al., 2008; Novak et al., 2004). In blood vessels, ET_AR is largely expressed in VSM, while a significant amount of ET_BR is expressed in the endothelium (Schneider et al., 2007). Also, ET_AR and ET_BR contribute differently to vascular function and hemodynamics, with ET_BR largely believed to mediate vasodilator responses that counteract ET_AR-mediated vasoconstriction (Nakashima and Vanhoutte, 1993; Tirapelli et al., 2005).

However, studies *in vivo* and *ex vivo* studies on isolated blood vessels have shown variable effects of ET_BR stimulation ranging from vasodilation, to vasoconstriction, to both effects. In the anaesthetized rabbit, injection of the ET_BR agonist IRL-1620 into the left ventricle induces a biphasic response; a transient depressor and vasodilator response followed by a rise in blood pressure and systemic vasoconstriction (McMurdo et al., 1994). Also, we have shown that ET-1 induced vasoconstriction is reduced and ET_BR-mediated vasodilation is enhanced in mesenteric microvessels of pregnant rats compared with virgin rats (Mazzuca et al., 2013). IRL-1620 and ET-3 cause endothelium-dependent hyperpolarization and vasodilation in the rat mesenteric artery by activating ET_BR (Nakashima and Vanhoutte, 1993). On the other hand, the ET_BR agonists ET-3 and [Ala1,3,11,15]ET-1 cause contraction in isolated rabbit jugular vein (Sumner et al., 1992). IRL-1620 causes contraction in the aorta of male deoxycorticosterone acetate (DOCA)-salt hypertensive rats (Tostes et al., 2000) and placental arteries from pregnant women (Sand et al., 1998). Also, S6c causes contraction in porcine coronary artery and isolated human internal mammary artery and vein (Seo et al., 1994), and vasoconstriction in endothelium-denuded mesenteric arteries from spontaneously hypertensive and Wistar-Kyoto rats (Touyz et al., 1995a). These variable effects of ET_BR agonists in different vascular preparations could be due to the presence of different ET_BR subtypes or signaling mechanisms in various cell types in the blood vessel. Blood vessels comprise three histologically distinct layers, intima, media and adventitia, containing different cell types including endothelium, VSM and fibroblasts, in addition to connective and adipose tissue, collagen and elastin fibers, and other components of the extracellular matrix. ET-1 also activates ET_AR and possibly ET_BR in VSM to induce vasoconstriction and endothelial ET_BR to induce relaxation. This makes it difficult to determine if the reduced ET-1 constriction previously shown in rat blood vessels during pregnancy (Mazzuca et al., 2013) is solely due to upregulation of endothelial ET_BR or could also involve down-regulation of VSM ET_AR and ET_BR. One approach to address this point is to study ET-1 constriction in endothelium-denuded vessels. Experiments on freshly

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isolated VSMCs could provide a more stringent approach to circumvent this difficulty and test the effects of ET-1 and the specific role of VSM ET_AR and ET_BR in the absence of any contributing factors from the endothelium, fibroblasts and other cell types, or connective tissue, extracellular matrix and other components of the vascular wall.

Using modulators of ET_AR and ET_BR we attempted to dissect the ET_AR-dependent responses from ET_BR-dependent contraction in isolated aortic VSMCs. ET-1 induced contraction in VSMCs of virgin rats appeared to involve both ET_AR and ET_BR because: 1) ET-1 induced contraction was significantly reduced in the presence of the ET_AR antagonist BQ123, 2) ET-1 induced contraction was also reduced, although to a lesser extent, by the ET_BR antagonist BQ788, 3) ET-1 induced contraction was almost abolished in the presence of both ET_AR and ET_BR antagonists, and 4) The ET_BR agonists IRL-1620 and S6c caused significant contraction in VSMCs of virgin rats.

ET_AR-mediated aortic VSMC contraction appeared to decrease during the course of pregnancy because in VSMCs treated with the ET_BR antagonist BQ788, ET-1 induced contraction was still reduced in late-Preg compared with mid-Preg and virgin rats. Although ET_BR-mediated VSMC contraction was much smaller (~1/3) than that mediated by ET_AR, it also decreased during pregnancy. This is supported by the observations that: 1) in VSMCs pretreated with the ET_AR antagonist BQ123, ET-1 contraction was significantly reduced in cells of late-Preg compared with mid-Preg and virgin rats, 2) VSMC contraction in response to the ET_BR agonist IRL-1620 was significantly reduced in late-Preg compared with mid-Preg and virgin rats, and 3) VSMC contraction in response to the ET_BR agonist S6c was similarly reduced in late-Preg compared with mid-Preg and virgin rats.

One possible explanation for the pregnancy-associated reduction of ET_AR-mediated VSMC contraction is pregnancy-related downregulation of ET_AR. Using RT-PCR analysis, we detected little change in ET_AR mRNA expression in late-Preg rats. Although Western blots revealed prominent ET_AR immunoreactivity, we could not detect significant changes in the protein amount of ET_AR in whole tissue homogenates from late-Preg compared with virgin rats. On the other hand, our immunohistochemistry analysis revealed that ET_AR was mainly localized in the tunica media and the immunofluorescence experiments demonstrated significant ET_AR staining in VSMCs, consistent with a role of ET_AR in vascular contraction. The ET_AR immunohistochemistry staining of the tunica media and VSM layer was significantly reduced in aortic sections of late-Preg compared with mid-Preg and virgin rats. Also, ET_AR immunofluorescence signal was less in VSMCs of late-Preg than mid-Preg and virgin rats. The significant decrease of ET_AR observed in aortic media as indicated by immunohistochemistry and in VSMCs as indicated by immunofluorescence despite the lack of changes in ET_AR mRNA expression in RT-PCR and protein amount in Western blot experiments could be related to differences in the sensitivity of the assays. The differences could also be related to the specific location of ET_AR whereby the immunohistochemistry and immunofluorescence studies measure ET_AR in aortic media and VSMCs, while the RT-PCR and Western blots measure ET_AR in whole tissue homogenate including different layers and cell types in the blood vessel.

The differences in the results between the whole tissue expression and the tissue and cellular localization approaches were even more apparent with the ET_BR. Although ET_BR-mediated contraction was also reduced in aortic VSMCs of late-Preg rats, RT-PCR analysis revealed an increase in ET_BR mRNA expression, and Western blot analysis revealed an increase in the protein amount of ET_BR in tissue homogenates of endothelium-intact aorta of late-Preg rats. This is likely because the VSMC contraction response largely involves VSM ET_BR, while the RT-PCR and Western blot experiments on whole tissue homogenate may not distinguish between endothelial and VSM ET_BR. To further distinguish between endothelial

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and VSM ET_BR, we performed immunohistochemistry in tissue sections, Western blots on tissue homogenate from endothelial-denuded vessels, and immunofluorescence in single VSMCs. Our immunohistochemistry data showed prominent ET_BR staining in the intima and endothelial cells compared with the tunica media and VSM layer. The prominent ET_BR staining in the intima is consistent with its role in endothelium-dependent relaxation and vasodilation (Nakashima and Vanhoutte, 1993; Novak et al., 2004; Tirapelli et al., 2005). Importantly, the ET_BR signal in the intima was increased in late-Preg compared with virgin rats, suggesting increased endothelial ET_BR expression during pregnancy. In contrast, the amount of VSM ET_BR appears to be reduced during pregnancy because: 1) immunohistochemistry analysis revealed less ET_BR staining in the media of late-Preg than virgin rats, 2) the amount of ET_BR was reduced in tissue homogenate of endothelium-denuded aorta of late-Preg compared with virgin rats, and 3) the average ET_BR immunofluorescence signal was reduced in aortic VSMCs of late-Preg compared with virgin rats.

During the course of pregnancy the distribution of ET_AR and ET_BR appeared to decrease in the cell periphery and to increase in the cytosol and nuclear region, suggesting that the pregnancy-related changes in ET_AR and ET_BR-mediated VSMC contraction may involve receptor redistribution from the cell surface to intracellular compartments. Both ET_AR and ET_BR are known to internalize upon agonist stimulation, and be directed to Rab5 positive early endosomes (sorting endosomes), then targeted to different intracellular fates. ET_AR is directed to the pericentriolar recycling compartment and subsequently reappears at the plasma membrane (Paasche et al., 2001). Ligand-occupied ET_BR is internalized, but instead of recycling like ET_AR it is sorted to the late endosomal/lysosomal pathway for degradation (Oksche et al., 2000). This may explain the ET-1 “clearance” function of not only endothelial ET_BR (Fukuroda et al., 1994; Johnstrom et al., 2005; Kedzierski and Yanagisawa, 2001), but possibly ET_BR in VSMCs (Honore et al., 2005). The decreased total amount and the changes in ET_AR and ET_BR distribution in aortic VSMCs of pregnant rats may reflect increased ET receptor turnover within the different cellular compartments during pregnancy.

An important question is how the changes in ET_AR and ET_BR expression/activity could affect the vascular contraction mechanisms and contribute to the increased systemic vasodilation observed during pregnancy. We have previously shown that vascular contraction to the α-adrenergic agonist Phe is reduced in aortic strips of late-Preg compared with virgin rats (Crews et al., 1999; Khalil et al., 1998). Consistent with these reports we found that VSMC contraction to Phe was reduced in late-Preg compared with virgin rats. Phe is known to stimulate both Ca^{2+} release from the intracellular stores and Ca^{2+} entry from the extracellular space (Khalil and van Breemen, 1988), and the pregnancy-associated decrease in Phe-induced VSMC contraction could be related to reduction in Ca^{2+} mobilization mechanisms. Membrane depolarization by high KCl solution activates Ca^{2+} entry through voltage-gated Ca^{2+} channels (Khalil and van Breemen, 1988), and the observed decrease in KCl contraction in VSMCs of late-Preg rats suggests reduction in these Ca^{2+} entry mechanisms. ET-1 has been shown to stimulate Ca^{2+} entry through plasma membrane channels (Miwa et al., 2005; Peppiatt-Wildman et al., 2007), and the pregnancy-associated reduction in ET-1 induced VSMC contraction may not only be due to decreased ET_AR and ET_BR expression, but could also involve reduction in post ET_AR and ET_BR Ca^{2+} entry pathways. Also, in addition to increasing $[Ca^{2+}]_i$, ET-1 may activate $[Ca^{2+}]_i$ sensitization pathways in VSM (Cain et al., 2002; Schiffrin and Touyz, 1998). The interaction of ET-1 with ET_AR or ET_BR in VSM is coupled to increased inositol 1,3,4-trisphosphate (IP_3) and diacylglycerol (DAG) (Schiffrin and Touyz, 1998). IP_3 releases Ca^{2+} from the intracellular stores, while DAG activates protein kinase C (PKC) (Kanashiro and Khalil, 1998; Nishizuka, 1992), which in turn increases the Ca^{2+} sensitivity of the

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contractile proteins (Khalil et al., 1994; Khalil et al., 1992; Salamanca and Khalil, 2005). ET-1 could also activate Rho-kinase which in turn inhibits myosin light chain phosphatase, and thus increases myosin phosphorylation and VSM contraction (Lee et al., 2004; Somlyo and Somlyo, 2004). Thus the pregnancy-associated reduction in ET-1 induced VSMC contraction could also involve reduction in post ET_AR and ET_BR Ca^{2+} sensitization pathways, and these mechanisms should be further examined in future studies.

An important observation is that the total aortic wall thickness was reduced in late-Preg rats, and the relative intima/total and media/total wall thickness were greater in late-Preg compared with virgin rats. These vascular structural changes are consistent with our previous report (Stennett et al., 2009), and may be related to the pregnancy-associated increases in cardiac output and hemodynamic forces. This may also be related to pregnancy-associated alterations in matrix metalloproteinases and extracellular matrix proteins including collagen and elastin, and the outward hypertrophic remodeling in maternal vessels to allow sufficient perfusion to the uteroplacental circulation and developing fetus (Osol and Mandala, 2009). Whether these structural changes are also related to the changes in the expression and distribution of vascular ET_AR and ET_BR and possible effects on vascular cell growth and proliferation during pregnancy should be further examined. Importantly, while the aortic wall was thinner, the individual cell size was not different in aortic VSMCs from pregnant compared with virgin rats. This could be related to pregnancy-associated changes in hemodynamic forces and pressure on the vessel wall that are eliminated during cell isolation, changes in the number of VSM layers in aortic media, the number and size of other cell types in various layers of the vascular wall, vascular remodeling and changes in connective tissue, collagen and elstin fibers, and other components of the extracellular matrix, and these changes should be examined in future studies.

The present findings in aortic VSMCs should not minimize the role of endothelium-dependent vasodilators in pregnancy-associated vasodilation. Activation of endothelial ET_BR is coupled to the release of vasodilator substances, which cause vascular relaxation. ET_BR could activate eNOS, NO production and the NO-cGMP pathway (Novak et al., 2004; Tirapelli et al., 2005; Verhaar et al., 1998), as well as PGI_2 synthesis and the PGI_2-cAMP pathway (McMurdo et al., 1994); both pathways decrease VSM $[Ca^{2+}]_i$ (Hynynen and Khalil, 2006). ET_BR could also mediate the release of EDHF leading to vascular tissue hyperpolarization, reduction of Ca^{2+} influx into VSM and vascular relaxation (Nakashima and Vanhoutte, 1993; Tirapelli et al., 2005). The observed increase in ET_BR staining in the intima of aortic tissue sections of late-Preg rats is consistent with a role of endothelial ET_BR in pregnancy-associated vasodilation.

The causes of the pregnancy-associated decreases in VSM ET_AR and ET_BR expression/activity are unclear, but could be partly related to the marked hormonal changes that occur during pregnancy. For instance, pregnancy is associated with significant increases in plasma estradiol and estriol levels (Risberg et al., 2009), and estrogen has been shown to decrease ET_AR expression in rabbit aorta (Pedersen et al., 2009) and to regulate ET_BR expression in the hearts of female spontaneously hypertensive rats (Nuedling et al., 2003). Other sex hormones such as progesterone are also elevated during pregnancy (Risberg et al., 2009). Other studies have suggested an emerging role of corpus luteal hormone relaxin on vascular ET-1 levels and ET_BR activity (Conrad, 2011). Studying the effects of sex hormones and other pregnancy-associated hormones on vascular ET_AR and ET_BR expression/activity should represent an important area for future investigations.

We should note that in the presence of both ET_AR and ET_BR antagonists ET-1 caused a small VSMC contraction (~5%). The cause of this minimal contraction is unclear but could be related to the possibility that the maximal doses of antagonists were not sufficient to

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completely block ET_AR and ET_BR. We used peptidic ET_AR antagonist BQ123 and ET_AR antagonist BQ788. Other potent and highly selective non-peptidic modulators of ET_AR and ET_BR have been developed or being developed and their effects on isolated VSMCs should be examined in future studies. Another possibility is that ET-1 may activate an ET receptor subtype that is resistant to ET_AR and ET_BR antagonists. Interestingly, studies suggested the presence of ETcR subtype in Xenopus laevis dermal melanophores (Karne et al., 1993), and while the information on this ET receptor subtype is still scant, its localization and function in blood vessels should be examined. Also, while this study focused on ET receptors, that should not minimize the role of ANG II and adrenergic receptors in the regulation of blood pressure and vascular reactivity during pregnancy, and their contribution relative to ET receptors should be examined using receptor-specific pharmacological and genetic interventions. The present study was also performed on VSMCs from rat aorta because we have established the cell isolation protocol and consistently isolated viable VSMCs with adequate cell yield from this tissue (Ma et al., 2010; Murphy and Khalil, 2000). The observed decrease in ET-1 induced contraction in aortic VSMCs of late pregnant rats is consistent with our report that ET-1 constriction is reduced in intact mesenteric microvessels of pregnant rats (Mazzuca et al., 2013). It would be important to examine if similar changes in the responses to ET-1, IRL-1620 and other ET_BR agonists also occur in VSMCs from mesenteric microvessels, and further clarify the role of ET_BR in the regulation of vascular function and the relative distribution of ET_AR and ET_BR in VSMCs from resistance vessels.

Perspective

ET-1 is a major endothelium-derived factor that activates not only ET_AR and ET_BR in VSM to induce vasoconstriction (Schiffrin and Touyz, 1998; Seo et al., 1994; Sumner et al., 1992), but also endothelial ET_BR to induce the release of vasodilators and promote relaxation (Hynynen and Khalil, 2006; LaDouceur et al., 1993). Unbalanced activation of VSM ET_AR and ET_BR by endogenous ET-1 would stimulate VSM contraction mechanisms (Lee et al., 2004; McNair et al., 2004; Schroeder et al., 2000; Sirous et al., 2001; Smith et al., 2003) and may lead to increased vasoconstriction and hypertension (Schiffrin, 2001). The present observation that ET-1 induced aortic VSMC contraction via ET_AR and ET_BR is reduced in late pregnant rats, is consistent with our report that ET-1 induced constriction was reduced in mesenteric vessels of pregnant rats (Mazzuca et al., 2013). Together these findings suggest that the reduced VSM ET_AR and ET_BR activity may play a role in the reduced vasoconstriction, enhanced vasodilation and increased blood flow during normal pregnancy. An imbalance between ET_AR and ET_BR expression/activity may be associated with vascular complications during pregnancy. Preeclampsia is a major complication of pregnancy characterized by hypertension and proteinuria, and is a major cause of maternal and fetal morbidity and mortality (Khalil and Granger, 2002; Roberts et al., 2003; Stennett and Khalil, 2006). Although the vascular mechanisms of preeclampsia are unclear, studies have suggested a role of various vasoactive factors and vascular mediators such as ET-1 in hypertension of pregnancy (Alexander et al., 2001; LaMarca et al., 2005). Whether VSM ET_AR and ET_BR expression/activity are altered and the ET-1 induced vasoconstriction is enhanced in pregnancy-associated disorders such as hypertension-in-pregnancy and preeclampsia should be examined in future studies. ET_AR antagonists, and perhaps specific VSM ET_BR antagonists as they become available, could be useful in reducing the vasoconstriction, and when combined with increasing the activity of endothelial ET_BR could further enhance vasodilation and prevent the increases in blood pressure in preeclampsia.

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List of Abbreviations

ANG II	angiotensin II
EDHF	endothelium-derived hyperpolarizing factor
ET-1	endothelin-1
ET_AR	endothelin receptor type A
ET_BR	endothelin receptor type B
late-Preg	late-pregnant
mid-Preg	mid-pregnant
NO	nitric oxide
PGI_2	prostacyclin
Phe	phenylephrine
PKC	protein kinase C
S6c	sarafotoxin 6c
VSMC	vascular smooth muscle cell

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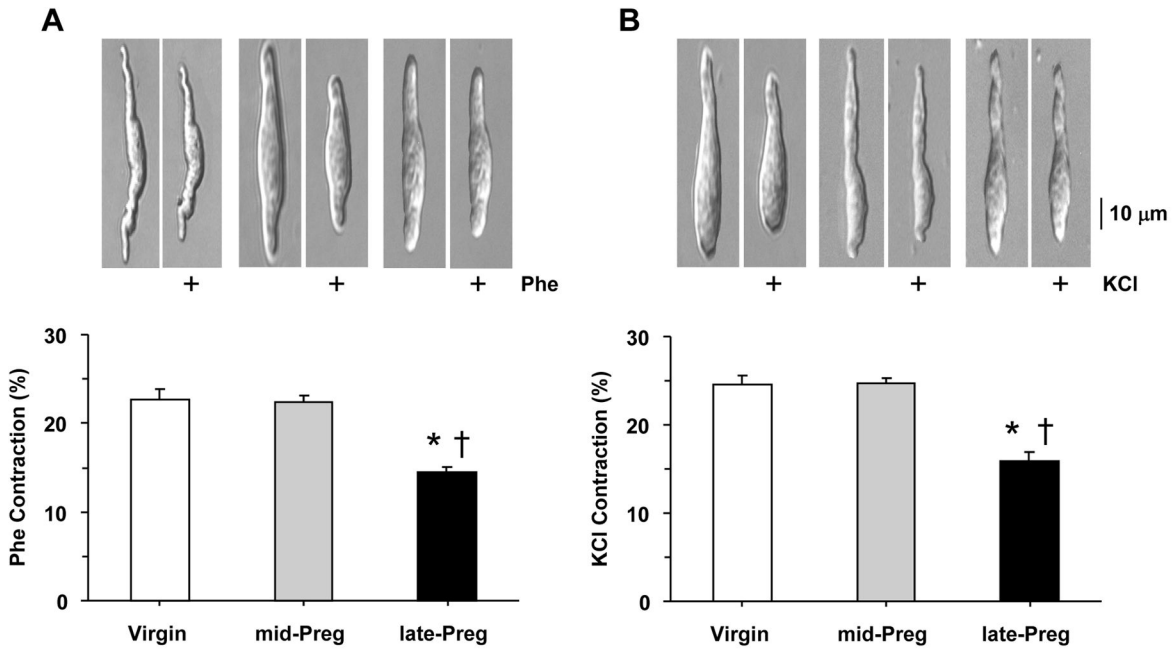

Fig. 1.

Effects of Phe and KCl on contraction of aortic VSMCs from virgin and pregnant rats. Freshly isolated aortic VSMCs from virgin, mid-Preg (day 12) and late-Preg rats (day 19) were viewed on the stage of a Nikon microscope and images of the single cells at rest were first acquired. The cells were then stimulated with Phe (10^{-5} M) (A) or KCl (51 mM) (B) for 10 min and the cell contraction was measured. Each representative cell is presented in two images, at rest and plus Phe or KCl. Cumulative data from different VSMCs isolated from 7 to 12 different rats were presented as means±SD in bar graphs.

* Significantly different ($p<0.05$) from corresponding measurements in virgin rats.

† Significantly different ($p<0.05$) from corresponding measurements in mid-Preg rats.

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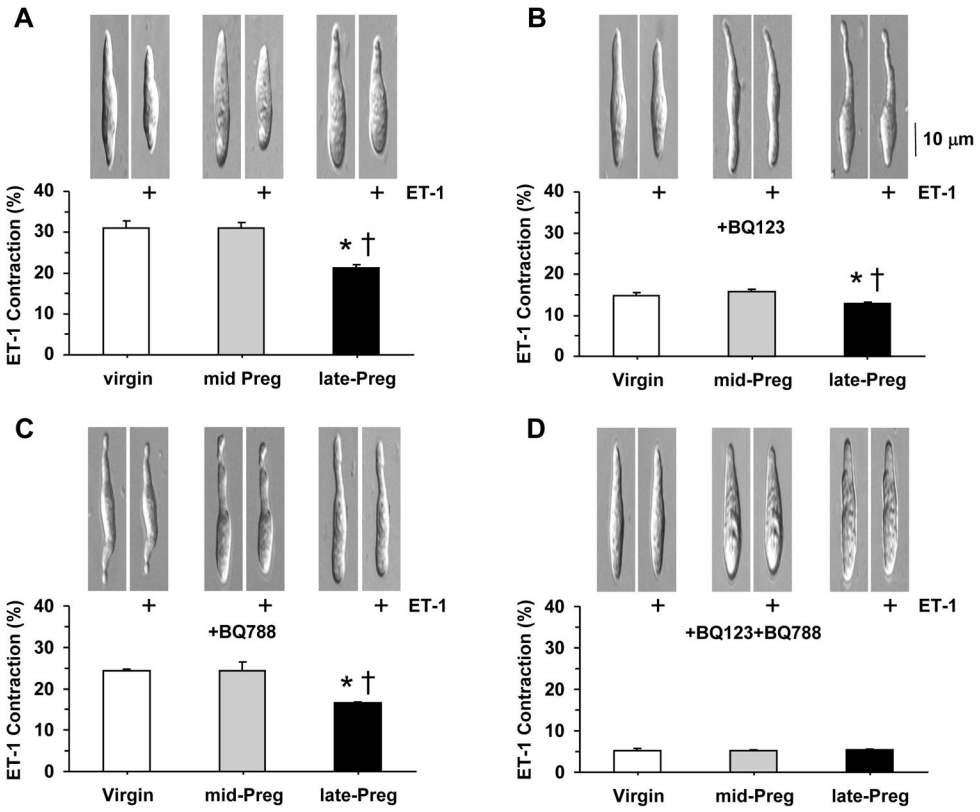

Fig. 2.

Effects of modulators of ET_AR and ET_BR on ET-1 induced contraction in aortic VSMCs from virgin and pregnant rats. Aortic VSMCs of virgin, mid-Preg, and late-Preg rats were stimulated with ET-1 (10^{-6} M) in the absence (A), or presence of ET_AR antagonist BQ123 (10^{-6} M) (B), ET_BR antagonist BQ788 (10^{-6} M) (C), or BQ123+BQ788 (D) and the cell contraction was measured. Each representative cell is presented in two images, at rest and after stimulation with ET-1 for 10 min. Cumulative data from different VSMCs isolated from 6 to 12 different rats were presented as means±SD in bar graphs.

* Significantly different ($p<0.05$) from corresponding measurements in virgin rats.

† Significantly different ($p<0.05$) from corresponding measurements in mid-Preg rats.

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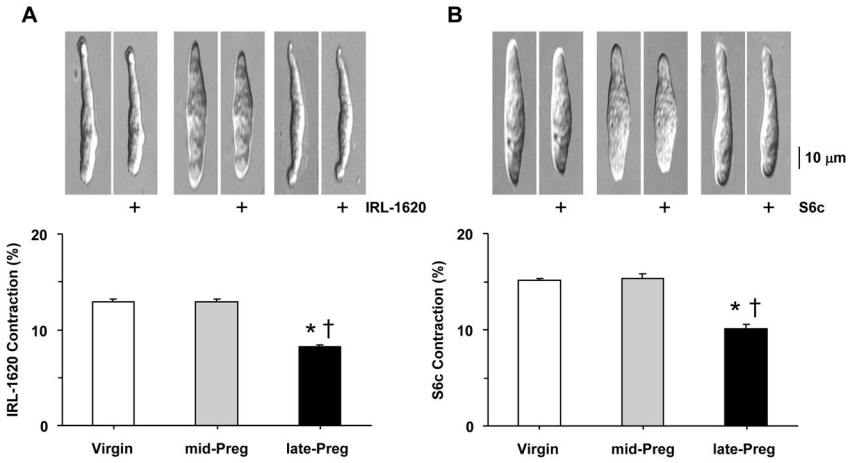

Fig. 3.

Effects of ET_BR agonists on contraction of aortic VSMCs from virgin and pregnant rats. Aortic VSMCs from virgin, mid-Preg, and late-Preg were stimulated with the ET_BR agonist IRL-1620 (10^{-6} M) (A) or S6c (10^{-6} M) (B) for 10 min and the cell contraction was measured. Each representative cell is presented in two images, at rest and after stimulation with IRL-1620 or S6c. Cumulative data from different VSMCs isolated from 7 to 11 different rats were presented as means±SD in bar graphs.

* Significantly different ($p<0.05$) from corresponding measurements in virgin rats.

† Significantly different ($p<0.05$) from corresponding measurements in mid-Preg rats.

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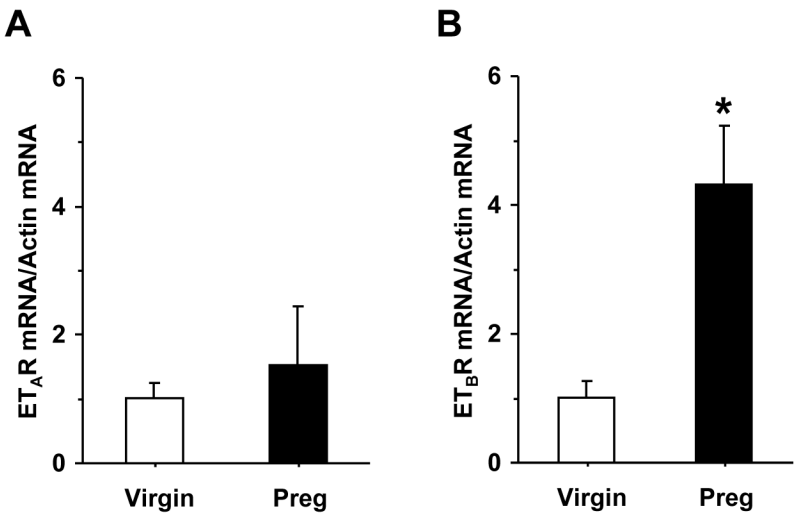

Fig. 4.

ET_AR and ET_BR mRNA expression in aortic tissue homogenate of virgin and pregnant rats. Real time RT-PCR was used to measure mRNA expression of ET_AR (A) and ET_BR (B) in aortic tissue homogenate of virgin and late-Preg rats. Measurements were normalized to the house keeping gene actin. Data represent means±SD (n = 4). * $p<0.05$.

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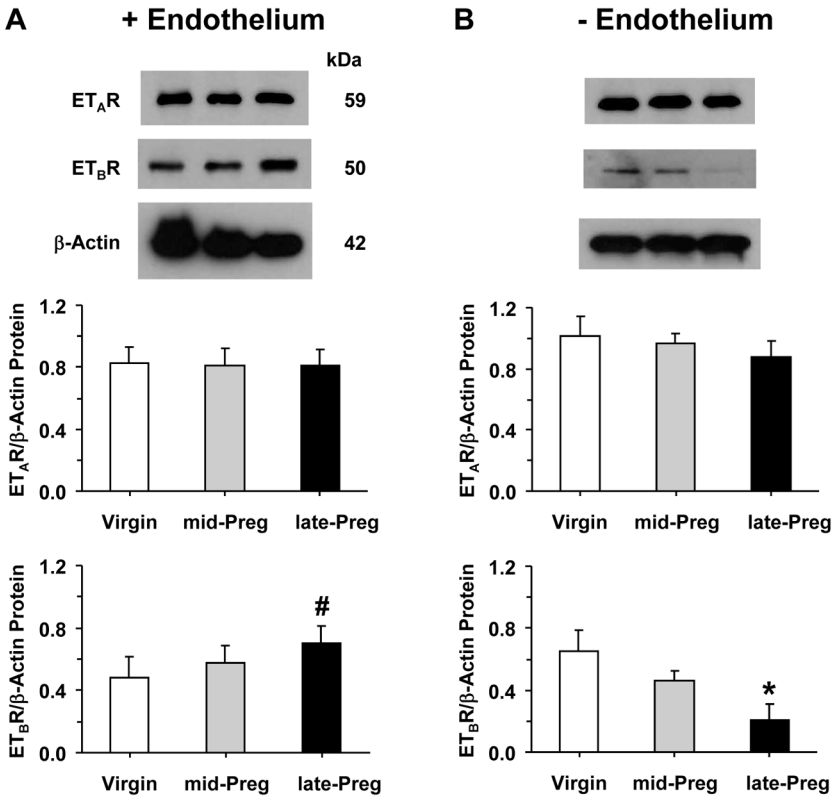

Fig. 5.

Protein amount of ET_AR and ET_BR in aortic tissue homogenate from virgin and pregnant rats. Tissue homogenate from intact (+Endothelium) (A) and endothelium-denuded (−Endothelium) aorta (B) isolated from virgin, mid-Preg and late-Preg rats were prepared for Western blot analysis using antibodies to ET_AR and ET_BR. The optical density of the immunoreactive bands corresponding to ET_AR and ET_BR was measured and normalized to the house-keeping protein actin. Data represent means±SD (n=5).

* Significantly reduced ($p<0.05$) compared with corresponding measurements in virgin rats.

Significantly greater ($p<0.05$) than corresponding measurements in virgin rats.

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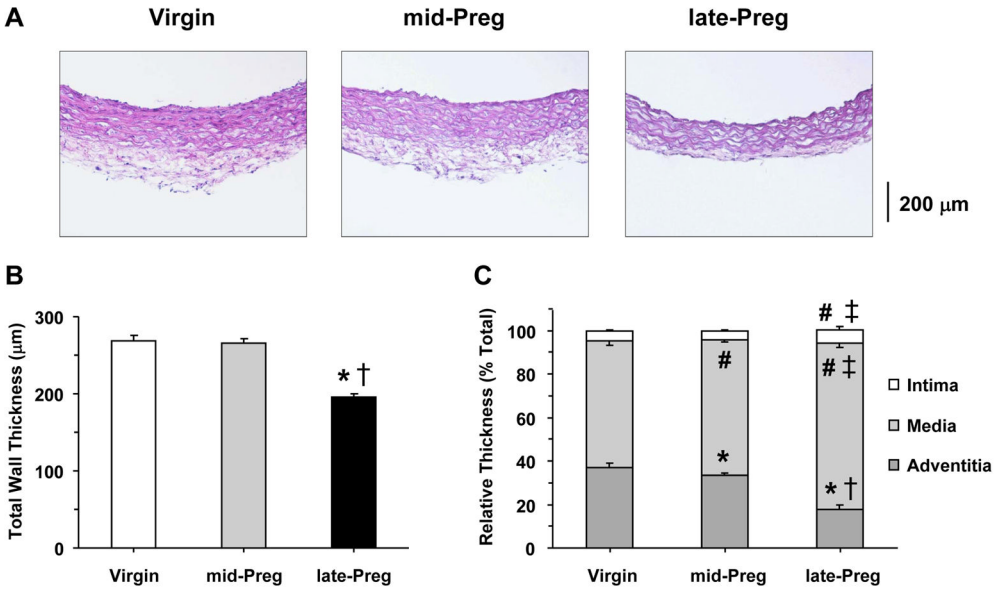

Fig. 6.

Histology of aorta from virgin and pregnant rats. Cryosections (6 μm) of aortic rings from virgin, mid-Preg and late-Preg rats were stained with hematoxylin & eosin (A). Total aortic wall thickness was measured (B). The aortic wall layers intima, media and adventitia were defined and their thickness was presented as % of total wall thickness (C). Total magnification = 200x. Data represent means±SD, n=6. * $p<0.05$.

* Significantly reduced ($p<0.05$) compared with corresponding measurements in virgin rats.

† Significantly reduced ($p<0.05$) compared with corresponding measurements in mid-Preg rats.

Significantly greater ($p<0.05$) than corresponding measurements in virgin rats.

‡ Significantly greater ($p<0.05$) than corresponding measurements in mid-Preg rats.

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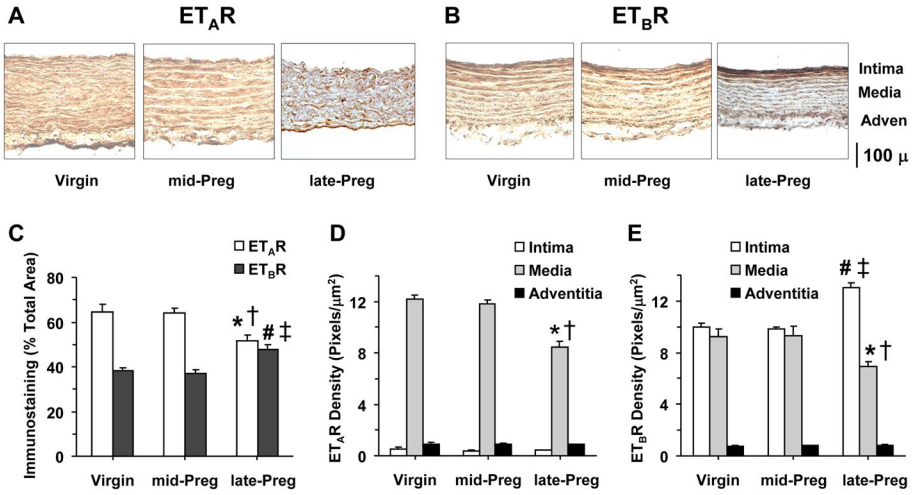

Fig. 7.

Distribution of ET_AR and ET_BR in aorta of virgin and pregnant rats. Cryosections (6 μm) of aortic rings from virgin, mid-Preg and late-Preg rats were labeled with ET_AR (A) or ET_BR antibody (B), immunostained with ABC Elite Kit and counterstained with hematoxylin. Images of tissue sections were acquired then analyzed using ImageJ software. The total number of pixels in the tissue section image was first defined, then the number of brown spots (pixels) was counted and presented as % of total pixels (C). The number of pixels in the specific vascular layer (intima, media and adventitia) was also defined and transformed into the area in μm^2 using a calibration bar. The number of brown spots (pixels) representing ET_AR (D) and ET_BR (E) in each vascular layer was then counted and presented as number of pixels/μm^2. Data represent means±SD, n=4 to 5.

* Significantly reduced ($p<0.05$) compared with corresponding measurements in virgin rats.

† Significantly reduced ($p<0.05$) compared with corresponding measurements in mid-Preg rats.

Significantly greater ($p<0.05$) than corresponding measurements in virgin rats.

‡ Significantly greater ($p<0.05$) than corresponding measurements in mid-Preg rats.

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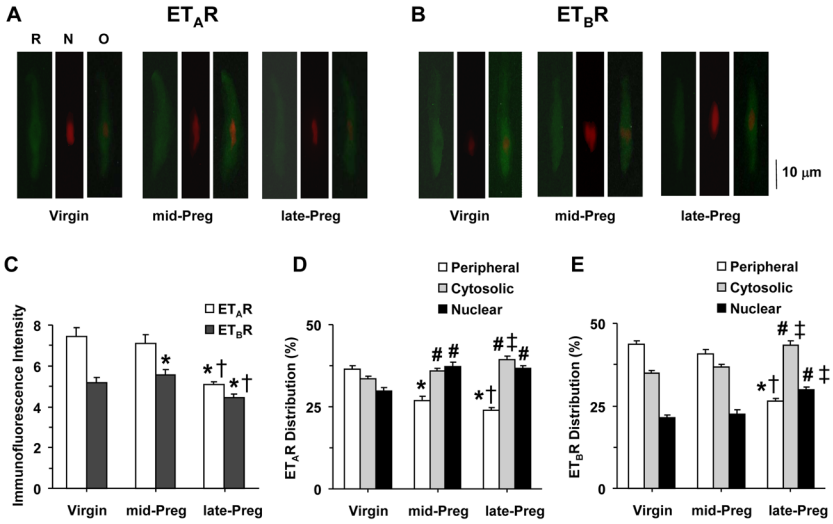

Fig. 8.

Immunofluorescence of ET_AR and ET_BR in VSMCs from virgin and pregnant rats. Aortic VSMCs from virgin, mid-Preg and late-Preg rats were fixed, permeabilized and labeled with primary antibodies to ET_AR (A) and ET_BR (1:500) (B), and FITC-labelled IgG (Green). Images of fluorescently-labeled cells were acquired and analyzed using ImageJ. Total cell fluorescence was divided by the number of pixels in the cell image, and the average cell fluorescence was measured (C). The center of the cell was determined by co-staining with the DNA marker ethidium dimer (Red). The images of ET receptor and ethidium dimer were merged in order to determine the overlay area of the specific ET receptor in the nuclear region and presented in yellow. The cell image was then dissected into 3 regions: the cell surface, the center or nuclear region, and the cytosol [total fluorescence − (peripheral +central fluorescence)] and the average pixel intensity in each region was measured. Data represent means±SD of measurements in different cells from 4 to 6 different rats.

R, ET Receptor; N, Nuclear; O, Overlay

* Significantly reduced ($p<0.05$) compared with corresponding measurements in virgin rats.

† Significantly reduced ($p<0.05$) compared with corresponding measurements in mid-Preg rats.

Significantly greater ($p<0.05$) than corresponding measurements in virgin rats.

‡ Significantly greater ($p<0.05$) than corresponding measurements in mid-Preg rats.