Downregulation of Microvascular Endothelial Type B Endothelin Receptor is a Central Vascular Mechanism in Hypertensive Pregnancy

(Methods Supplement)

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Animals

Time-pregnant (day 11) Sprague-Dawley rats (12 week of age) were purchased from Charles River Laboratories (Wilmington, MA). The rats were housed in the animal facility and maintained on ad libitum standard rat chow and tap water in 12-hr light-dark cycle. On day 13 of pregnancy, rats were either sham-operated (Norm-Preg) or underwent surgical procedure to reduce uteroplacental perfusion pressure (RUPP) by placing a silver clip (0.203 mm ID) on lower abdominal aorta above iliac bifurcation and two clips (0.1 mm ID) on uterine branches of the ovarian artery.\(^1\)\(^-\)\(^3\) This procedure reduces uterine perfusion pressure in the gravid rat by $\approx 40\%$.\(^4\) While the timing of surgery was slightly earlier, these procedures produced comparable results to those reported in Sprague-Dawley rats from either Charles River Laboratories\(^2\),\(^5\),\(^6\) or Harlan Inc (Indianapolis, IN)\(^1\),\(^3\) when the RUPP surgery was performed on day 14 of pregnancy and blood pressure measured on day 19. However, the timing of surgery in relation to the actual date of measurement of blood pressure and other maternal and fetal parameters is important and could have potential implications as other studies showed that when the RUPP surgery was performed on day 12 and blood pressure measured on day 17 a milder form of hypertension was observed.\(^6\) RUPP rats in which the clipping procedure resulted in maternal death or total reabsorption of the fetuses were excluded from the data analyses.

To test the role of ET\(_B\)R in HTN-Preg, a separate group of pregnant rats (day 14) were infused with the ET\(_B\)R antagonist BQ-788 100 µg/kg/day subcutaneously for 5 days using osmotic minipump. Also, some of the RUPP rats were infused with the ET\(_B\)R agonist IRL-1620 100 µg/kg/day subcutaneously for 5 days using osmotic minipump.\(^7\) All surgical procedures were performed using aseptic technique and proper anesthetics and analgesics in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, and the guidelines of the Harvard Medical Area Standing Committee on Animals.

Measurement of Arterial Pressure

On day 19 of pregnancy, rats were anaesthetized with isoflurane, a PE-50 catheter was inserted in the carotid artery, and exteriorized at the back of the neck. The rats were allowed to recover from anesthesia for at least 1 hr. The carotid arterial catheter was connected to a pressure transducer attached to an amplifier and blood pressure (BP) recorder (Living System Instrumentation, Burlington, VT). BP in conscious rats was recorded every 20 min for 1 hr and the average mean arterial BP was measured.\(^8\),\(^9\)

Tissue Preparation

After measuring BP, rats were euthanized by inhalation of CO\(_2\), the uterus was excised, and the litter size and individual pup weight were recorded. Also, the 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 third order mesenteric microvessels outside diameter (OD) $\leq 300$ µm were carefully isolated and cleaned of fat and adipose tissue and used for microvascular functional studies. Similar size microvessels have been considered as resistance arteries in other studies\(^10\) and have been reported to contribute to peripheral vascular resistance in conscious rats\(^11\) and to show pregnancy-associated alterations in reactivity.\(^2\) Attempts were made to dissect the microvessels as close as possible to the wall of the gut with the aim of isolating at least a 4-5 mm-long complete vessel segment, specifically avoiding injuring or having any branches or holes in the microvessel, which would interfere with the vessel pressurization. The remainder of mesenteric vessels were used to measure nitrate/nitrite production or stored at -80°C for Western blots. The thoracic aorta was also excised for measurement of nitrate/nitrite production and RT-PCR.

Microvessel Cannulation and Pressurization
Mesenteric microvessels ~4-5 mm in length were transferred to a 5 ml temperature-controlled perfusion chamber, mounted between two glass micropipettes (cannulas), and secured with 10–0 ophthalmic nylon monofilament (Living Systems Instrumentation) as previously described.\(^5\),\(^12\) One end of the microvessel was mounted on the first glass micropipette and the lumen was gently rinsed with Krebs solution to remove any remaining blood, then the other end of the microvessel was mounted on the second cannula and tied in place. The microvessel segment in the perfusion chamber was placed on an inverted microscope (TE300, Nikon, Melville, NY). A stopcock located distal to the vessel was closed, the proximal end was connected to a pressure transducer and pressure servo control system (Living Systems Instrumentation). The microvessel was pressurized under 60 mmHg and maintained at constant pressure using the pressure-servo control unit.\(^5\),\(^12\) The microvessel was bathed in 5 ml of Krebs bubbled with 95% O\(_2\) and 5% CO\(_2\) at 37°C and was continuously superfused with fresh Krebs at a rate of 1 ml/min using a peristaltic mini-pump (Master-Flex; Cole-Parmer, Vernon Hills, IL). The microvessel 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 acetylcholine (ACh 10\(^{-5}\) M). Microvessels were unacceptable 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) and digitized at 1 Hz using a personal computer as previously described.\(^5\),\(^12\) 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).

**Fura-2 Loading and [Ca\(^{2+}\)] Recording**

For measurement of intracellular free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]), microvessels were incubated in Krebs solution containing the cell permeable Ca\(^{2+}\) indicator fura-2/AM (5 \(\mu\)M) and the mild detergent cremophor EL (0.25%) for 1 hr as previously described.\(^5\),\(^12\) The microvessel was washed 3 times in Krebs to remove extracellular fura-2/AM and incubated in normal Krebs for an additional 30 min to allow for de-esterification 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, and the emitted light was collected at 510 nm every 1 sec and the fluorescence signal was measured using Felix Fluorescence data acquisition and analysis software (Photon Technology International, Birmingham, NJ). The 340/380 ratio was calculated and represented the changes in [Ca\(^{2+}\)]. The signal-to-noise ratio was improved by averaging 10 consecutive 340/380 fluorescence ratio readings.

**Simultaneous Measurement of Microvessel Diameter and [Ca\(^{2+}\)]**

In all experiments, the microvessel from Norm-Preg or RUPP rats was first stimulated with 51 mM KCl and the simultaneous changes in constriction and 340/380 ratio (indicative of [Ca\(^{2+}\)]) were recorded. Our initial experiments demonstrated that KCl- and Phe-induced changes in [Ca\(^{2+}\)] were rather small. Also, 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\(^{2+}\)] measurements. Therefore, to accurately compare the [Ca\(^{2+}\)]-dependent constriction induced by various agonists, we used maximal agonist concentration and an 8-min exposure time. The maximal concentrations of ET-1 (10\(^{-7}\) M), Phe (10\(^{-5}\) M) and KCl (51 mM) used were based on previous reports from our laboratory.\(^5\),\(^12\) To measure the effects of ET-1, Phe and KCl on the Ca\(^{2+}\) sensitivity of VSM contractile proteins, we calculated the ratio of
vasoconstriction/ (Δ change in [Ca^{2+}]), i.e. stimulated [Ca^{2+}] – basal [Ca^{2+}]) as previously described. Dose-dependent microvascular constriction to ET-1 (10^{-11}-10^{-7} M), Phe (10^{-9}-10^{-5} M) and KCl (16 to 96 mM) were also measured. We should note that the plasma ET-1 levels are in the range of 3 to 12 pmol/L in Norm-Preg women and 6 to 23 pmol/L in preeclamptic women. However, the endogenous ET-1 released from the endothelium mainly functions as an autocrine or paracrine factor on the ET_{b}R and ET_{a}R receptors on the adjacent endothelial and VSM cells. Because ET-1 secretion is directional, with a larger proportion of the peptide being released on the basolateral side of the endothelium, ET-1 levels in the circulation may not accurately reflect the tissue levels of ET-1. To block ET_{b}R-mediated relaxation, in some experiments, microvessels were pretreated with the ET_{b}R antagonist BQ-788 (10^{-6} M), and the ET-1 induced ETAR-mediated vasoconstriction and [Ca^{2+}] were measured.

To test for endothelial function, microvessels were submaximally preconstricted (~70% of maximum) with Phe (6×10^{-6} M), then stimulated with increasing concentrations of ACh (10^{-9}-10^{-5} M) added cumulatively 2 min for each concentration, and the simultaneous changes in microvessel diameter and [Ca^{2+}] (340/380 ratio) were recorded. Phe (6×10^{-6} M) produced similar preconstriction in microvessels from Norm-Preg and RUPP rats. To test ET_{b}R-mediated relaxation, microvessels were pretreated with the ET_{a}R antagonist BO-123 (10^{-6} M) for 30 min to block ET_{a}R-mediated vasoconstriction, preconstricted with Phe (6×10^{-6} M), then stimulated with increasing concentrations of ET-1 (10^{-12}-10^{-7} M). To further test ET_{b}R-mediated relaxation, mesenteric microvessels were preconstricted with Phe (6×10^{-6} M) then stimulated with increasing concentrations of the ET_{b}R agonist sarafotoxin 6c (S6c, 10^{-12}-10^{-7} M) or IRL-1620 (10^{-12}-10^{-7} M). The various agonist concentrations were 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 Nω-nitro-L-arginine methyl ester (L-NAME, 3×10^{-4} M) to block nitric oxide synthase (NOS) and indomethacin (INDO, 10^{-6} M) to block cyclooxygenase (COX). Previous studies have shown minimal contribution of PGI_{2} to vasodilation in rat mesenteric arteries. Therefore, in all experiments, both NOS and COX activity 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, in some experiments 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 preconstricted with Phe (6×10^{-6} M), then stimulated with increasing concentrations of the exogenous NO donor sodium nitroprusside (SNP, 10^{-9}-10^{-5} M), applied cumulatively, allowing each relaxation to plateau between successive doses, and the simultaneous changes in microvessel diameter and [Ca^{2+}], (340/380 ratio) were recorded.

Nitrate/Nitrite (NOx) Measurement

Endothelium-intact thoracic aorta or mesenteric artery segments were placed in 2 ml Krebs solution aerated with 95% O_{2} 5% CO_{2} at 37°C for 30 min, and samples for basal accumulation of nitrite (NO_{2}⁻) formed from released NO were taken. Vascular segments were treated with ACh (10^{-5} M), BQ-123+ET-1 (10^{-7} M), S6c (10^{-7} M) or IRL-1620 (10^{-7} M) for 30 min, then rapidly removed, dabbed dry with filter paper and weighed. The incubation solutions were assayed for the stable end product of NO, NO_{2}⁻. Samples of incubation solution (50 μl, in triplicate) were mixed in 96-well microplate with 100 μl Griess reagent. The chromophore generated from the
reaction with NO$_2^-$ was detected spectrophotometrically (535 nm) using SpectraMAX microplate reader (Molecular Devices, Sunnyvale CA). The concentration of NO$_2^-$ was calculated using a calibration curve with known concentrations of NaNO$_2$.\textsuperscript{1}

**Real-Time RT-PCR Analysis**

We could not measure mRNA in mesenteric microvessels, and therefore used the thoracic aorta for RT-PCR analysis. Because Western blots require large amount of protein, we dissected the whole mesenteric arterial arcade in order to collect sufficient tissue from each animal for the protein analysis. Also, in order to delineate endothelial vs. VSM ET$_B$R, we divided the mesenteric vessels into endothelium-intact and -denuded mesenteric vessels. Because of the intricate mesenteric arterial tree, the dissection of mesenteric arteries from adipose tissue and adjacent veins is somewhat tedious and takes a long time and therefore could interfere with the integrity of the rapidly degrading mRNA. Because the aorta is easier to isolate and dissect, we opted to use the aorta to rapidly collect sufficient tissue with intact RNA for the mRNA measurements. Total RNA was extracted from thoracic aorta 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 preproET, ET$_A$R and ET$_B$R, and 1:25 for $\alpha$-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 preproET, ET$_A$R and ET$_B$R (Integrated DNA Technologies (IDT), Coralville, IA), and the Bio-Rad iQ SYBR Green Supermix for amplicon detection (Bio-Rad, Hercules, CA). $\alpha$-Actin primer was included in the RT-PCR reaction as internal standard to normalize the results.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>PreproET</td>
<td>Forward 5'- GAGGCCATCAGCAACAGCATCA -3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'- TCCGAGGCCCATCCCCAGAC -3'</td>
</tr>
<tr>
<td>ET$_A$R</td>
<td>Forward 5'- CAGCCTGGCCCTTGGAGACCTTAT -3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'- TTCTGTGCTGCTGCCCTTGTATT -3'</td>
</tr>
<tr>
<td>ET$_B$R</td>
<td>Forward 5'- GATACGACAACCTCCGCTCCA- 3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'- GTCCAGGATGAGGACAATGAG- 3'</td>
</tr>
<tr>
<td>$\alpha$-actin</td>
<td>Forward 5'- GACACCGGGATGTTGGA-3'</td>
</tr>
<tr>
<td></td>
<td>Reverse 5'- GTTAGCAAGGTCGGATGTC-3'</td>
</tr>
</tbody>
</table>

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. Relative quantification of gene expression was performed by the comparative CT ($\Delta\Delta$CT) method with $\alpha$-actin as endogenous control as previously described.\textsuperscript{18}

**Western Blot Analysis**

Three samples of endothelium-intact mesenteric arteries were prepared; each sample containing arteries pooled from 4 rats per group. In other samples, the endothelium was removed by passing air bubbles through the lumen of the artery while mounted in the arterial chamber or by pinning down the excised mesenteric arteries in a petri dish, inserting a thin wire (~150 µm ID) into the vessel lumen and carefully rubbing the vessel 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). The homogenate was centrifuged at 10,000 g for 5 min. The supernatant was collected, and protein concentration was determined using a protein assay kit (Bio-Rad). 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. Membranes were incubated in 5% nonfat dried milk in TBS-Tween for 1 hr and then overnight at 4°C with rabbit polyclonal anti-ET AR antibody (sc-33536) or anti-ET BR antibody (sc-33538) (1:1000; Santa Cruz Biotechnology, Dallas, TX). Negative control experiments were performed with the omission of primary antibody, and showed no detectable immunoreactive bands. Membranes were washed 5 times 15 min each in TBS-Tween then incubated with horseradish peroxidase-conjugated secondary antibody (1:1000) for 1.5 hr, and the immunoreactive bands were detected using enhanced chemiluminescence (ECL) Western blotting detection reagent (GE Healthcare Bio-Sciences, Piscataway, NJ). The blots were subsequently reprobed for β-actin (1:2000). Data were analyzed by optical densitometry and ImageJ software (National Institutes of Health, Bethesda, MD). The densitometry values represented the pixel intensity normalized to β-actin to correct for loading as previously described.12

Histology and Quantitative Morphometry

Morphometric analysis of the dimensions of cannulated pressurized 3rd order mesenteric microvessels using light microscopy showed no significant differences between Norm-Preg and RUPP rats in the microvessel outside diameter (OD Norm-Preg 297±4 vs. RUPP 293±7 μm), lumen or internal diameter (ID, Norm-Preg 266±5 vs. RUPP 259±7 μm), or total wall thickness (Norm-Preg 15.7±1.0 vs. RUPP 16.8±1.2 μm).

To assess if HTN-Preg is associated with adaptive vascular tissue changes in the relative thickness of the intima, media and adventitia, mesenteric arteries from Norm-Preg and RUPP rats were cryopreserved in Tissue-Tek 4583 optimal cutting temperature compound (OCT, Sakura Finetek Inc., Torrance, CA) and stored at -80°C. Because of the difficulty in embedding the small 3rd order microvessels in OCT and in keeping the cylindrical arterial segment upright and the lumen open so that it fills with enough OCT during the embedding process, we used 1st order mesenteric arterial segments upstream in the mesenteric arterial arcade. Transverse 6 μm thick cryosections were placed on glass slides and prepared for staining with hematoxylin and eosin. An equal number of arteries from Norm-Preg and RUPP groups were stained at the same time under identical conditions. Stained sections were coded and labeled in a blinded fashion. Images were analyzed by two independent examiners blinded to the study group. Images were acquired on a Nikon microscope (Nikon, Tokyo, Japan) with digital camera mount, photographed at ×40 magnification using the same light intensity, camera gain and Nikon NIS Elements software, and analyzed using ImageJ software (National Institute of Health). Outlines of the vessel lumen, internal elastic lamina and external microvessel wall were defined and the total wall thickness and relative thickness of the intima, media and adventitia were measured as previously described.19

Detailed histological and morphometric analysis in 1st order mesenteric artery tissue sections of Norm-Preg vs. RUPP rats showed no significant difference in total wall thickness (99±12 vs.79±8 μm) or thickness of tunica intima (6±1 vs. 8±1 μm), media (55±6 vs. 52±3 μm) or adventitia (39±6 vs. 40±2 μm). Although the average total wall thickness in RUPP vessels was roughly 20% less than in Norm-Preg, the SEM was large and the difference did not reach statistical significance (P=0.21).
**Immunohistochemistry**

To determine the tissue distribution of ET receptor subtypes, transverse cryosections (6 μm in thickness) were prepared from OCT embedded mesenteric artery of Norm-Preg and RUPP rats. Immediately before immunostaining, cryosections were thawed and fixed in ice-cold acetone for 10 min and rehydrated in phosphate buffered saline (PBS) containing 0.25% Triton X-100 for 15 min at room temperature. Endogenous peroxidase activity was quenched with 1.5% H₂O₂ (Sigma) in methanol (Sigma) for 10 min, and nonspecific binding was blocked in 10% horse serum (VectaStain Elite ABC Kit, Vector Laboratories, Burlingame, CA) in PBS for 30 min. Tissue sections were incubated with rabbit polyclonal ET AR or ET BR primary antibody (1:100, Santa Cruz Biotechnology) for 1 hr then washed with PBS. Tissue sections were then incubated with biotinylated anti-rabbit secondary antibody for 30 min, rinsed with PBS, then incubated with avidin-labeled peroxidase (VectaStain Elite ABC Kit) for 30 min, followed by a rinse in PBS for 5 min. Positive labeling was visualized using diaminobenzidine (DAB) and appeared as brown spots. Negative control slides were run simultaneously with no primary antibody, and showed no detectable immunostaining. Sections were counterstained with Gill’s hematoxylin for 30 sec and cover slipped with cytoseal 60 mounting medium (8310; Richard-Allen Scientific, Kalamazoo, MI). Images of 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 (NIH).

Total ET AR or ET BR content (visualized as brown spots) was quantified using bright-field illumination and images were background subtracted using ImageJ software. For quantification of ET AR and ET BR immunostaining, 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 area. The number of pixels in the specific vascular layer (intima, media and adventitia) was also defined, then the ET AR or ET BR brown immunostaining was counted and presented as percentage of the respective layer area.¹⁹

**Solution and Drugs**

Krebs solution contained in mM: 120 NaCl, 5.9 KCl, 25 NaHCO₃, 1.2 NaH₂PO₄, 11.5 dextrose, 2.5 CaCl₂, 1.2 MgCl₂, at pH 7.4, and bubbled with 95% O₂ and 5% CO₂. High KCl (51 mM) solution was prepared as Krebs solution with equimolar substitution of NaCl with KCl. Stock solutions of Phe, ACh, SNP, ET-1, L-NAME (10⁻¹ M; Sigma-Aldrich), S6c and IRL-1620 (10⁻³ M; Tocris Bioscience, Islington, MO) were prepared in distilled water. TEA (30 mM, Sigma) was prepared as Krebs solution with equimolar substitution of NaCl with TEA. Stock solution of indomethacin (INDO, 10⁻² M; Sigma), 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) were prepared in DMSO. Further dilutions of BQ-123 or BQ-788 were made either in saline (for in vivo infusion) or in distilled water (for ex vivo experiments). The final concentration of DMSO in the experimental solution was <0.1%. All other chemicals were of reagent grade or better.

**Statistical Analysis**

Experiments were conducted on mesenteric vessels isolated from 4–12 different rats per group, and cumulative data were presented as means±SEM, with the “n” value representing the number of rats per group. Time-dependent constriction was measured as: [(resting diameter – constriction diameter) / resting diameter] X 100. Time-dependent relaxation was measured as [(relaxation diameter – Phe preconstriction diameter) / (resting diameter – Phe preconstriction diameter)] X 100. Concentration-dependent contraction and relaxation were expressed as percentage of maximum response of the specific agonist, concentration-response 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). Data were first analyzed using repeated measures ANOVA. When a statistical difference was observed, data were further analyzed using Bonferroni’s post hoc correction for multiple comparisons. Student’s unpaired t-test was used for comparison of two means. Differences were statistically significant when P < 0.05.

REFERENCES


