Preeclampsia

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

Marc Q. Mazzuca, Wei Li, Ossama M. Reslan, Peng Yu, Karina M. Mata, Raouf A. Khalil

Abstract—Preeclampsia is a pregnancy-related disorder characterized by hypertension with an unclear mechanism. Studies have shown endothelial dysfunction and increased endothelin-1 (ET-1) levels in hypertensive pregnancy (HTN-Preg). ET-1 activates endothelin receptor type-A (ET\(_R\)) in vascular smooth muscle to induce vasoconstriction, but the role of vasodilator endothelium-derived relaxing factor (EDRF) in the changes in blood pressure (BP) and vascular function in HTN-Preg is unclear. To test whether downregulation of endothelial ET\(_R\) expression/activity plays a role in HTN-Preg, BP was measured in normal pregnancy (Norm-Preg) rats and rat model of HTN-Preg produced by reduction of uteroplacental perfusion pressure (RUPP), and mesenteric microvessels were isolated for measuring diameter, [Ca\(^{2+}\)], and endothelin receptor type-A and ET\(_R\) levels. BP, ET-1– and potassium chloride–induced vasoconstriction, and [Ca\(^{2+}\)]\(_i\) were greater in RUPP than in Norm-Preg rats. Endothelium removal or microvessel treatment with ET\(_R\) antagonist BQ-788 enhanced ET-1 vasoconstriction and [Ca\(^{2+}\)]\(_i\) in Norm-Preg, but not RUPP, suggesting reduced vasodilator ET\(_R\) in HTN-Preg. The ET-1+endothelin receptor type-A antagonist BQ-123 and the ET\(_R\) agonists sarafotoxin 6c and IRL-1620 caused less vasorelaxation and nitrate/nitrite production in RUPP than in Norm-Preg. The nitric oxide synthase inhibitor N\(\omega\)-nitro-L-arginine methyl ester reduced sarafotoxin 6c– and IRL-1620–induced relaxation in Norm-Preg but not in RUPP, supporting that ET\(_R\)-mediated nitric oxide pathway is compromised in RUPP. Reverse transcription polymerase chain reaction, Western blots, and immunohistochemistry revealed reduced endothelial ET\(_R\) expression in RUPP. Infusion of BQ-788 increased BP in Norm-Preg, and infusion of IRL-1620 reduced BP and ET-1 vasoconstriction and [Ca\(^{2+}\)]\(_i\) in Norm-Preg, but not RUPP, suggesting reduced vasodilator ET\(_R\) in HTN-Preg. Thus, downregulation of microvascular vasodilator ET\(_R\) is a central mechanism in HTN-Preg, and increasing ET\(_R\) activity could be a target in managing preeclampsia. (Hypertension. 2014;64:632-643.) ● Online Data Supplement

Key Words: calcium ■ endothelins ■ hypertension ■ nitric oxide ■ pre-eclampsia

Normal pregnancy (Norm-Preg) is associated with increased plasma volume and cardiac output, decreased vascular resistance, and slight or no change in blood pressure (BP).\(^1\) Preeclampsia is a major disorder affecting 5% to 8% of pregnancies in the United States and 8 million pregnancies worldwide.\(^2\) Preeclampsia is manifested as maternal hypertension\(^3,4\) and, if untreated, could lead to eclampsia with severe hypertension and seizures. Preeclampsia may be associated with intrauterine growth restriction and could lead to in utero programming of cardiovascular, renal, and metabolic disorders and adult-onset hypertension and diabetes mellitus.\(^3\) Although preeclampsia poses serious consequences to the health of mother and fetus, the mechanisms involved are unclear. Placental ischemia could be an initiating event\(^1\) leading to release of various bioactive factors in the maternal circulation including cytokines, anti-angiogenic factors, angiotensin receptor type-1 agonistic auto-antibody, reactive oxygen species, and hypoxia-inducible factor.\(^6,8\) These circulating factors are thought to cause generalized endotheliosis in the peripheral, glomerular, and cerebral vessels leading to some of the hallmarks of preeclampsia/eclampsia, namely hypertension, proteinuria, and seizures, respectively; however, the central endothelial cell target involved is unclear.

One of the major factors released by endothelium is endothelin-1 (ET-1). ET-1 is a potent vasoconstrictor in some forms of hypertension including hypertensive pregnancy (HTN-Preg).\(^3,10\) Also, plasma ET-1 levels are increased in preeclamptic women.\(^3,11\) Because of the difficulty to perform mechanistic studies in pregnant women, we and others have utilized animal models of HTN-Preg such as the rat model of placental ischemia produced by reduction of uteroplacental perfusion pressure (RUPP).\(^6,8,12\) RUPP rats mimic some of the features of preeclampsia in women, including hypertension, endothelial...
dysfunction, and increased vascular reactivity.\textsuperscript{3,4,12} RUPP rats also exhibit increased expression of preproET mRNA in the renal medulla and cortex.\textsuperscript{13} ET-1 activates endothelin receptor type-A (ET\textsubscript{A}R) and type-B (ET\textsubscript{B}R). ET\textsubscript{B}R is mainly expressed in vascular smooth muscle (VSM) to induce vasoconstriction, whereas ET\textsubscript{A}R is predominantly expressed in endothelial cells to induce the release of vaso dilator substances.\textsuperscript{14,15} and also plays a role in ET-1 clearance.\textsuperscript{16} Although a role of ET-1 and its vasoconstrictor ET\textsubscript{A}R in modulation of cardiovascular-renal function during HTN-Preg has been suggested,\textsuperscript{3,17–19} the role of ET\textsubscript{BR} in HTN-Preg, other pregnant rats were infused with the ETBR agonist IRL-1620 $\mu$g/kg per day subcutaneously for 5 days. All experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. See details in Methods in the online-only Data Supplement.

Animals

Time-pregnant (day 13) Sprague–Dawley rats were either sham-operated (Norm-Preg) or underwent surgical RUPP by placing a silastic catheter connected to a pressure transducer.

Blood Pressure

On day 19 of gestation, BP was measured via a PE-50 carotid arterial catheter connected to a pressure transducer.

Tissue Preparation

Rats were euthanized by CO\textsubscript{2} inhalation, the uterus was excised, and the litter size and individual pup weight were recorded. Third-order mesenteric arteries (≤300 μm OD) were dissected for measuring microvascular function, nitrate/nitrite production, and Western blots. The thoracic aorta was used for measuring nitrate/nitrite production and reverse transcription polymerase chain reaction (RT-PCR).

Measurement of Microvessel Diameter and [Ca\textsuperscript{2+}]i

Mesenteric microvessels were mounted between 2 glass cannulae (Living Systems Instrumentation, Burlington, VT), pressurized under 60 mmHg,\textsuperscript{20,21} and the vessel diameter was measured using automatic edge-detection system. For measurement of [Ca\textsuperscript{2+}], microvessels were incubated in Krebs solution containing fura-2/AM (5 μmol/L) for 1 hour.\textsuperscript{20,21}

Nitrates/Nitrites (NO\textsubscript{x}) Production

Endothelium-intact aortic and mesenteric artery segments were treated with acetylcholine (Ach, 10\textsuperscript{−6} mol/L), BQ-123+ET-1, sарafenoxin 6c (56c), or IRL-1620 (10\textsuperscript{−7} mol/L) for 30 minutes and the incubation solution was assayed for the stable end product of NO, NO\textsubscript{3} using Griess reagent.\textsuperscript{17}

Reverse Transcription Polymerase Chain Reaction

Total RNA was extracted from thoracic aorta using RNeasy Fibrous Tissue Mini Kit (QIAGEN, Valencia, CA), 1 μg total RNA was used for reverse transcription to synthesize single-strand cDNA, and 2 μL of cDNA dilution (1:5 for preproET, ET\textsubscript{A}R and ET\textsubscript{B}R, and 1:25 for α-titulin) was applied to 20 μL RT-PCR reaction. Gene expression was measured using quantitative RT-PCR system (MX4000, Stratagene, La Jolla, CA), published oligonucleotide primers for preproET, ET\textsubscript{A}R, ET\textsubscript{B}R (Integrated DNA Technologies, Coralville, IA), and IQSYBR Green Supermix (Bio-Rad, Hercules, CA). Relative mRNA expression was calculated by the comparative ΔΔCT method, with α-titulin as internal control.\textsuperscript{22}

Western Blots

Microvessels were homogenized and proteins were separated by electrophoresis and transferred to nitrocellulose membranes. Membranes were probed with polyclonal anti-ET\textsubscript{A}R (sc-33536) or anti-ET\textsubscript{B}R antibodies (sc-33538; 1:1000, Santa Cruz Biotechnology, Dallas, TX) at 4°C overnight. Immunoreactive bands were visualized using the ECL detection kit (GE Healthcare Bio-Sciences) and the intensity was normalized to β-actin.\textsuperscript{20}

Immunohistochemistry

Transverse 6-μm-thick cryosections of mesenteric artery were labeled with polyclonal ET\textsubscript{A}R and ET\textsubscript{B}R antibodies (1:100). Vessel images were acquired on a Nikon microscope and analyzed using ImageJ (National Institutes of Health). The total wall thickness, relative thickness of the intima, media and adventitia, and the amount of ET\textsubscript{A}R and ET\textsubscript{B}R in the whole vessel wall and in each layer were measured.

Statistical Analysis

Experiments were conducted on vessels from n=4 to 12 rats per group. Data were presented as means±SEM. Concentration-dependent responses were fitted to sigmoidal curves using the least squares method and pD\textsubscript{2} (−log EC\textsubscript{50}) was calculated using Graphpad Prism 5.01. Data groups were compared with the Student t test for multiple comparisons or Student t test for comparison of 2 means. Differences were statistically significant when $P<0.05$.

Results

On gestation day 19, the mean arterial BP was greater in RUPP (130±3 mmHg) than in Norm-Preg rats (92±6 mmHg). RUPP rats showed smaller litter size (8±1 pups) and average pup weight (1.59±0.12 g) than Norm-Preg (13±1 pups, average weight 2.24±0.25 g). In mesenteric microvessels of Norm-Preg (Figure 1A) and RUPP rats (Figure 1B), ET-1 (10\textsuperscript{−6} mol/L) caused maintained decrease in diameter, associated with an initial peak followed by smaller increase in [Ca\textsuperscript{2+}]i (Figure 1C and 1D). Basal [Ca\textsuperscript{2+}]i was not different in Norm-Preg (0.82±0.06) versus RUPP (0.83±0.03), whereas ET-1–induced vasoconstriction (Figure 1E), [Ca\textsuperscript{2+}]i (Figure 1F), and Δ constriction/Δ [Ca\textsuperscript{2+}]i (Figure 1G) were greater in RUPP than in Norm-Preg. Also, ET-1–induced concentration-dependent constriction showed...
greater maximum constriction in RUPP (86.2±3.8%) than Norm-Preg (58.1±5.2%; P=0.004) with no difference in sensitivity (pD2 RUPP 8.88±0.10 versus Norm-Preg 8.74±0.19; P=0.57; Figure 1H).

To test whether the enhanced vasoconstriction is specific to ET-1 or involves mechanisms shared by other vasoconstrictors, the α-adrenergic receptor agonist phenylephrine (10⁻⁵ mol/L) caused significant decrease in microvessel diameter and an initial spike followed by smaller increase in [Ca²⁺] (Figure 2A and 2B). Cumulative data showed no differences in phenylephrine-induced constriction, [Ca²⁺] or Δ constriction/Δ [Ca²⁺] in microvessels of Norm-Preg and RUPP (Figure 2A and 2B). Also, phenylephrine (10⁻⁵–10⁻⁴ mol/L) caused concentration-dependent constriction that was not different in vessels of RUPP (Eₘₐₓ=56.3±3.9%; pD₂=6.55±0.30) and Norm-Preg (Eₘₐₓ=49.5±2.8%; pD₂=6.59±0.33; Figure 2).

High KCl is known to cause membrane depolarization and to stimulate Ca²⁺ entry. In microvessels of Norm-Preg (Figure 2C) and RUPP rats (Figure 2D), KCl (51 mmol/L) caused maintained decrease in diameter and an initial spike followed by maintained [Ca²⁺]. Potassium chloride (KCl)-induced constriction and [Ca²⁺] were greater in RUPP than in Norm-Preg, but Δ constriction/Δ [Ca²⁺] was not different in Norm-Preg and RUPP (Figure 2). Also, KCl (16–96 mmol/L) caused concentration-dependent vasoconstriction that was greater in microvessels of RUPP (Eₘₐₓ=79.3±5.0%) than of Norm-Preg (Eₘₐₓ=65.3±4.9%; Figure 2).

To test the role of endothelium, ET-1–induced constriction and [Ca²⁺] were enhanced in endothelium-denuded versus intact microvessels of Norm-Preg (Figure 3A and 3C) but not RUPP (Figure 3B and 3D), suggesting that endothelial function is already compromised in RUPP. Phenylephrine- and KCl-induced constriction and [Ca²⁺] were not different in endothelium-denuded versus intact vessels of Norm-Preg or RUPP (data not shown), supporting that the effects of endothelium removal are specific to an ET-1–stimulated receptor/signaling pathway. ETₐR antagonist BQ-788 (10⁻⁶ mol/L) enhanced ET-1 constriction and [Ca²⁺] in microvessels of Norm-Preg (Figure 3E and 3G) but not RUPP (Figure 3F and 3H), suggesting an intact vasodilator ETₐR activity in Norm-Preg rats that is reduced in RUPP.

To test further the role of endothelium, in microvessels of Norm-Preg (Figure 4A) and RUPP (Figure 4B) preconstricted with phenylephrine, ACh caused concentration-dependent increases in diameter and simultaneous decreases in [Ca²⁺]. ACh was less potent in inducing relaxation in RUPP (pD₂=6.06±0.10) than in Norm-Preg (pD₂=6.91±0.12), with no differences in maximal relaxation (Figure 4). Also, ACh was less potent in decreasing [Ca²⁺] in RUPP (pD₂=5.86±0.40) than in Norm-Preg (pD₂=6.67±0.26), with no difference in

![Figure 1. Effect of endothelin-1 (ET-1) on vasoconstriction and [Ca²⁺]. ET-1–induced changes in diameter (A and B) and [Ca²⁺] (C and D) were recorded simultaneously in microvessels from normal pregnancy rats (Norm-Preg; A and C) and rats with reduced uteroplacental perfusion pressure (RUPP; B and D). Cumulative ET-1–induced constriction (E), [Ca²⁺] (F), constriction/Δ [Ca²⁺] (G), and concentration-dependent constriction (H) were measured. Data represent means±SEM (n=5–12). *P<0.05, RUPP vs Norm-Preg.]
maximal effect on $[Ca^{2+}]_i$ (Figure 4). To test the responsiveness of VSM to vasodilators, the NO donor sodium nitroprusside caused concentration-dependent increases in diameter and decreases in $[Ca^{2+}]_i$, and was equally potent in inducing relaxation in vessels of Norm-Preg ($pD_2=6.32\pm0.20$; Figure 4C) and RUPP rats ($pD_2=6.41\pm0.30$; Figure 4D) with no difference in maximal relaxation or underlying $[Ca^{2+}]_i$ (Figure 4).

In microvessels preconstricted with phenylephrine, ET-1 in the presence of the ET$_A$ antagonist BQ-123 ($10^{-6}$ mol/L; Figure 5A), or the selective ET$_B$ agonists S6c (Figure 5B) and IRL-1620 (Figure 5C) caused concentration-dependent relaxation that was less in RUPP than in Norm-Preg, with no difference in $[Ca^{2+}]_i$ (Figure 5D, 5E, and 5F).

To investigate the vascular mediators released during microvascular relaxation, the NO synthase inhibitor $N\omega$-nitro-L-arginine methyl ester (L-NAME) ($3\times10^{-4}$ mol/L) plus cyclooxygenase inhibitor indomethacin (INDO, $10^{-6}$ mol/L) caused a shift to the right in ACh concentration–relaxation curve in Norm-Preg ($pD_2$ control=$6.91\pm0.12$; +L-NAME+INDO=$6.13\pm0.15$; Figure 6A), but not in RUPP ($pD_2$ control=$6.06\pm0.10$; +L-NAME+INDO=$6.19\pm0.11$; Figure 6B), suggesting that an NO-mediated vasodilator component is active in Norm-Preg but reduced in RUPP. L-NAME+INDO also reduced ACh-induced relaxation in Norm-Preg (Figure 6C) but not in RUPP (Figure 6D). The L-NAME+INDO–resistant component of ACh-induced relaxation and decreased $[Ca^{2+}]_i$ was similarly abolished by the K$^+$ channel blocker tetraethylammonium (30 mmol/L) or in endothelium-denuded microvessels of both Norm-Preg (Figure 6A and 6C) and RUPP (Figure 6B and 6D), suggesting a similar contribution of EDHF.

To test the vascular factors involved in ET$_B$-mediated relaxation, L-NAME+INDO reduced S6c- and IRL-1620–induced relaxation in microvessels of Norm-Preg (Figure 6E and 6G), but not in RUPP (Figure 6F and 6H). Additional treatment with tetraethylammonium or endothelium removal abolished S6c- and IRL-1620–induced relaxation in Norm-Preg (Figure 6E and 6G) and RUPP (Figure 6F and 6H). To test further the role of NO, we first measured nitrate/nitrite production in aortic segments as previously described. Basal, ACh ($10^{-6}$ mol/L), BQ-123+ET-1-, S6c-, and IRL-1620 ($10^{-7}$ mol/L)–induced nitrate/nitrite production were reduced in aorta of RUPP versus Norm-Preg (Figure 6I).
ET<sub>R</sub> was not different (Figure 7B), the estimated endothelial ET<sub>R</sub> was less in RUPP than in Norm-Preg (Figure 7C).

Immunohistochemistry revealed that total ET<sub>R</sub> immunostaining was not different and total ET<sub>B</sub><sub>R</sub> was insignificantly reduced (P=0.10) in mesenteric vessels of RUPP versus Norm-Preg (Figure 7D and 7E). ET<sub>R</sub> staining was not different in intima, media, or adventitia of RUPP versus Norm-Preg (Figure 7F). ET<sub>B</sub><sub>R</sub> staining was significantly reduced in intima and endothelium, insignificantly increased in media, and not different in adventitia of mesenteric vessels of RUPP versus Norm-Preg (Figure 7G).

Infusion of ET<sub>R</sub> antagonist BQ-788 for 5 days in Norm-Preg rats resulted in increased BP compared with control nontreated Norm-Preg (Figure 8A). Infusion of RUPP rats with IRL-1620 for 5 days reduced BP compared with nontreated RUPP (Figure 8A). Also, ET-1 (10<sup>-7</sup> mol/L)–induced constriction and [Ca<sup>2+</sup>]<sub>i</sub> were compared in endothelium-nontreated or ETBR antagonist BQ-788 (10<sup>-6</sup> mol/L) pretreated and DB, and in AC rats and rats with reduced and denuded (-Endo) and intact (+Endo) microvessels of normal and RUPP rats.19,21 The differences may be related to regional differences along the arterial tree.25 (First-order mesenteric arteries and HTN-Preg to generalized endotheliosis,9 decreased hypertension have shown many of the features of preeclampsia.4,6,8,12 Consistent with these reports, we found an increase in aortic ET-1 plasma levels and preproET mRNA and ETAR protein expression in RUPP versus Norm-Preg rats. Also, RUPP rats exhibit increased renal tissue expression of preproET.13 Chronic hypoxia during pregnancy in rats is associated with preeclampsia-like manifestations and increased ET-1 plasma levels and preproET mRNA and ET<sub>R</sub> protein in the kidneys and placenta.24 Also, serum from RUPP rats increases ET-1 production by cultured endothelial cells.17 Consistent with these reports, we found an increase in aortic preproET mRNA expression in RUPP versus Norm-Preg rats, supporting a role for enhanced ET-1 system in placental ischemia-induced hypertension.

To test the microvascular effects of ET-1 in HTN-Preg, ET-1–induced constriction was enhanced in RUPP versus Norm-Preg. These data are different from reports that ET-1 produces similar vascular contraction in Norm-Preg and RUPP rats.19,21 The differences may be related to regional differences along the arterial tree (first-order mesenteric arteries versus the present third-order mesenteric microvessels) or the method of measuring vascular function (wire myography versus the present pressurized microvessels). Importantly, we observed enhanced ET-1 constriction in endothelium-intact vessels of RUPP versus Norm-Preg (Figure 7G).

Discussion

Preeclampsia is manifested as hypertension and often intrauterine growth restriction.22 Although the mechanisms of preeclampsia are unclear, placental ischemia is thought to be an initiating event. Studies in animal models of HTN-Preg such as the RUPP rat model of placental ischemia-induced hypertension have shown many of the features of preeclampsia.4,6,8,12 Consistent with these reports, the present study showed that RUPP in late pregnant rats was associated with increased BP, decreased litter size, and average pup weight. Studies have related the main characteristics of preeclampsia and HTN-Preg to generalized endotheliosis,9 decreased release of vasodilator substances such as NO and EDHF, and increased release of vasoconstrictors such as ET-1. Plasma ET-1 levels are increased in preeclamptic women. Also, RUPP rats exhibit increased renal tissue expression of preproET.13 Chronic hypoxia during pregnancy in rats is associated with preeclampsia-like manifestations and increased ET-1 plasma levels and preproET mRNA and ET<sub>R</sub> protein in the kidneys and placenta. Also, serum from RUPP rats increases ET-1 production by cultured endothelial cells. Consistent with these reports, we found an increase in aortic preproET mRNA expression in RUPP versus Norm-Preg rats, supporting a role for enhanced ET-1 system in placental ischemia-induced hypertension.
but not -denuded vessels of RUPP versus Norm-Preg rats. Although we measured endothelium-dependent relaxation in our pressurized microvessels, other studies did not measure endothelial function, and the endothelium integrity could be compromised during microdissection or passage of wire in the vessel in the wire myography studies.

To test whether the enhanced contraction is specific to ET-1, the α-adrenergic receptor agonist phenylephrine caused

**Figure 4.** Effect of acetylcholine (ACh) and sodium nitroprusside (SNP) on relaxation and [Ca^{2+}]i in microvessels of normal pregnancy (Norm-Preg; A and C) rats and rats with reduced uteroplacental perfusion pressure (RUPP; B and D). Microvessels were preconstricted with phenylephrine (Phe; 6×10^{-6} mol/L) and ACh- and SNP-induced changes in diameter and [Ca^{2+}]i, cumulative concentration-relaxation and [Ca^{2+}]i curves, and pD_{2} were measured. Data represent means±SEM (n=10–12). *P<0.05, RUPP vs Norm-Preg.

**Figure 5.** Effect of endothelin receptor type-B (ET_{B}R) activation on microvascular relaxation in normal pregnancy (Norm-Preg; A) rats and rats with reduced uteroplacental perfusion pressure (RUPP; C) and then treated with endothelin-1 (ET-1; 10^{-12}–10^{-7} mol/L) plus the endothelin receptor type-A antagonist BQ-123 (10^{-6} mol/L; A and D) or with the ET_{B}R agonists sarafotoxin 6c (S6c; B and E) and IRL-1620 (C and F) and % relaxation of Phe constriction (A–C) and underlying [Ca^{2+}]i (D–F) were compared in Norm-Preg and RUPP rats. Data represent means±SEM (n=10–12). *P<0.05, RUPP vs Norm-Preg.
Figure 6. Role of nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factor in microvascular relaxation.

Microvessels of normal pregnancy (Norm-Preg; A and C) rats and rats with reduced uteroplacental perfusion pressure (RUPP; B and D) were preconstricted with Phe (6×10^{-6} mol/L), and acetylcholine (ACH; 10^{-9}–10^{-5} mol/L)-induced relaxation and underlying [Ca^{2+}] in the absence or presence of L-NAME (3×10^{-4} mol/L)+indomethacin (INDO, 10^{-6} mol/L) and tetraethylammonium (TEA; 30 mmol/L) and in endothelium-denuded vessels (-Endo) were recorded. Concentration-relaxation curves to sarafotoxin 6c (S6c) and IRL-1620 were also constructed in microvessels of Norm-Preg (E and G) and RUPP rats (F and H). Basal, ACh (10^{-6} mol/L)-, BQ-123+ET-1-, S6c-, and IRL-160 (10^{-7} mol/L)-stimulated nitrate/nitrite (NOx) production were also measured in aortic (I) and mesenteric arterial segments (J). Data represent means±SEM (n=4–12). *P<0.05, vs control measurements in absence of blockers. #P<0.05, vs measurements in presence of L-NAME+INDO. †P<0.05, vs basal measurements. ‡P<0.05, RUPP vs Norm-Preg.
increases in constriction and [Ca^{2+}], that were not different in microvessels of Norm-Preg and RUPP. The lack of difference in phenylephrine contraction is in accordance with other reports in mesenteric arteries,21,26 and suggests that the enhanced vasoconstriction in RUPP rats is specific to ET-1-activated receptor and signaling mechanisms and not generalized to receptor-mediated vasoconstrictor stimuli.

The enhanced ET-1 vasoconstriction in RUPP versus Norm-Preg rats was associated with greater initial peak [Ca^{2+}], likely because of Ca^{2+} release from the intracellular stores, and greater maintained [Ca^{2+}], likely because of Ca^{2+} influx.27,28 High KCl causes membrane depolarization and stimulates Ca^{2+} entry through voltage-gated Ca^{2+} channels. The greater KCl-induced constriction and [Ca^{2+}], in RUPP

Figure 7. Expression, protein amount, and localization of endothelin receptor type-A (ET_{A}R) and endothelin receptor type-B (ET_{B}R) in vessels of normal pregnancy (Norm-Preg) rats and rats with reduced uteroplacental perfusion pressure (RUPP). Reverse transcription polymerase chain reaction was performed on aortic tissue homogenate and mRNA expression of preproET, ET_{A}R, and ET_{B}R were measured (A). Western blots were performed to compare protein levels of ET_{A}R and ET_{B}R in endothelium-intact vs -denuded mesenteric vessels of Norm-Preg and RUPP rats (B and C, top). To estimate endothelial ET_{A}R and ET_{B}R, the protein level in endothelium-denuded vessels was subtracted from that in endothelium-intact vessels (B and C, bottom). The distribution of ET_{A}R and ET_{B}R immunostaining was measured in vessel sections (D). Arrow indicates prominent staining in the intima. The total number of pixels in the tissue section was first defined, then the number of brown spots (pixels) was counted, and presented as % of total area (E). The number of pixels in the intima, media, and adventitia was also defined, then ET_{A}R (F) and ET_{B}R brown staining (G) was counted, and presented as % of the respective layer area. Data represent means±SEM (n=4–5). *P<0.05, RUPP vs Norm-Preg. #P<0.05, -Endo vs +Endo.
versus Norm-Preg rats support a role of increased microvascular Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels in HTN-Preg, although ET-1 induced Ca\(^{2+}\) influx may also involve receptor- and store-operated Ca\(^{2+}\) channels.\(^{27}\)

We have used the ratio of vasoconstriction/Δ [Ca\(^{2+}\)] as a measure of Ca\(^{2+}\) sensitivity of VSM contractile proteins.\(^{20}\) KCl vasoconstriction/Δ [Ca\(^{2+}\)] was similar in RUPP and Norm-Preg rats, supporting that KCl-induced contraction is Ca\(^{2+}\) dependent. Although maximal phenylephrine-induced constriction was similar to that of KCl, [Ca\(^{2+}\)] was smaller, and the constriction/Δ [Ca\(^{2+}\)] was greater with phenylephrine than KCl (Figure 2), suggesting activation of additional mechanisms that increase the myofilament force sensitivity to [Ca\(^{2+}\)]. Likewise, ET-1–induced constriction/Δ [Ca\(^{2+}\)] was greater in microvessels of RUPP than of Norm-Preg, suggesting that the enhanced ET-1–induced vasoconstriction in RUPP may not be exclusively because of VSM [Ca\(^{2+}\)], but could also involve Ca\(^{2+}\) sensitization pathways such as protein kinase-C, Rho-kinase, and mitogen-activated protein kinase.\(^{29}\)

Although ET-1 is known for its vasoconstrictor effects, intravenous injection of ET-1 causes transient hypotension followed by delayed increase in BP,\(^{30,31}\) which has been attributed to its 2 receptor subtypes, ET\(_{A}\)R and ET\(_{B}\)R.\(^{32,33}\) ET\(_{A}\)R is largely localized in VSM causing vasoconstriction, increased [Ca\(^{2+}\)], and activation of other pathways of VSM contraction and growth.\(^{34}\) In contrast, ET\(_{B}\)R is located predominantly in the endothelium and mediates vasodilation.\(^{35,36}\) Studies have shown that treatment of RUPP rats with an ET\(_{A}\) antagonist decreases BP, and suggested a role for ET\(_{A}\)R in HTN-Preg.\(^{13,18,24,36}\) However, the decreased BP in RUPP rats treated with ET\(_{A}\)R antagonist can also be explained by the possibility that most ET-1 would be directed toward endothelial ET\(_{B}\)R to promote vasodilation, and thus made it important to examine the role of vascular ET\(_{A}\)R in normotensive and HTN-Preg.

We have recently shown that upregulation of endothelial ET\(_{A}\)R may be responsible for the blunted ET-1 vasoconstriction and enhanced ET\(_{A}\)R-mediated vasodilation in Norm-Preg rats.\(^{30}\) The present microvascular function studies suggest that the enhanced ET-1 constriction in HTN-Preg produced by RUPP could be as a result of downregulation of vasodilator endothelial ET\(_{B}\)R because (1) endothelium removal enhanced ET-1 constriction and [Ca\(^{2+}\)] in microvessels of RUPP but not in Norm-Preg, suggesting that the enhanced ET-1–induced vasoconstriction in RUPP may not be exclusively because of VSM [Ca\(^{2+}\)], but could also involve Ca\(^{2+}\) sensitization pathways such as protein kinase-C, Rho-kinase, and mitogen-activated protein kinase.\(^{29}\)

**Figure 8.** In vivo effects of endothelin receptor type-B (ET\(_{B}\)R) antagonists and agonists. Normal pregnancy (Norm-Preg) rats were infused with ET\(_{B}\)R antagonist BQ-788, and rats with reduced uteroplacental perfusion pressure (RUPP) were infused with ET\(_{B}\)R agonist IRL-1620. On day 19 of pregnancy, blood pressure was measured (A), microvessels were isolated, and endothelin-1 (ET-1; 10\(^{-7}\) mol/L)–induced constriction (B) and [Ca\(^{2+}\)] (D), ET-1–induced concentration-dependent constriction (G), and sarafotoxin 6c (S6c; E) and IRL-1620–induced concentration-dependent relaxation (F) were recorded. Data represent means±SEM (n=5–12). †P<0.05, vs Norm-Preg. *P<0.05, RUPP infused with IRL-1620 vs RUPP.
mRNA expression in aorta of RUPP versus Norm-Preg, (2) Western blots revealed that the estimated endothelial ET_\(_R\) was less in microvessels of RUPP than of Norm-Preg, and (3) immunohistochemistry demonstrated prominent localization of ET_\(_R\) in the mesenteric vessel intima of Norm-Preg that was reduced in RUPP rats. The observations that ET_\(_R\) mRNA, protein level, and immunohistochemical distribution were not different in RUPP and Norm-Preg rats support that the enhanced ET-1 contraction in microvessels of RUPP rats is not because of primary increase in VSM ET_\(_R\) and is more likely secondary to downregulation of endothelial ET_\(_R\) vasoconstrictor activity.

The endothelium releases NO, prostacyclin, and EDHF. NO diffuses into VSM where it increases cGMP, which causes vascular relaxation by decreasing VSM [Ca^{2+}] and Ca^{2+} sensitivity of contractile proteins. In agreement with studies of mesenteric arteries, aorta, and aortas of RUPP rats, Ach was less potent in inducing relaxation and decreasing [Ca^{2+}] in microvessels of RUPP than of Norm-Preg rats. Similar to Ach, the decreased ET_\(_R\)-mediated relaxation in RUPP rats is partly as a result of decreased endothelium-derived NO and the NO-cGMP relaxation pathway because (1) endothelium removal abolished Ach-, S6c-, and IRL-1620–induced relaxation, (2) Blocking NO synthesis by L-NAME partly reduced Ach-induced relaxation and changes in [Ca^{2+}], as well as S6c- and IRL-1620–induced relaxation in Norm-Preg, but not in RUPP. ET_\(_R\) agonists-induced microvascular relaxation was not associated with decrease in [Ca^{2+}], suggesting that ET_\(_R\)-mediated activation of NO-cGMP may function by decreasing Ca^{2+}-sensitivity of VSM contractile proteins. (3) Basal, Ach-, S6c-, and IRL-1620–induced nitrate/nitrite production were less in the aorta and mesenteric arteries of RUPP than of Norm-Preg. (4) Sodium nitroprusside–induced relaxation and [Ca^{2+}] were not different in microvessels of RUPP and Norm-Preg rats, supporting that the impaired Ach- and ET_\(_R\) agonists-induced relaxation was not because of increased sensitivity of VSM to NO-cGMP. (5) While EDHF is an important vasodilator in resistance arteries and may explain the L-NAME+INDO-resistant component of Ach-, S6c-, and IRL-1620–induced relaxation, its blockade with tetraethylammonium caused similar inhibition of Ach- and ET_\(_R\)-agonist–induced relaxation in RUPP and Norm-Preg rats, supporting an intact EDHF and equally active EDHF-dependent ET_\(_R\)-mediated pathway in both groups. The reduced ET_\(_R\)-mediated NO but not EDHF pathway in RUPP rats could be related to possible uncoupling of ET_\(_R\) from NO but not EDHF, or the presence of sub-population of ET_\(_R\) or other ET-1 receptor subtype(s) with different post-receptor NO or EDHF signaling through receptor heterodimerization or cross-talk mechanisms.

Our in vivo findings also support a role of ET_\(_R\) downregulation in HTN-Preg. Infusion of ET_\(_R\) antagonist BQ-788 in pregnant rats caused an increase in BP, likely because during ET_\(_R\) blockade most of endogenous ET-1 will bind to ET_\(_R\) and cause vasoconstriction. ET_\(_R\) is also involved in ET-1 clearance, and ET_\(_R\) blockade is predicted to allow more endogenous ET-1 to activate ET_\(_R\) and increase vasoconstriction and BP. A study by Madsen et al showed that infusion of ET_\(_R\) antagonist A-192621 caused maintained increases in BP in virgin rats and dose-dependently increased maternal BP, although these increases were not sustained in pregnant rats. Although we observed comparable elevation in maternal BP, the cause of the differences in maintained BP is unclear and could be partly related to the ET_\(_R\) antagonist used (ie, A-192621 versus BQ-788). Also, BP regulation involves not only vascular but also renal, neuronal, and hormonal mechanisms, and increased BP via the vascular mechanism could be compensated for by other control mechanisms. For instance, ET_\(_R\) in the renal medulla may regulate salt and water excretion, plasma volume, and BP.31 Also, changes in vascular reactivity in vivo may be compensated for by cardiac baroreceptor reflexes and mask any vascular effects on BP.25 We should note that the Madsen study did not measure vascular function, whereas we measured the direct ex vivo effects of ET_\(_R\) agonists and antagonists on microvessels and demonstrated a role of decreased endothelial ET_\(_R\) in the decreased vasodilation in HTN-Preg. Furthermore, we showed that infusion of ET_\(_R\) agonist IRL-1620 reduced BP in RUPP rats and that ET-1 constriction and [Ca^{2+}] were reduced, and S6c- and IRL-1620–induced relaxation were enhanced in microvessels of RUPP rats infused with IRL-1620, supporting that increasing ET_\(_R\) activity improves ET_\(_R\)-mediated vasodilation, reduces vasoconstriction, and decreases BP in HTN-Preg. Whether the effects of in vivo modulation of ET_\(_R\) using ET_\(_R\) agonists and antagonists on BP and microvascular activity reflect changes in the sensitivity, mRNA expression, protein levels, vascular tissue localization of ET_\(_R\), or all of the items listed should be examined in future studies.

An important question is what causes downregulation of endothelial ET_\(_R\) in HTN-Preg. Preeclampsia and HTN-Preg are associated with increased vasoactive factors such as cytokines, antiangiogenic factors, and angiotensin receptor type-1 agonistic auto-antibody. Serum tumor necrosis factor-α is increased in RUPP rats, and infusion of pregnant rats with tumor necrosis factor-α is associated with decreased vascular relaxation, increased contraction, HTN-Preg,7 and increased preproET mRNA expression in the placenta, aorta, and kidneys.9 Also, in preeclampsia there is an imbalance between the angiogenic factors vascular endothelial growth factor and placental growth factor, and the anti-angiogenic factor soluble fms-like tyrosine kinase-1.9 RUPP rats also show increased soluble fms-like tyrosine kinase-1, and infusion of soluble fms-like tyrosine kinase-1 in pregnant rats results in increased BP, decreased plasma vascular endothelial growth factor, and increased tissue levels of ET-1.41 In preeclampsia, there is also an increase in angiotensin receptor type-1 agonistic auto-antibody, which could activate angiotensin type-1 receptor in VSM leading to enhanced vasoconstriction and HTN-Preg, and infusion of angiotensin receptor type-1 agonistic auto-antibody in pregnant rats causes increases in renal and placental ET-1. Future studies should test whether the vasoactive factors released during HTN-Preg affect the expression/activity of endothelial ET_\(_R\). ET_\(_R\) has been identified not only in endothelium, but may also be expressed in VSM, where it could promote vasoconstriction.15,22,23 Our Western blots showed greater ET_\(_R\) levels in endothelium-denuded microvessels and immunohistochemistry showed insignificant
increase ($P=0.10$) in ET$_R$R in tunica media of mesenteric vessels of RUPP versus Norm-Preg rats, suggesting a role of VSM ET$_R$R in hypertension-Preg, and the functionality and potential vasoconstrictive effects of these receptors need to be further evaluated in endothelium-denuded vessels and isolated VSM cells.

Perspectives

Endothelial ET$_R$R expression/activity is reduced in pregnant rats with RUPP and may explain the increased BP and ET-1 vasoconstriction and reduced ET$_R$R-mediated relaxation in placental ischemia-induced hypertension. Although the data should be interpreted in a more circumspect fashion because there are forms of HTN-Preg and preeclampsia that may not be adequately represented by the RUPP model, the results suggest that ET$_R$R could be an important target in HTN-Preg, and upregulation of endothelial ET$_R$R, using pharmacological agonists or genetic manipulation, may represent a novel approach in managing preeclampsia.

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Disclosures

None.

References

What Is New?

- The present study provides new information on the role of microvascular endothelial endothelin receptor type-B (ETBR) in placental ischemia-induced hypertension, and first evidence that its downregulation is a major causative mechanism in hypertensive pregnancy (HTN-Preg).
- The results highlight the importance of targeting vascular endothelial ETBR in treatment of HTN-Preg and could have an impact on future therapeutic strategies of preeclampsia.

What Is Relevant?

- HTN-Preg and preeclampsia are major disorders affecting ≈5% to 8% of pregnancies in the United States, but the underlying vascular mechanisms are unclear.

Novelty and Significance

- Reduction in uteroplacental perfusion pressure could be an initiating event, but the central vascular target is unclear.
- Endothelin-1 is a potent vasoconstrictor in some forms of hypertension acting via endothelin receptor type-A in vascular smooth muscle, but could also affect ETBR in the endothelium.
- Although the role of endothelin receptor type-A in vasoconstriction has been studied, the role of vasodilator endothelial ETBR, particularly during HTN-Preg, is poorly understood.

Summary

Downregulation of microvascular endothelial vasodilator ETBR is a critical causative mechanism in HTN-Preg. Increasing ETBR activity could be a target in managing HTN-Preg and preeclampsia.
Downregulation of Microvascular Endothelial Type B Endothelin Receptor Is a Central Vascular Mechanism in Hypertensive Pregnancy
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Downregulation of Microvascular Endothelial Type B Endothelin Receptor is a Central Vascular Mechanism in Hypertensive Pregnancy

(Methods Supplement)

Marc Q. Mazzuca, Wei Li, Ossama M. Reslan, Peng Yu,
Karina M. Mata, Raouf A. Khalil

Division of Vascular and Endovascular Surgery, Brigham and Women's Hospital
and Harvard Medical School, Boston, Massachusetts 02115

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Correspondence and Reprints:
Raouf A. Khalil, MD, PhD
Brigham and Women’s Hospital
Division of Vascular Surgery
75 Francis Street
Boston, MA  02115
Tel: (617)525-8530
Fax: (617)264-5124
E-mail: raouf_khalil@hms.harvard.edu
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 \(\sim 40\%\).\(^4\) 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) \(\leq 300\ \mu\text{m}\) 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. 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. 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 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. 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 [Ca2+]i Recording**

For measurement of intracellular free Ca2+ concentration ([Ca2+]i), microvessels were incubated in Krebs solution containing the cell permeable Ca2+ indicator fura-2/AM (5 μM) and the mild detergent cremophor EL (0.25%) for 1 hr as previously described. 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 Ca2+-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 [Ca2+]i. The signal-to-noise ratio was improved by averaging 10 consecutive 340/380 fluorescence ratio readings.

**Simultaneous Measurement of Microvessel Diameter and [Ca2+]i**

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 [Ca2+]i) were recorded. Our initial experiments demonstrated that KCl- and Phe-induced changes in [Ca2+]i 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 [Ca2+]i measurements. Therefore, to accurately compare the [Ca2+]i-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. To measure the effects of ET-1, Phe and KCl on the Ca2+ 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_{a}R and ET_{b}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_{a}R antagonist BQ-788 (10^{-6} M), and the ET-1 induced ET_{a}R-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+}], 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 BQ-123 (10^{-6} M) for 30 min to block ET_{b}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_{a}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 vasoconstrictive 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$_2$.$^1$

**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$\beta$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 $\mu$g 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$\alpha$R and ET$\beta$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$\alpha$R and ET$\beta$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>
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<tr>
<th>Primer</th>
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| PreproET | Forward 5'- GAGGCATCAGCAACAGCATCA -3'
| Reverse 5'- TCCAGGCCCATCCGCA -3' |
| ET$\alpha$R | Forward 5'- CAGCCTGGGCCCTTGGAGACCTTAT -3'
| Reverse 5'- TTCTGTGCTGCTCGCCCTTGTATT -3' |
| ET$\beta$R | Forward 5'- GATACGAACTTCCGCTCCA -3'
| Reverse 5'- GTCCACGATGAGGACAATGAG- 3' |
| $\alpha$-actin | Forward 5'- GACACCAGGGATGTATGGTT -3'
| Reverse 5'- GCTAGGTTAGTGGCTGCTA -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. Relative quantification of gene expression was performed by the comparative CT ($\Delta\Delta$CT) method with $\alpha$-actin as endogenous control as previously described.$^{18}$

**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 $\mu$m 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 ETAR or ETBR 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 diaminobenzadine (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 Gills 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 ETAR or ETBR content (visualized as brown spots) was quantified using bright-field illumination and images were background subtracted using ImageJ software. For quantification of ETAR and ETBR 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 ETAR or ETBR 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, Illsville, 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 ETAR antagonist BQ-123, the ETBR 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


