Prenatal Hypoxia Causes Long-Term Alterations in Vascular Endothelin-1 Function in Aged Male, but Not Female, Offspring

Stephane L. Bourque, Ferrante S. Gragasin, Anita L. Quon, Yael Mansour, Jude S. Morton, Sandra T. Davidge

Abstract—Prenatal hypoxia can alter the growth trajectory of the fetus and cause lasting health complications including vascular dysfunction. We hypothesized that offspring that were intrauterine growth restricted (IUGR) because of prenatal hypoxia would exhibit altered vascular endothelin-1 (ET-1) signaling in later life. Isolated mesenteric artery responses to big ET-1 (bET-1) and ET-1 were assessed by using wire myography. Male IUGR offspring had 3-fold greater bET-1–induced vasoconstriction compared with controls (n=7 per group; P<0.001); NO synthase inhibition with L-NG-nitroarginine-methyl ester potentiated bET-1–induced vasoconstriction, albeit this effect was 2-fold greater (P<0.05) in male control compared with IUGR offspring. Vascular responses to bET-1 were similar between female IUGR and control offspring (n=9–11 per group). In the presence of L-NG-nitro-arginine-methyl ester, pretreatment with the chymase inhibitor chymostatin, the gelatinase inhibitor GM6001, or the neutral endopeptidase inhibitor thiorphan did not alter responses to bET-1; however, the ET-converting enzyme inhibitor CGS35066 almost completely abolished vascular responses to bET-1 in control and IUGR groups. Systolic blood pressure in IUGR male offspring was more responsive to ET-1 antagonism in vivo compared with controls (−9 versus −4 mm Hg; n=5 per group; P=0.02); no such differences were observed in female offspring (n=5–6 per group). These results demonstrate that vascular ET-1 function is programmed by prenatal hypoxia and provide further insights into the sex differences in the long-term vascular effects of developmental stressors. (Hypertension. 2013;62:00-00.) • Online Data Supplement

Key Words: endothelin-1 ■ fetal hypoxia ■ intrauterine growth retardation ■ nitric oxide ■ vascular resistance

Fetal hypoxia is believed to be an important pathogenetic factor in a number of pregnancy-related complications such as preeclampsia and intrauterine growth restriction (IUGR). Numerous epidemiological and animal studies have shown that stressors such as hypoxia can influence the growth and developmental trajectories of the fetus, thereby increasing its susceptibility to long-term health complications, including cardiovascular diseases (reviewed in Rueda-Clausen et al2). In support of this, we and others have shown that prenatal hypoxia is associated with long-term alterations in vascular function, characterized by reduced NO-mediated vasodilation, as well as increased responsiveness to adrenergic vasoconstrictors.3–4

ET-1 is a polypeptide that plays an integral role in vascular function. By virtue of its potent vasoconstrictor properties and its capacity to induce vascular remodeling, ET-1 signaling is believed to be important in the progression of diseases where vascular dysfunction plays a role.5,6 The vasoactive peptide ET-1 is synthesized as an inactive precursor, big ET-1 (bET-1), which is then subsequently cleaved by 1 of several enzymes, including the endothelin (ET)-converting enzymes (ECE), certain members of the matrix-metalloproteinase (MMP) family (notably the gelatinases), chymase and neutral endopeptidase.7 Recently, we showed that NO plays a role in regulating the conversion of bET-1 to active ET-1,8 and consequently conditions of NO deficiency, such as IUGR, may also be associated with increased ET-1 activity. Collectively, these studies provided us the impetus to investigate whether prenatal hypoxia confers on the offspring an altered circulatory phenotype, characterized by increased ET-1 signaling, and determine whether male and female offspring were similarly affected.

Methods

Expanded methods are available in the online-only Data Supplement. Briefly, the experimental protocols described herein were approved by the University of Alberta Health Sciences Animal Policy and Welfare Committee in accordance with the Canadian Council on Animal Care guidelines. IUGR was induced in rats by placing pregnant dams in a hypoxic chamber (set at 11% O2) from gestational day 15 to gestational day 21, as previously described.9 At birth, litters were reduced to 8 offspring (4 males and 4 females) to standardize postnatal conditions. At postnatal...
day 21, 2 male and 2 female offspring that had representative body weights of their litter were selected, and housed (2 rats/cage) until 14 months of age. Vascular function studies were performed on isolated mesenteric vessels by using wire myography, according to established procedures. Blood pressures (BP) were assessed by tail-cuff plethysmography (ITC Life Sciences, Woodland Hills, CA). For BP responsiveness to the dual ET$_A$ and ET$_B$ (ET$_{AB}$) receptor antagonist tezosentan (10 mg/kg, IV), rats were instrumented with indwelling aortic catheters under isofluorane anesthesia. Data sets between control and IUGR groups were compared by Student t test, or ANOVA with Bonferroni post hoc test for multiple comparisons; n values reflect number of litters represented.

**Results**

**Offspring Characteristics**

The detailed effects of prenatal hypoxia on fetal outcomes, as well as growth patterns, have been previously reported. In summary, exposure to a hypoxic environment during the last week of pregnancy causes asymmetrical growth restriction, characterized by reduced fetal body and liver weights, and increased brain and heart weights. To confirm that prenatal hypoxia–induced IUGR in the present study, birth weights (control: 866±26 g, n=18; IUGR: 832±34 g, n=14; P=0.024); male offspring (control: 539±21 g, n=15; IUGR: 506±20 g, n=14; P=0.40); female offspring (control: 539±21 g, n=15; IUGR: 506±20 g, n=15; P=0.40).

**Vascular Reactivity to bET-1**

All vascular responses were normalized to the maximum constriction obtained in the presence of phenylephrine (10 μmol/L). There was no difference between control and IUGR vascular responses to maximal constrictor doses of PE for male (Control: 9.65±0.61 mN/mm, n=9; IUGR: 9.39±0.50 mN/mm, n=7; P=0.77) or female offspring (Control: 8.54±0.32 mN/mm, n=11; IUGR: 8.48±0.34 mN/mm, n=9; P=0.76).

Concentration–response curves to bET-1 were generated in isolated resistance arteries of male (Figure 1A) and female (Figure 1B) offspring. Importantly, bET-1 requires conversion to active ET-1 to induce vasoconstriction. bET-1–induced vasoconstriction was greater in IUGR males compared with controls, corresponding to a 3-fold greater area under the curve (P<0.01; Figure 1A, inset); no difference was observed in females (Figure 1B, inset). Control male bET-1 area under the curve was not different from control female bET-1 area under the curve (P=0.09 after Bonferroni correction). To determine whether bET-1 and ET-1 expression levels were increased in the vasculature of aged offspring, immunofluorescence was performed on mesenteric artery sections. Both bET-1 (Figure S1 in the online-only Data Supplement) and ET-1 (Figure S2) immunofluorescence were elevated in mesenteric vascular sections in male and female IUGR offspring compared with their respective controls (P<0.05 for all parameters).

We subsequently investigated the role of NO in the vascular responses to bET-1 and ET-1, based on (1) our previous observations that the contribution of NO to vasodilation in IUGR offspring is altered and (2) our previous observations concerning the interaction between NO and ET-1 signaling. Pretreatment with the NO synthase inhibitor L-NAME (L-N)-nitro-arginine-methyl ester (L-NAME) potentiated the vasoconstrictor response to bET-1 administration in both control and IUGR male offspring (P=0.001; Figure 1C), although this effect was less pronounced in IUGR offspring, as indicated by a 4-fold greater constriction in the controls, versus 2-fold increase in IUGR offspring (P<0.05; Figure 1D).

![Figure 1](Image)

**Figure 1.** Vascular responses to big endothelin-1 (bET-1) in control and intrauterine growth–restricted (IUGR) offspring in the presence and absence of L-NAME (LN). Cumulative concentration–response curves to bET-1 were generated in (A) male control (n=7) and IUGR offspring (n=7) and (B) female control (n=9) and IUGR offspring (n=11). Cumulative concentration–response curves to bET-1 were generated in the presence of LN (100 μmol/L) in (C) male control (n=7) and IUGR offspring (n=7) and (E) female control (n=9) and IUGR offspring (n=11). Data are shown as percentage of phenylephrine (PE) maximal constriction. Insets show summarized data, calculated as areas under the curve (AUC) *P<0.05, **P<0.01 compared with respective controls. D and F, Fold increase in AUC caused by LN, expressed as a fraction of the AUC of bET-1 with LN to AUC of bET-1 with vehicle.
Figure 1D). Similarly, L-NAME potentiated the effects of bET-1 in female offspring (Figure 1E), albeit no differences in constriction were observed between control and IUGR groups (Figure 1F).

Cleavage of bET-1 to active ET-1 has been shown to occur principally via the ECE. However, alternative pathways have been identified, including those mediated by matrix-metalloproteinase, chymase, and neutral endopeptidase. To gain insights into the source of bET-1 conversion to active ET-1, we performed concentration–response curves to bET-1 in the presence of inhibitors of these enzymes, as well as L-NAME. The rationale for using L-NAME was 2-fold: (1) in control male offspring, vascular responses to bET-1 with intact NO synthase (ie, without L-NAME treatment) were relatively low, and almost completely absent in some cases; consequently, removal of inhibitory NO was believed to be necessary to accurately assess the relative contributions of bET-1 cleaving enzymes; (2) we sought to remove NO as a confounding factor in the differences in bET-1 conversion between control and IUGR offspring because we observed that NO had different modulatory effects on bET-1 in control and IUGR offspring (Figure 1). Treatment with the ECE inhibitor CGS35066 almost completely abolished the vasoconstrictor responses in both male (Figure 2A) and female offspring (Figure 2B), irrespective of prenatal treatment. In contrast, treatment with GM6001 (Figure 2C and 2D), chymostatin (Figure 2E and 2F), or thiorphan (data not shown) did not have any effect in mesenteric vessels. Interestingly, mesenteric vascular expression levels of ECE were not different between control and IUGR groups in either male (ECE expression levels normalized to β-actin; control: 0.92±0.13, n=4; IUGR: 0.91±0.13, n=6; P=0.97) or female offspring (ECE expression levels normalized to β-actin; control: 0.82±0.09, n=4; IUGR: 0.85±0.10, n=7; P=0.84).

Vascular Reactivity to ET-1
We subsequently investigated potential changes in receptor signaling associated with IUGR to account for the possibility that the observed differences in bET-1 constriction could stem from an altered interaction of ET-1 with its receptors. Cumulative concentration–response curves to ET-1 were not different between control and IUGR in male offspring (Figure 3A) or female offspring (Figure 3B). The effects of NO synthase inhibition on ET-1–induced constriction were also investigated; L-NAME caused similar potentiation of ET-1 constrictions in control and IUGR groups in both male (Figure S3A) and female offspring (Figure S3B). To investigate the role of specific ET-1 receptors, cumulative concentration–response curves to ET-1 were generated in the presence of BQ-123 or BQ-788, antagonists of ET A and ET B receptors, respectively. As expected, treatment with the selective ET A receptor antagonist BQ-123 significantly decreased ET-1–mediated constriction in all groups analyzed, corresponding to a rightward shift in the concentration–response curve, and hence decreased in pEC 50 values (Table S1); there were no effects of prenatal treatment. Treatment with the ET B receptor antagonist BQ-788 had little effect on ET-1 curves, and no differences between control and IUGR were observed in either male or female offspring (Table S1).

Figure 2. Vascular responses to big endothelin-1 (bET-1) in control and intrauterine growth–restricted (IUGR) offspring in the presence of L-NAME (LN) and converting enzymes inhibitors. Cumulative concentration–response curves to bET-1 were generated in control and IUGR offspring in the presence of LN (100 μmol/L) alone, or LN in combination with inhibitors; inhibitors included (A and B) the endothelin-converting enzyme selective inhibitor CGS35066 (CGS; 25 μmol/L), (C and D) the gelatinase inhibitor GM6001 (GM; 30 μmol/L), and (E and F) the chymase inhibitor chymostatin (Chymo; 100 μmol/L). Data are shown as percentage of phenylephrine (PE) maximal constriction. Big ET-1 curves in the presence of LN alone are shown in all graphs for reference purposes. Insets show summarized data, calculated as the ratio of the areas under the curve (AUC) of bET-1 in the presence of LN and inhibitors to the AUC of bET-1 in the presence of LN alone. n=4 to 8 in each group.
Vascular responses to active endothelin-1 (ET-1) in control and intrauterine growth–restricted (IUGR) offspring. Cumulative concentration–response curves to ET-1 were generated in (A) male control (n=10) and IUGR offspring (n=7) and (B) female control (n=10) and IUGR offspring (n=6). Data are shown as percentage of phenylephrine (PE) maximal constriction. Insets show summarized data, calculated as areas under the curve (AUC); pEC50 values are shown in Table S1 in the online-only Data Supplement.

**Discussion**

Here, we show that male, but not female, offspring that were exposed to prenatal hypoxia develop altered circulatory and vascular function that implicates the ET-1 system. To summarize, we found the following: (1) IUGR males had increased conversion of bET-1 to active ET-1 compared with controls, and this effect was partially normalized with L-NAME treatment; (2) no differences in bET-1 conversion to active ET-1 were observed between aged control and IUGR female offspring; (3) no differences in ET-1 interaction with its receptors were observed between control and IUGR offspring in either male or female offspring; (4) male IUGR offspring were hypertensive, and this increase in BP was partially mitigated by treatment with the dual ET_{A/B} receptor antagonist tezosentan; (5) female IUGR offspring were not hypertensive compared with control offspring, and tezosentan treatment had little effect in either female treatment group. These results suggest that the ET-1 system, and possibly the conversion of bET-1 to ET-1, could be a modifiable target for the treatment of hypertension by early prenatal stressors. Furthermore, the differences observed between males and females identify the ET-1 system as a mediator in sexually dimorphic effects of early prenatal stressors.

The focus of the present study was on aged offspring rather than their young counterparts largely because the aging population reflects that which is at the greatest risk for cardiovascular events. Indeed, aging is an important risk factor for circulatory dysfunction, and recently it has been shown to exacerbate the correlation between birth weight and BP. In the present study, we report a hypertensive phenotype in aged male IUGR offspring, whereas no such phenotype was observed in young offspring. Although it is interesting that offspring from hypoxia-induced IUGR do not develop hypertension at a young age, in contrast to numerous models of developmental programming, there is nevertheless evidence of vascular changes in young offspring (see below for further discussion). The lack of hypertensive phenotype in young IUGR offspring is consistent with the notion that there is considerable physiological reserve in the young, and that aging constitutes a second hit that may expose an underlying circulatory dysfunction. In addition to these issues, we also studied aged offspring rather than young offspring because enhanced ET-1 signaling has been shown to be a mechanism involved in the increased vascular tone associated with aging.

A marked difference was observed between aged male and female offspring in vascular conversion of bET-1 to ET-1, as well as BP reliance on ET-1 signaling. We previously reported sex-based differences in vascular function in IUGR offspring as they aged, particularly with respect to NO signaling. IUGR male offspring exhibited an almost complete loss of NO-mediated vasodilation with age, whereas control males
maintained NO-dependent endothelial function. Because NO is known to modulate ET-1 signaling in the vasculature, these data support the present findings that intact NO was present in the vasculature of control males, which was antagonizing the conversion of bET-1 to ET-1, based on the observation that this conversion could be potentiated in the presence of L-NAME. IUGR males instead had enhanced bET-1 conversion, consistent with a loss of NO-dependent endothelial function. Interestingly, because L-NAME treatment did not completely equalize the bET-1 areas under the curve between male control and IUGR offspring (Figure 1B), NO-independent mechanisms may also be involved. In contrast, both control and IUGR females exhibited a similar loss of NO-dependent vasodilation at 12 months of age, consistent with no observable differences in bET-1 conversion in the females in the present study.

Numerous studies have reported sex-based differences in the susceptibility to fetal stressors in both young and aged offspring. These differences have been attributed, in large part, to the effects of sex hormones. Indeed, estrogen and testosterone have been shown to influence the susceptibility to cardiovascular dysfunction in the wake of prenatal stress, with the former being largely protective, and the latter being largely deleterious. In previous studies in our laboratory, ovariectomy in both young and aged rats exacerbated the conversion of bET-1 to active ET-1, and this effect was mitigated by estrogen replacement, suggesting that the presence of estrogen may be protective in female offspring. It may be that the presence of estrogen mitigates the signaling of the ET-1 system, even if conversion of bET-1 to ET-1 is enhanced in the IUGR females, possibly by interfering with ET-1 release from Weibel–Palade bodies. Hormonal modulation of ET-1 release from storage vesicles in the endothelium is a particularly intriguing notion because it could provide an explanation as to why we observed enhanced bET-1 and ET-1 staining in the vasculature of female IUGR offspring compared with their respective controls, despite the lack of enhanced vascular responsiveness to bET-1 and hemodynamic effects. In light of these studies, the interaction between estrogen, NO, and ET-1 in the present model of vascular dysfunction warrants further investigation. Irrespective of the underlying mechanism, it is clear that an enhanced ET-1 signaling in male rats underlies a deranged circulatory phenotype, based on the observation that the BP in the IUGR offspring was more responsive to ET receptor antagonism. The findings that tezosentan failed to elicit any marked reduction in BP in female offspring, coupled with no evidence of hypertension, are consistent with those on the lack of vascular differences observed in female offspring.

The data showing no differences in vascular reactivity to ET-1 between control and IUGR groups provide insights into the mechanism of altered ET-1 signaling in the vasculature of IUGR offspring. In particular, these data suggest that the programming of ET-1 function by prenatal hypoxia resides upstream of ET-1 interaction with its receptors, possibly via increased bET-1 conversion to active ET-1, but once ET-1 is released from the endothelium, the course of events that follows is similar irrespective of prenatal treatment. Accumulating evidence indicates that other bET-1–converting enzymes, including MMP-2, neutral endopeptidase, and chymase, are upregulated in models of vascular pathophysiology, which may contribute to enhanced ET-1 signaling. In the present study, we used a number of enzyme inhibitors and found that bET-1 conversion was largely attributable to ECE, based on the observations that the ECE inhibitor CGS35066 abrogated the majority of the contractile response to bET-1. These data suggest that ECE remains the predominant source of conversion from bET-1 to ET-1 in this model. Interestingly, no differences in vascular expression of ECE were observed between groups in either male or female offspring. Although we cannot provide a definitive explanation for this observation, it is tempting to speculate that the differences in ECE activity stem from post-translational modulation, rather than expression levels; this hypothesis is consistent with a modulatory role of NO on ECE activity and is the subject on ongoing investigation. It should also be noted that the present results do not preclude the involvement of the other aforementioned enzymes (eg, MMPs, chymase, etc) in the conversion of bET-1 to ET-1. It is possible that upregulation of these enzymes occurs under conditions such as inflammation or oxidative stress, thereby increasing conversion of bET-1 to active ET-1 in a more marked and consistent manner. We must also consider the possibility that systemic, rather than vascular, conversion of bET-1 to active ET-1 may play a role in the elevated BP in male IUGR offspring because inhibitors for chymase, neutral endopeptidase, and MMPs were not tested in vivo. It may be that conversion of bET-1 to ET-1 occurs to a large degree in distal organs or even large conductance vessels, leading to subsequent vasoconstriction in resistance arteries; studies investigating the role of systemic conversion of bET-1 are needed to fully elucidate the role of the ET system in this model of IUGR.

Perspectives

ET-1 is an important pathogenetic factor in a number of hypertensive complications, and the data presented here suggest an increased capacity of bET-1 processing could underlie enhanced ET-1 signaling in the vasculature of IUGR male offspring. Although the involvement of ET-1 in the sex-based differences in hypertension associated with IUGR is evident, its precise role in the long-term vascular changes requires further investigation. As discussed above, numerous factors are known to modulate activity of the ET-1 system, such as NO and sex hormones, and the dynamic interplay between these mediators may dictate susceptibility to vascular dysfunction. Concerning this, further insights may reveal why male offspring, more than female offspring, are susceptible to the programming effects of prenatal hypoxia. As we continue to identify factors that contribute to sex-based differences and gain a better understanding of disease mechanisms, we can begin to refine strategies to optimize therapeutic outcomes.

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the endothelin-1 system, which can be targeted using currently available drugs.

Summary

In summary, we demonstrate that endothelin-1 signaling is altered in the vasculature of aged intrauterine growth restriction offspring, and this underlies, at least in part, a hypertensive phenotype. Moreover, the present data may suggest that alterations in the endothelin-1 system may underlie the sex-based differences in the susceptibility to cardiovascular derangements characteristic of prenatal stressors.
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Prenatal hypoxia causes long-term alterations in vascular endothelin-1 function in aged male but not female offspring


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Methods

Animals and treatments

Sprague Dawley rats were purchased from Charles River (Wilmington, MA) at 12 wk of age, and housed in the Animal Care Facility at the University of Alberta. Rats were given at least 1 wk to acclimatize to their surroundings prior to experimentation. Rats were given *ad libitum* access to a standard rat chow and water throughout the experiments, and maintained in room with a 12:12h light dark cycle and an ambient temperature of 22±1°C. Dams were bred with male Sprague Dawley rats fed a standard grain-based rodent chow (Lab Diet, Oakville, ON). Vaginal smears were checked daily for the presence of sperm; the presence of sperm was considered gestational day (GD) 0. At GD15, pregnant dams were randomized to either the control or hypoxic groups. Rats in the hypoxic group were individually housed in a standard cage inside a Plexiglas chamber, and the environmental oxygen content was reduced to 11.5 ± 0.2% using a regulated infusion of nitrogen gas. Oxygen levels were monitored continuously throughout the treatment period using an oxygen analyzer (Hudson RCI, Temecula, CA). Control animals were housed in room air. At GD21, rats were removed from the hypoxic chamber, and allowed to give birth in a normal oxygen environment. At birth, litters were reduced to 8 offspring (4 males and 4 females) to standardize postnatal conditions. At postnatal day 21, 2 male and 2 female offspring that had representative body weights of their litter were separated from their mothers, and housed (2 rats/cage) until approximately 14 months of age.

Assessment of vascular function.

At approximately 14 months of age, offspring were anesthetized with isofluorane (dosed to effect by inhalation), and killed by exsanguination. Mesentery was rapidly excised and placed in iced HEPES-buffered physiological saline solution (PSS; NaCl-[142mmol/L], KCl [4.7mmol/L], MgSO4 [1.17mmol/L], CaCl2 [4.7mmol/L], K2PO4 [1.18mmol/L], HEPES [10mmol/L], and glucose [5.5 mmol/L], pH 7.4). Mesenteric arteries were carefully isolated from the surrounding adipose tissue using a binocular microscope, and arteries with internal diameters ranging 150-250 µm were mounted in an isometric myograph system (DMT, Copenhagen, Denmark), using 40 µm tungsten wire. Vessels were normalized through a series of stepwise increases in diameter to determine their optimal resting tension, set to 0.8 x IC100 (the internal circumference equivalent to a transmural pressure of 100 mmHg). Following tension optimization, vessels were rinsed with PSS and given 15 minutes to equilibrate prior to viability testing. To assess viability, vessels were twice treated with a dose of 10µmol/L phenylephrine (PE; Sigma). On the second PE administration, methylcholine (MCh; 3µmol/L; Sigma) was administered to all baths once maximal constriction with PE had been achieved, to assess functionality of the vascular endothelium. Vessels were then rinsed with PSS, and treated for a minimum of 30 minutes with antagonists: N^G^-nitro-L-arginine methyl ester (L-NAME; 100 µmol/L; Sigma), GM6001 (30µmol/L; Calbiochem); CGS35066 (CGS; 25µmol/L; Tocris Bioscience, Ellisville, MO), thiorphan (25µmol/L; Calbiochem), chymostatin (100µmol/L; Calbiochem), MnTBAP (50µmol/L; Calbiochem), BQ-123 (1µmol/L; Sigma), BQ788 (1µmol/L; Sigma). Stock solutions of MCh, PE, L-NAME, BQ-123, BQ788, bET-1 and ET-1 were prepared in ddH2O; stock solutions of GM6001, chymostatin, and thiorphan were prepared in dimethylsulfoxide; stock solutions of CGS was prepared in 100mM NaOH. After pre-incubation in antagonists, cumulative concentration response curves were generated with either ET-1 (Calbiochem; 1x10^−
9mol/L to 5x10⁻⁸mol/L), or bET-1 (Anaspec, Freemont CA; 1e-8mol/L to 3.1e-7mol/L). Only 1 concentration response curve was generated in each isolated vessel.

Blood Pressure Assessments

Blood pressures were assessed by tail-cuff plethysmography (ITC Life Sciences, Woodland Hills, CA). Rats were trained in restraint tubes for 10 minutes on 3 successive days prior to measurements of BP. Three consecutive measurements were taken and the mean was calculated for each rat.

For hemodynamic assessments in the presence of the ET-1 antagonist tezosentan, rats were implanted with indwelling arterial catheters under isoflurane anesthesia at approximately 15 months of age. Briefly, rats were anesthetized with isoflurane (induction 5% in air; maintenance 3% in air), and their descending aortas were cannulated using a 22G Insyte Autoguard shielded catheter (BD Biosciences, Mississauga, ON). A 24G catheter was also inserted into the femoral vein for drug and fluid delivery. Systolic BP was assessed for a minimum baseline period of 15 minutes. Tezosentan (10mg/kg; Actelion Pharmaceuticals, Allschwill, Switzerland) was dissolved in saline, and infused intravenously over a period of 30 seconds.

Statistical Analyses

Big ET-1 and ET-1 vascular responses were compared between control and IUGR groups by Student’s t test. The effects of antagonists were assessed by calculating the AUC in the presence of inhibitor as a fraction of the AUC of its respective vehicle-treated control, and comparing these between control and IUGR by Student’s t-test; this analysis avoided the problems of multiple comparisons using the same treatment group. pEC50 values from ET-1 concentration response curves were calculated by fitting to the Hill equation; comparisons between untreated vessels and those pre-incubated with antagonists were compared by ANOVA, with bonferroni correction. Due to our previous studies that show marked sex-based differences in vascular responses to bET-1 and ET-1 (unpublished results), males and females were, in most cases, not directly compared. BP measurements obtained by tail-cuff plethysmography were compared by Student’s t-test. The hemodynamic effects of ET-1 antagonism by tezosentan were calculated as the maximal change from baseline pressure that occurred within 5 minutes after infusion, and compared between groups by Student’s t-test. Data are presented as Mean±SEM. P<0.05 was considered significant.
Table S1: pEC50 values for ET-1 concentration response curves in mesenteric arteries of control and IUGR offspring in the presence and absence of ET receptor antagonists.

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*p<0.01 vs. vehicle treated in the same group by 1 way ANOVA with Bonferroni post hoc test.

Data are mean±SEM (N).
Figure S1: Big ET-1 (bET-1) immunofluorescence staining of mesenteric vessels of male and female offspring at approximately 14 months of age. (Left) representative images; (right) summarized data (n=4-7 in each group). Scale bar in upper panels denote 100µm. *P<0.05 compared to respective controls.
**Figure S2:** ET-1 immunofluorescence staining of mesenteric vessels of male and female offspring at approximately 14 months of age. (Left) representative images; (right) summarized data (n=4-7 in each group). Scale bar in upper panels denote 100µm. *P<0.05 compared to respective controls.
**Figure S3:** Vascular responses to ET-1 in the presence of L-NAME. Cumulative concentration response curves to ET-1 were generated in (A) male control (n=10) and IUGR offspring (n=6), and (B) female control (n=8) and IUGR (n=5) offspring. Insets show summarized data, calculated as area under the curve; pEC50 values are shown in Table S1. Right-hand panels depict ratios of area under curve (AUC) from vessels treated with L-NAME (LN; 100µmol/L) over AUC of vessels treated with vehicle; cumulative concentration response curves to ET-1 in the absence of LN are shown in Figure 3.