Gestational Hypoxia Induces Preeclampsia-Like Symptoms via Heightened Endothelin-1 Signaling in Pregnant Rats

Jianjun Zhou, Daliao Xiao, Yali Hu, Zhiqun Wang, Alexandra Paradis, Eugenia Mata-Greenwood, Lubo Zhang

Abstract—Preeclampsia is a life-threatening pregnancy disorder. However, its pathogenesis remains unclear. We tested the hypothesis that gestational hypoxia induces preeclampsia-like symptoms via heightened endothelin-1 (ET-1) signaling. Time-dated pregnant and nonpregnant rats were divided into normoxic and hypoxic (10.5% O₂ from the gestational day 6–21) groups. Chronic hypoxia had no significant effect on blood pressure or proteinuria in nonpregnant rats but significantly increased blood pressure on day 12 (systolic blood pressure, 111.7±6.1 versus 138.5±3.5 mmHg; P=0.004) and day 20 (systolic blood pressure, 103.4±4.6 versus 125.1±6.1 mmHg; P=0.02) in pregnant rats and urine protein (μg/μL)/creatinine (nmol/μL) ratio on day 20 (0.10±0.01 versus 0.20±0.04; P=0.04), as compared with the normoxic control group. This was accompanied with asymmetrical fetal growth restriction. Hypoxia resulted in impaired trophoblast invasion and uteroplacental vascular remodeling. In addition, plasma ET-1 levels, as well as the abundance of prepro-ET-1 mRNA, ET-1 type A receptor and angiotensin II type 1 receptor protein in the kidney and placenta were significantly increased in the chronic hypoxic group, as compared with the control animals. Treatment with the ET-1 type A receptor antagonist, BQ123, during the course of hypoxia exposure significantly attenuated the hypoxia-induced hypertension and other preeclampsia-like features. The results demonstrate that chronic hypoxia during gestation induces preeclamptic symptoms in pregnant rats via heightened ET-1 and ET-1 type A receptor–mediated signaling, providing a molecular mechanism linking gestational hypoxia and increased risk of preeclampsia. (Hypertension. 2013;62:00-00.)

Key Words: angiotensin II type I receptor ■ anoxia ■ endothelin-1 ■ hypertension ■ preeclampsia ■ receptor, endothelin A

Preeclampsia is a pregnancy-specific disorder that affects 2% to 8% of pregnant women, and is a leading cause of maternal and neonatal morbidity and mortality.1,2 Preeclampsia is defined by the onset of hypertension and proteinuria after 20 weeks of gestation and is often associated with fetal growth restriction (FGR). The pregnancy complications with abnormal fetal development not only significantly increase maternal and infant mortality and morbidity rates3–6 but also have long-term adverse effects on adult health, predisposing to cardiovascular and metabolic diseases.7–10 The hallmark of preeclampsia is a shallow trophoblast invasion and insufficient spiral artery (SA) remodeling, leading to persistent placental hypoxia and the release of various mediators into the maternal circulation resulting in preeclamptic symptoms.1

Although the causes of preeclampsia have not been clearly defined, a growing body of evidence supports the notion that hypoxia may be an important factor in the pathophysiology of preeclampsia and plays a pivotal role in the origins of preeclampsia.11–22 Previous studies have shown that the incidence of preeclampsia is significantly increased in women who are residing at high altitudes.12–15 However, whether gestational hypoxia causes the development of preeclampsia is controversial. Previous studies have demonstrated that exposure to 11% O₂ from days 6.5 to 13.5 gestation increased vascularity and potentiated trophoblast invasion in pregnant rats,23 but exposure to 9.5% O₂ from day 7.5 to day 17 induced hypertension and preeclampsia-like features in both wild-type and interleukin-10−/− pregnant mice.24 These studies suggest that timing and severity of hypoxia during the course of gestation may be important in the development of preeclampsia. In the early placental development, hypoxia may promote trophoblast proliferation rather than differentiation.25,26 However, the persistence of low oxygen tension and the presence of hypoxia-inducible factor-1α (HIF-1α) may cause a proliferative, noninvasive phenotype of the trophoblast and the development of preeclampsia.27–30 Furthermore, the molecular mechanisms underlying hypoxia-mediated preeclampsia remain largely unclear.

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From the Department of Obstetrics and Gynecology, Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing, China (J.Z., Y.H., Z.W.); and Division of Pharmacology, Department of Basic Sciences, Center for Perinatal Biology, Loma Linda University School of Medicine, Loma Linda, CA (D.X., A.P., E.M.-G., L.Z.).

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Correspondence to Lubo Zhang, Division of Pharmacology, Department of Basic Sciences, Center for Perinatal Biology, Loma Linda University School of Medicine, Loma Linda, CA 92350. E-mail lzhang@llu.edu

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Endothelin-1 (ET-1) is a peptide hormone with a potent vasoconstriction effect, and has been implicated in the pathogenesis of preeclampsia. Plasma ET-1 levels were increased in preeclamptic patients and were correlated with antiangiogenic factor soluble fms-like tyrosine kinase 1 and soluble endoglin levels in these patients. In addition, both renal and placental ET-1 expressions were increased in the animal model of preeclampsia induced by soluble fms-like tyrosine kinase 1 or angiotensin II type I receptor (AT1R)-agonistic autoantibodies. These studies suggest that increased ET-1 may be a key molecule linking adverse stimuli and the development of preeclampsia.

Hypoxia is one of the most potent inducers of ET-1 gene expression in endothelial cells and is a primary cause of heightened ET-1 signaling during cardiovascular ischemia. Therefore, in the present study we investigated whether gestational hypoxia induces hypertension and preeclamptic symptoms in pregnant rats, and tested the hypothesis that heightened ET-1 signaling is a key molecular mechanism underlying chronic hypoxia-induced preeclampsia.

**Materials and Methods**

An expanded Materials and Methods section is available in the online-only Data Supplement.

**Experimental Animals**

Six groups of female Sprague–Dawley rats were used: (1) normoxic control nonpregnant group; (2) hypoxic treatment nonpregnant group; (3) normoxic control time-dated pregnant group; (4) hypoxic treatment time-dated pregnant group, continuous exposure to 10.5% O2 from day 6 to day 21 of gestation; (5) normoxic pregnant rats treated with BQ123, an antagonist of ET-A receptor (ETAR), via osmotic mini-pumps (100 nmol/kg per day) from day 4 to day 21 of gestation; and (6) hypoxic pregnant rats treated with BQ123. The BQ123 treatment was started 2 days before the initiation of 10.5% O2 treatment to allow the recovery of animals from the surgical implantation of osmotic pumps before the hypoxia treatment. The BQ123 treatment sustained the course of hypoxia treatment. Rats were euthanized under isoflurane anesthesia on gestational day 21, and pups, placentas, and kidneys were isolated. All procedures and protocols used in the present study were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

**Measurement of Arterial Blood Pressure**

Rats were implanted with catheters in femoral arteries for recording of arterial blood pressure (BP) on gestational day 4. Arterial systolic BP, diastolic BP, and mean arterial BP were measured on days 12 and 20 of gestation, as described previously.

**Measurement of Proteinuria**

Protein and creatinine levels in 12-hour (from 07:00 pm to 07:00 am) urine samples were measured before hypoxia treatment on day 3 and after hypoxia treatment on day 20 of gestation.

**Determination of Plasma ET-1 and Renin Activity**

Plasma ET-1 was measured by ELISA kit. Renin activity was measured by a fluorometric method.

**Real-Time Polymerase Chain Reaction**

Placental and renal prepro–ET-1 mRNA abundance was determined by real-time polymerase chain reaction.

**Western Immunoblotting Analysis**

AT-R, angiotensin II type II receptor (AT2R), ET, R, and ET-B receptor (ET, B) protein abundance was measured in the placenta and kidney by Western blot analysis, and HIF-1α protein abundance was measured in the placenta.

**Histology and Immunohistochemistry**

Kidney slices were stained with hematoxylin and eosin and periodic acid Schiff (PAS) by standard techniques. Placentas with the associated mesometrial triangle (MT) were paraffin fixed and sections were cut step-serially from each implantation site parallel to the mesometrial-fetal axis. For each implantation site, one set of sections containing a central maternal arterial channel were selected for staining PAS as a fibrinoid tissue marker, cytokeratin as a trophoblast marker, α-actin as a vascular smooth muscle cells (VSMC) marker, as described previously. The degree of trophoblast invasion and SA remodeling was assessed using Image J analysis system. Briefly, the lumen of each SA cross section in the whole MT was manually delineated and stretches of trophoblast, fibrinoid, and VSMC were traced separately over the lumen contour tracing, then the percentages of cytokeratin staining, fibrinoid staining, and α-actin staining of the corresponding SA contour were calculated. The expression of AT-R, AT2-R, ET-R, ET-B, and HIF-1α in the placenta and kidney was also determined using corresponding antibodies.

**Data Analysis**

Results are expressed as mean±SEM. The differences were evaluated for statistical significance by ANOVA or Student t test, where appropriate. A 2-tailed P value of <0.05 was considered significant.

**Results**

**Gestational Hypoxia Increased ET-1 Expression**

As shown in Figure 1A, plasma ET-1 levels were significantly increased in the hypoxic group at midgestation (day 12) and near-term (day 20) pregnant rats, as compared with the normoxic control group. Although there was a tendency of increasing plasma ET-1 levels from day 12 to day 20 of gestation, they were not significantly different. In addition, tissue levels of ET-1 production measured as prepro–ET-1 mRNA in the kidney and placenta of hypoxic animals were significantly higher than those in the control animals (Figure 1B). Furthermore, chronic hypoxia significantly enhanced the protein expression of ET-R but not ET-A in both kidney and placental tissues (Figure 2). Immunohistochemistry study indicated that ET-R expression in the placenta was significantly enhanced by chronic hypoxia.
and ET\textsubscript{A}R were expressed throughout the placental tissue and mainly expressed in tubular but not in glomerulus cells of the kidney (Figure S1 in the online-only Data Supplement).

**Gestational Hypoxia Increased AT\textsubscript{1}R Expression**

Chronic hypoxia had no significant effect on maternal plasma renin activity (Figure S2). However, the protein abundance of AT\textsubscript{1}R in both placenta and kidney was significantly increased in the hypoxic group, as compared with the normoxic group (Figure 3). In contrast, AT\textsubscript{2}R protein abundance was significantly decreased in the kidney but not in the placenta in the hypoxic animals, as compared with the normoxic control (Figure 3). Immunochemistry study indicated that AT\textsubscript{1}R and AT\textsubscript{2}R were expressed throughout the placental tissue (Figure S1). In the kidney, AT\textsubscript{1}R was expressed mainly in tubular but not in glomerulus cells, whereas AT\textsubscript{2}R expressed in both tubular and glomerulus cells (Figure S1).

**Gestational Hypoxia Increased HIF-1\(\alpha\) Expression in the Placenta**

As shown in Figure S3, HIF-1\(\alpha\) protein abundance in the placenta was significantly increased in the hypoxic group, as compared with the normoxic group.

**BQ123 Abrogated the Hypoxia-Induced Increase in BP**

As shown in Figure 4, chronic hypoxia significantly increased systolic BP, diastolic BP, and mean arterial pressure in midgestation (day 12) and near-term (day 20) pregnant rats, as compared with normoxic control animals. Inhibition of ET\textsubscript{A}R with BQ123 abrogated the hypoxia-mediated increase in BP in midgestation and near-term pregnant rats (Figure 4). Chronic hypoxia for the same duration had no significant effect on BP in nonpregnant rats (Figure S4).

**BQ123 Blocked the Hypoxia-Induced Renal Damage and Proteinuria**

As shown in Figure 5A, chronic gestational hypoxia significantly increased urine protein levels in near-term (day 20) pregnant rats, which was blocked by BQ123. In contrast, the hypoxia treatment of nonpregnant rats had no effect on urinary protein levels (Figure S5). Histological examining of the kidney indicated that chronic hypoxia caused extensive endothelial swelling, narrowing, and occlusion of capillary lumen in glomeruli (Figure 5B). PAS stain of hypoxia-treated animals showed PAS-negative swollen cytoplasm of endocapillary cells. BQ123 treatment blocked the hypoxia-induced renal damage (Figure 5B).
BQ123 Reversed the Hypoxia-Induced Impairment of Trophoblast Invasion

Trophoblast-associated vascular remodeling and trophoblast invasion were evaluated in the MT. Figure 6A shows the representative implantation site, including the placenta and its associated MT staining with cytokeratin, showing a maternal spiral arterial channel crossing the placenta. The maternal spiral arterial channel was used as a marker for each slide that was used in the subsequent quantification. Figure 6B shows representative PAS staining (solid arrows), cytokeratin staining (solid arrowheads), and α-actin staining (hollow arrows) in spiral arteries in the MT. PAS staining revealed the deposition of fibrinoid in the

Figure 4. BQ123 abrogated chronic hypoxia-induced increase in blood pressure (BP). Systolic BP (SBP), diastolic BP (DBP), and mean arterial BP (MBP) were measured in hypoxic and normoxic control pregnant rats at day 12 (gd 12) and day 20 (gd 20) of gestation, in the absence or in the presence of BQ123. Data are mean±SEM, n=5 to n=6. *P<0.05 vs normoxia.
As shown in Figure 6C, when expressed as percentages of the corresponding total SA contour length, there were significantly fewer trophoblasts in the hypoxic group than in the normoxic control. The amount of fibrinoid wall, expressed as a percentage of the total lumen contour, was also significantly decreased in the hypoxic group as compared with the normoxic control. In contrast, the length of VSMC, expressed as a percentage of the total lumen contour, was significantly greater in the hypoxic group than that in the normoxic group. These hypoxia-mediated impairments of trophoblast invasion and vascular remodeling of spiral arteries were abrogated by BQ123 (Figure 6C).

**BQ123 Inhibited the Hypoxia-Induced FGR**

As shown in Figure 7, chronic hypoxia resulted in fetal asymmetrical growth restriction by decreasing the fetal body weight and increasing the brain-to-body weight ratio. BQ123 significantly attenuated hypoxia-mediated effects and partially restored the fetal body weight and the brain-to-body weight ratio, and recovered the heart-to-body weight ratio (Figure 7). Chronic hypoxia did not affect the placental weight (0.56±0.02 versus 0.52±0.02 g; P>0.05) but significantly increased the placenta-to-fetal weight ratio (0.21±0.01 versus 0.13±0.01; P<0.05), which was attenuated by BQ123 (Figure S6). In addition, although chronic hypoxia did not affect the litter size (Figure S7), it significantly increased the number of resorbed fetuses, which was blocked by BQ123 (Figure S8).

**Discussion**

The present study demonstrates that exposure to 10.5% O₂ during day 6 through day 21 of gestation causes the development of preeclamptic symptoms, including hypertension and proteinuria in a model of pregnant rats. Of importance, the findings provide new evidence that the heightened ET-1 signaling is a key molecular mechanism in the pathogenesis of chronic hypoxia-induced preeclampsia.

The previous studies have shown that chronic hypoxia impairs normal adaptation of the uteroplacental circulation and enhances uterine vascular tone in pregnancy, which may contribute to the pathogenesis of preeclampsia and FGR. The present findings that chronic hypoxia significantly increased arterial BP in pregnant rats suggest that gestational hypoxia enhances arterial vascular resistance and induces hypertension in pregnancy. In addition to hypertension, maternal hypoxia also increased proteinuria in pregnant rats. This is likely resulting from hypoxia-induced renal damage as the kidney of hypoxia-treated animals showed extensive endothelial swelling, narrowing, and occlusion of capillary lumen. The finding of asymmetrical FGR in hypoxic animals is in agreement with the previous findings in humans showing FGR with preeclamptic pregnancy.

Growing evidence shows a key role of hypoxia in the pathophysiology of preeclampsia. Preeclampsia is associated with shallow trophoblast invasion and inadequate SA remodeling, which are widely believed to lead to placental hypoxia, and the local hypoxia ultimately results in the maternal manifestations of the disease. The present finding that chronic hypoxia increased HIF-1α protein abundance in the placenta provides evidence of placental hypoxia. However, whether maternal hypoxia exposure during gestation is a major factor and a key pathogenesis of preeclampsia remains unclear and controversial. Although previous studies have shown that the incidence of preeclampsia is significantly increased in women who are residing at high altitudes, not all pregnant women at high altitudes develop preeclampsia.

The mechanisms behind this are not fully understood but may be, in part, because of different abilities of the compensatory adaptation to chronic hypoxia among different individuals. A previous study has demonstrated that maternal hypoxia (11% O₂) between days 6.5 and 13.5 of gestation significantly increases the vascularity and trophoblast invasion in pregnant rats, and have suggested that maternal hypoxia during early stages of placentation may activate the invasive endovascular trophoblast cell lineage and promotes uterine vascular remodeling. Although it is known that local physiological hypoxia during normal pregnancy is important in the normal placentation and SA remodeling, it is questionable that...
exaggerated hypoxia further induced by maternal hypoxia may improve the physiology of the placenta and vascular remodeling. Because BP, kidney function, and urine protein levels were not measured in this study, the functional significance of these findings was not clear. Indeed, a more recent study has demonstrated that maternal exposure to 9.5% O₂ from gestational day 7.5 to day 17 induces clear preeclampsia-like symptoms with hypertension and proteinuria in both wild-type and interleukin-10−/− pregnant mice. The present study provides additional support that gestational hypoxia decreases trophoblast invasion and impairs uterine arterial remodeling, resulting in the development of preeclampsia-like symptoms in pregnant rats.

The question arises as to how chronic hypoxia during gestation provokes these preeclamptic symptoms. Recent studies showed that ET-1 was a key pathological factor in preeclampsia. ET-1 has been implicated in a diverse range of signaling events in a wide variety of target tissues. ET-1 was first identified as a potent endothelium-derived vasoconstrictor, the most potent vasoconstrictor known. ET-1 is derived from a longer 203-aa precursor known as prepro–ET-1, the active peptide proteolytically cleaved into its final 21-amino acid form. The important role of ET-1 signaling in the pathogenesis of preeclampsia has been widely demonstrated in clinic and different animal studies. Previous studies have demonstrated that an ETₐR antagonist blocks hypertension induced by purified ATₐR-agonistic autoantibodies from a transgenic rat model of preeclampsia or preeclamptic patients. In addition, ET-1 was increased in reduced uterine perfusion pressure model of preeclampsia, and the administration of an ETₐR antagonist blocked hypertension. In the present study, we found that chronic hypoxia increases maternal plasma ET-1 levels in midgestation (day 12) and near-term (day 21) pregnant rats. In addition, prepro–ET-1 mRNA levels in both placenta and kidney were also elevated with the hypoxia treatment. Hypoxia is a well-known inducer of ET-1 expression and a HIF-1α-binding site has been identified on the 5'-promoter region of the prepro–ET-1 gene. These findings suggest that the heightened ET-1 signaling contributes to the pathogenesis of chronic hypoxia-induced preeclampsia.

ET-1 mediates its physiological effects mainly through 2 seven-transmembrane G-protein–coupled endothelin receptors, the ETₐR, and ETₐR. Both receptor types are widely distributed in noncardiovascular and cardiovascular tissues. The activation of ETₐR results in sustained vasoconstriction. In contrast, the activation of ETₐR induces vasodilatation. The finding that gestational hypoxia selectively upregulated ETₐR expression in both kidney and placenta reinforces the notion that ET-1 may contribute to the pathogenesis of hypoxia-induced preeclampsia through ETₐR signaling. Indeed, the present finding that the sustained infusion of an ETₐR selective antagonist, BQ123, during the course of hypoxia treatment abrogated hypoxia-induced preeclampsia-like symptoms in pregnant rats, indicates a causative role of the heightened ET-1/ETₐR-mediated signaling in the pathogenesis of preeclampsia.

Figure 6. BQ123 reversed chronic hypoxia-induced impairment of trophoblast invasion and spiral artery (SA) remodeling. Placentas were obtained in hypoxic and normoxic control pregnant rats at day 21 of gestation, in the absence or in the presence of BQ123. A, The representative implantation site including the placenta and its associated mesometrial triangle (MT) stained with cytokeratin (CK; ×40). A SA channel is crossing the placenta. B, Representative periodic acid Schiff (PAS) staining of fibrinoid (solid arrows), CK staining of trophoblast invasion (solid arrowheads), and α-actin staining of vascular smooth muscle (VSM; hollow arrows) in spiral arteries in the MT (×200). C, The percentage of fibrinoid, trophoblast, and VSM of total SA contour length was determined. Data are mean±SEM, n=49 to n=53. *P<0.05 vs normoxia.
pathogenesis of hypoxia-induced preeclampsia. Future studies of blocking ET-R at different time points may provide more information detailing the role of ET-1 in the maternal hypoxia-induced preeclampsia symptoms.

The renin angiotensin system is known to stimulate ET-1 production. Previous studies have demonstrated that AT₁R-agonistic autoantibodies increases ET-1 by activation of AT₁R. In the present study, we found that plasma renin activity was not significantly altered by gestational hypoxia. This suggests that circulating angiotensin II may not contribute to the increased ET-1 in this animal model. However, the present findings that chronic hypoxia significantly increased AT₁R protein expressions in both kidney and placenta but decreased AT₂R protein expression in the kidney in pregnant rats suggest that the increased AT₁R/AT₂R ratio may contribute to the ET-1 elevation and preeclampsia-like symptoms in pregnant rats.

Perspectives

Growing evidence indicates a role of gestational hypoxia in preeclampsia. Yet whether hypoxia is a causal factor and is involved in the pathogenesis of preeclampsia remains unclear. The present study demonstrates that chronic hypoxia during gestation induces preeclampsia-like symptoms in pregnant rats via heightened ET-1 and ET-R-mediated signaling, providing a molecular mechanism linking gestational hypoxia and increased risk of preeclampsia. Although caution should be always observed in extrapolating the findings of animal studies directly to the humans, the present finding has a translational potential, and provides a mechanistic understanding worthy of investigation in humans. This is because hypoxia is a common insult during pregnancy, and preeclampsia is one of the most common complications of pregnancy.

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Disclosures

None.

References

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### Novelty and Significance

#### What Is New?
- Chronic hypoxia during gestation induces preeclampsia-like symptoms in an animal model of pregnant rats.
- Gestational hypoxia impairs the trophoblast invasion and uteroplacental vascular remodeling.
- Inhibition of the endothelin-1/ET-A receptor signaling abrogates hypoxia-induced preeclampsia-like features.

#### What Is Relevant?
- Chronic hypoxia is a common insult during pregnancy.

#### Summary

The present study provides new evidence in an animal model linking gestational hypoxia and the increased risk of preeclampsia, and reveals a mechanistic understanding of the heightened endothelin-1/ET-A receptor signaling in the pathogenesis of preeclampsia.
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Gestational hypoxia induces preeclampsia-like symptoms via heightened endothelin-1 signaling in pregnant rats

by

Jianjun Zhou, Daliao Xiao, Yali Hu, Zhiqun Wang, Alexandra Paradis, Eugenia MataGreenwood and Lubo Zhang

Department of Obstetrics and Gynecology (J.J.Z., Y.L.H., Z.Q.W.), Nanjing Drum Tower Hospital, Nanjing University Medical School, Nanjing 210008, China; Center for Perinatal Biology (D.X., A.P., E.M., L.Z.), Division of Pharmacology, Department of Basic Sciences, Loma Linda University School of Medicine, Loma Linda, CA 92350;

Running title: Chronic hypoxia and Preeclampsia

Corresponding author:
Lubo Zhang, Ph.D.
Center for Perinatal Biology
Division of Pharmacology
Department of Basic Sciences
Loma Linda University
School of Medicine
Loma Linda, CA 92350
Tel: 909-558-4325
Fax: 909-558-4029
Email: lzhang@llu.edu
Materials and Methods

Experimental animals. Six groups of female Sprague-Dawley rats were used: 1) normoxic control non-pregnant group; 2) hypoxic treatment non-pregnant group; 3) normoxic control time-dated pregnant group; 4) hypoxic treatment time-dated pregnant group, continuous exposure to 10.5% O₂ from day 6 through day 21 of gestation; 5) normoxic pregnant rats treated with BQ123, an antagonist of ET-A receptor (ETₐR), via osmotic minipumps (100 nmol/kg/d) from day 4 through day 21 of gestation; 6) hypoxic pregnant rats treated with BQ123. All the rats were purchased from Charles River Laboratories (Portage, MI). Hypoxia was induced by a mixture of nitrogen gas and air as described previously.¹ Previous studies showed that an ambient oxygen level of 10.5% lowered maternal arterial oxygen tension to 50 mm Hg.² The normoxic group was housed identically with room air flowing through chambers. Water and food were provided as desired. All procedures and protocols used in the present study were approved by the Institutional Animal Care and Use Committee of Loma Linda University and followed the guidelines in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Urine was collected using metabolic cages before the hypoxia treatment (on gd 3) and after the hypoxia treatment (on gd 20). On gestational day 12, blood samples were taken through the implanted catheter. Rats were euthanized under isoflurane anesthesia on gd 21, and a midline ventral incision was made to isolate the abdominal aorta for blood collection. The uterus was exteriorized, the number of viable and resorbed pups was counted and recorded, and the pups and placenta were excised, blotted dry, and weighed. Then pups were euthanized by cervical dislocation under anesthesia, their brains, hearts and kidneys were weighed. Blood was taken for laboratory assays, placentas and kidneys were snap frozen according to standard procedures and stored at -80 °C for real time RT-PCR and western immunoblotting analysis. Placenta and kidney tissues were also fixed in 4% (wt/vol) paraformaldehyde overnight at 4 °C for histological and immunohistochemical analysis.

Measurement of arterial blood pressure. Rats were implanted with catheters in femoral arteries for recording of arterial blood pressure (BP) on gestational day 4, as we described previously.³,⁴ Arterial systolic BP (SBP), diastolic BP (DBP), mean arterial pressure (MAP) were recorded continuously in conscious animals for 60 minutes with data acquisition software (Powerlab 16/SP and Chart version 4, ADInstruments, Colorado Springs, CO) on gd 12 and gd 20.

Measurement of proteinuria. Urine samples were collected before the hypoxia treatment (on gd 3) and after the hypoxia treatment (on gd 20) for 12 hours (07:00 pm to 07:00 am the next day), with the animals housed individually in metabolic cages in the absence of food to eliminate contamination of urinary protein measurements by fallen food particles. Urine protein concentrations were measured using the pyrogallol red method (Total Protein Kit, Micro Pyrogallol Red Method; Sigma, St Louis, MO). Urinary creatinine concentrations were determined using a Creatinine Companion kit (Exocell, Inc., Philadelphia, PA). Urinary protein was normalized to creatinine excretion, and was presented as micrograms of protein per nanomole of creatinine.

Determination of plasma ET-1 Level and renin activity. ET-1 levels in plasma at
gd 12 and gd 21 were determined using Endothelin-1 Quantikine ELISA Kit (R&D Systems, Minneapolis, MN. Sensitivity of the assay was 0.2 pg/mL. Coefficient of variation for intra-assay was 1.9% - 4%, and inter-assay 5.3% - 7.6%. Plasma renin activity at gd 21 was determined using SensoLyte® 520 Rat Renin Assay Kit (AnaSpec, Fremont, CA).

**Real-time RT-PCR.** Placental and renal RNA was extracted from tissue samples using TRIzol reagents (Invitrogen, Carsbad, CA). PreproET-1 mRNA levels were determined by real-time RT-PCR using the iCycler Thermal cycler (BioRad, Hercules, CA). Specific PreproET-1 primers were 5'-CTAGGTCTAAGCGATCTTGAA-3’ (forward) and 5'-CTTGATGCTGTTGCTGATGG-3’ (reverse). Real-time RT-PCR was performed in a 25 μl-reaction mixture according to the instruction of iScript one-step RT-PCR kit (BioRad). RT-PCR was carried out under the following conditions: 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 55 °C for 10 sec and 72 °C for 20 sec. GAPDH was used as an internal reference. The relative amount of gene expression was calculated by comparison of cycle thresholds with the housekeeping gene of GAPDH.

**Western immunoblotting analysis.** AT1R, AT2R, ETAR and ETBR protein abundance was measured in isolated placentas and kidneys from each group. Tissues were homogenized in a lysis buffer containing 150 mM NaCl, 50 mM Tris HCl, 10 mM EDTA, 0.1% Tween 20, 0.1% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml leupeptin, and 5 μg/ml aprotinin, PH 7.4. Homogenates were then centrifuged at 4°C for 10 min at 10,000g and the supernatants were collected. Nuclear extracts were prepared using the NXTRACT CellLytic Nuclear Extraction Kit (Sigma) with few modifications. Proteins were quantified in the supernatant with a protein assay kit (Bio-Rad). Samples with equal proteins were loaded onto 10% (AT1R, AT2R, ETAR and ETBR) or 7.5% (HIF-1α) polyacrylamide gel with 0.1% sodium dodecyl sulfate (SDS), and were separated by electrophoresis at 100 V for 2 h. Proteins were then transferred onto nitrocellulose membranes. Nonspecific binding sites in the membranes were blocked by an overnight incubation at 4°C in Tris-buffered saline solution (TBS) containing 5% dry milk. The membranes were incubated with primary antibodies against AT1R (1:250), AT2R (1:250), ETAR (1:500), ETBR (1:500) and HIF-1α (1:500) antibodies (Santa Cruz Biotechnology; Santa Cruz, CA). After washing, membranes were incubated with secondary horseradish peroxidase-conjugated antibodies. Proteins were visualized with enhanced chemiluminescence reagents, and blots were exposed to Hyper film. Results were quantified with the Kodak electrophoresis documentation and analysis system and Kodak ID image analysis software.

**Histology and immunohistochemistry.** Kidneys and placentas were harvested from gd 21 pregnant rats. Kidneys were fixed and processed. Briefly, the tissues were fixed in 4% formaldehyde solution (Fisher Scientific) for 36–48 hours at room temperature, washed with PBS for 30 minutes, dehydrated, infiltrated, and embedded in paraffin. Five-micron serial sections were cut from blocks and stained with H&E and Periodic Acid Schiff (PAS) by standard techniques. Placentas with their associated mesometrial triangle were paraffin fixed and parallel sections were cut step-serially from each implantation site parallel to the mesometrial-fetal axis, as described previously. For each implantation site one set of parallel sections containing a central maternal arterial channel (Figure 6A) were selected for further study. Stained for PAS as a fibrinoid tissue marker, for cytokeratin as a trophoblast marker and for α-actin as a vascular smooth muscle cell (VSMC) marker. For immunohistochemical
analysis, heat-induced antigen retrieval was accomplished with 2% ethylenediaminetetra-acetic acid at 95°C for 10 min. Endogenous peroxidase was blocked with 3% H₂O₂ for 15 min at room temperature. Anti-cytokeratin (1:50, clone MNF116, Santa Cruz Biotechnology; Santa Cruz, CA) and anti-α-actin (1:200, clone 1A4, Santa Cruz Biotechnology; Santa Cruz, CA) was applied at 4 °C for overnight. Then was detected using Anti-mouse Ig HRP Detection kit (BD Biosciences, San Jose, CA). The degree of trophoblast invasion and spiral artery (SA) remodeling were assessed using Image J analysis system as described earlier, briefly, the lumen of each SA cross-section in the whole MT was manually delineated and stretches of trophoblast, fibrinoid and VSMC were traced separately over the lumen contour tracing, the percentages of CK staining, fibrinoid staining and α-actin staining of the corresponding spiral artery contour were calculated. The expression of AT₁R, AT₂R, ET₁R, ET₂R and HIF-1α (1:100) in the placenta and kidney was also determined by using corresponding antibodies.

**Data analysis.** Results were expressed as means ± SEM. For real-time PCR and Western blotting analyses, the mean values of preproET-1 mRNA and proteins were first determined in the normoxic group, which were subsequently used to normalize values in both normoxic and hypoxic groups. The differences were evaluated for statistical significance by ANOVA or Student t-test, where appropriate. A two-tailed P-value of less than 0.05 was considered significant.

**References**


Online Figure S1. Expression of AT₁R, AT₂R, ETₐR and ET₇R in the placenta and kidney in normoxic pregnant rats at day 21 of gestation (×200).
Online Figure S2. **Chronic hypoxia had no effect on plasma renin activity.** Plasma renin activity was measured in hypoxic and normoxic control pregnant rats at day 21 of gestation. Data are means ± SEM, n = 6.
Online Figure S3. Gestational hypoxia increased HIF-1α expression in the placenta. A, Protein abundance of HIF-1α was determined in the placenta of hypoxic and normoxic control pregnant rats at day 21 of gestation. Data are means ± SEM, n = 6. * P < 0.05 versus normoxia. B, The placenta histology with HIF-1α staining (×200) was examined in hypoxic and normoxic control pregnant rats at day 21 of gestation.
Online Figure S4. Hypoxia treatment had no effect on blood pressure in non-pregnant rats. Non-pregnant rats were treated with normoxic control or 10.5% O₂, and systolic (SBP), diastolic (DBP) and mean (MBP) arterial blood pressure were measured at 6 days (equivalent to the duration of hypoxic treatment of pregnant rats at gd 12) and 14 days (equivalent to the duration of hypoxic treatment of pregnant rats at gd 20). Data are means ± SEM, n = 5.
Online Figure S5. Hypoxia treatment had no effect on proteinuria in non-pregnant rats. Non-pregnant rats were treated with normoxic control or 10.5% O₂, and 12-hour protein and creatinine levels were determined at 14 days (equivalent to the duration of hypoxic treatment of pregnant rats at gd 20). Data are means ± SEM, n = 5.
Online Figure S6. BQ123 partially reversed chronic hypoxia-induced increase in the placenta to fetal weight ratio. Placenta to fetal weight ratio was determined in hypoxic and normoxic control pregnant rats at day 21 of gestation, in the absence or presence of BQ123. Data are means ± SEM, n = 29-53. * P < 0.05, versus normoxia; + P < 0.05, versus -BG123.
Online Figure S7. Hypoxia treatment had no effect on litter size. Litter size was determined in hypoxic and normoxic control pregnant rats at day 21 of gestation, in the absence or presence of BQ123. Data are means ± SEM, n = 5-6.
Online Figure S8. BQ123 blocked chronic hypoxia-induced fetal resorption. Fetal resorption was determined in hypoxic and normoxic control pregnant rats at day 21 of gestation, in the absence or presence of BQ123. Data are means ± SEM, n = 5-6. * P < 0.05, versus normoxia.