

Online Supplement

**Gestational hypoxia induces preeclampsia-like symptoms *via* heightened endothelin-1 signaling in pregnant rats**

by

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## 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% O<sub>2</sub> from day 6 through day 21 of gestation; 5) normoxic pregnant rats treated with BQ123, an antagonist of ET-A receptor (ET<sub>A</sub>R), *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.<sup>1</sup> Previous studies showed that an ambient oxygen level of 10.5% lowered maternal arterial oxygen tension to 50 mm Hg.<sup>2</sup> 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.<sup>3,4</sup> Arterial systolic BP (SBP), diastolic BP (DBP), mean arterial pressure (MBP) 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 kit 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, Carlsbad, 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'-CTAGGTCTAAGCGATCCTTGAA-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, 50 °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.** AT<sub>1</sub>R, AT<sub>2</sub>R, ET<sub>A</sub>R and ET<sub>B</sub>R 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% (AT<sub>1</sub>R, AT<sub>2</sub>R, ET<sub>A</sub>R and ET<sub>B</sub>R) 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 AT<sub>1</sub>R (1:250), AT<sub>2</sub>R (1:250), ET<sub>A</sub>R (1:500), ET<sub>B</sub>R (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.<sup>5</sup> 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

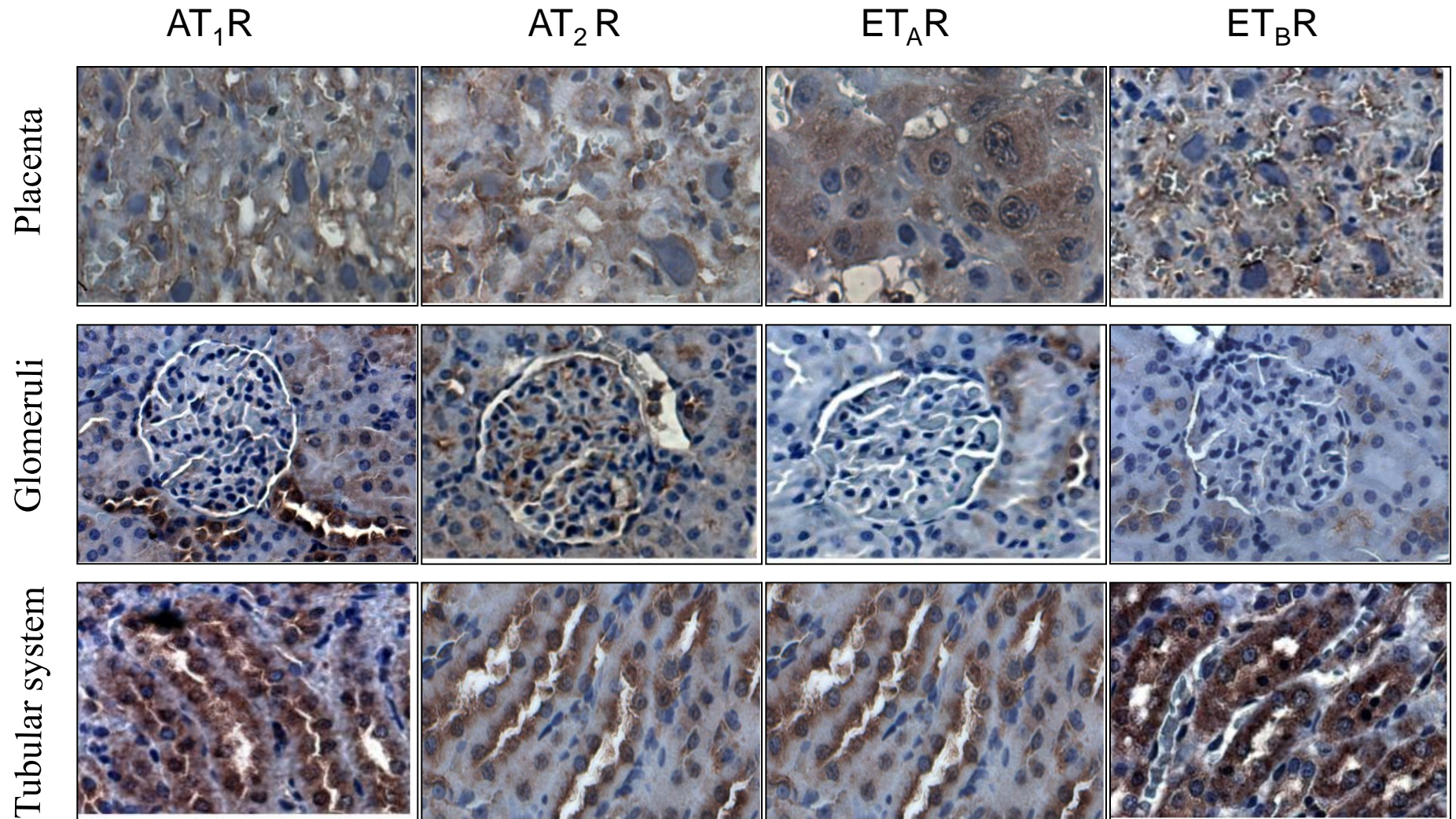
analysis, heat-induced antigen retrieval was accomplished with 2% ethylenediaminetetra-acetic acid at 95°C for 10 min. Endogenous peroxidase was blocked with 3% H<sub>2</sub>O<sub>2</sub> for 15 min at room temperature. Anti-cytokeratin (1:50, clone MNF116, Santa Cruz Biotechnology; Santa Cruz, CA) and anti- $\alpha$ -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,<sup>6-8</sup> 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  $\alpha$ -actin staining of the corresponding spiral artery contour were calculated. The expression of AT<sub>1</sub>R, AT<sub>2</sub>R, ET<sub>A</sub>R, ET<sub>B</sub>R and HIF-1 $\alpha$  (1:100) in the placenta and kidney was also determined by using corresponding antibodies.

**Data analysis.** Results were expressed as means  $\pm$  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.

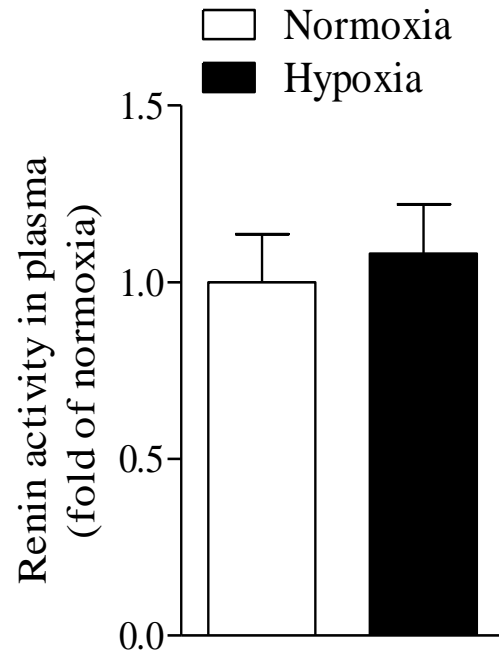
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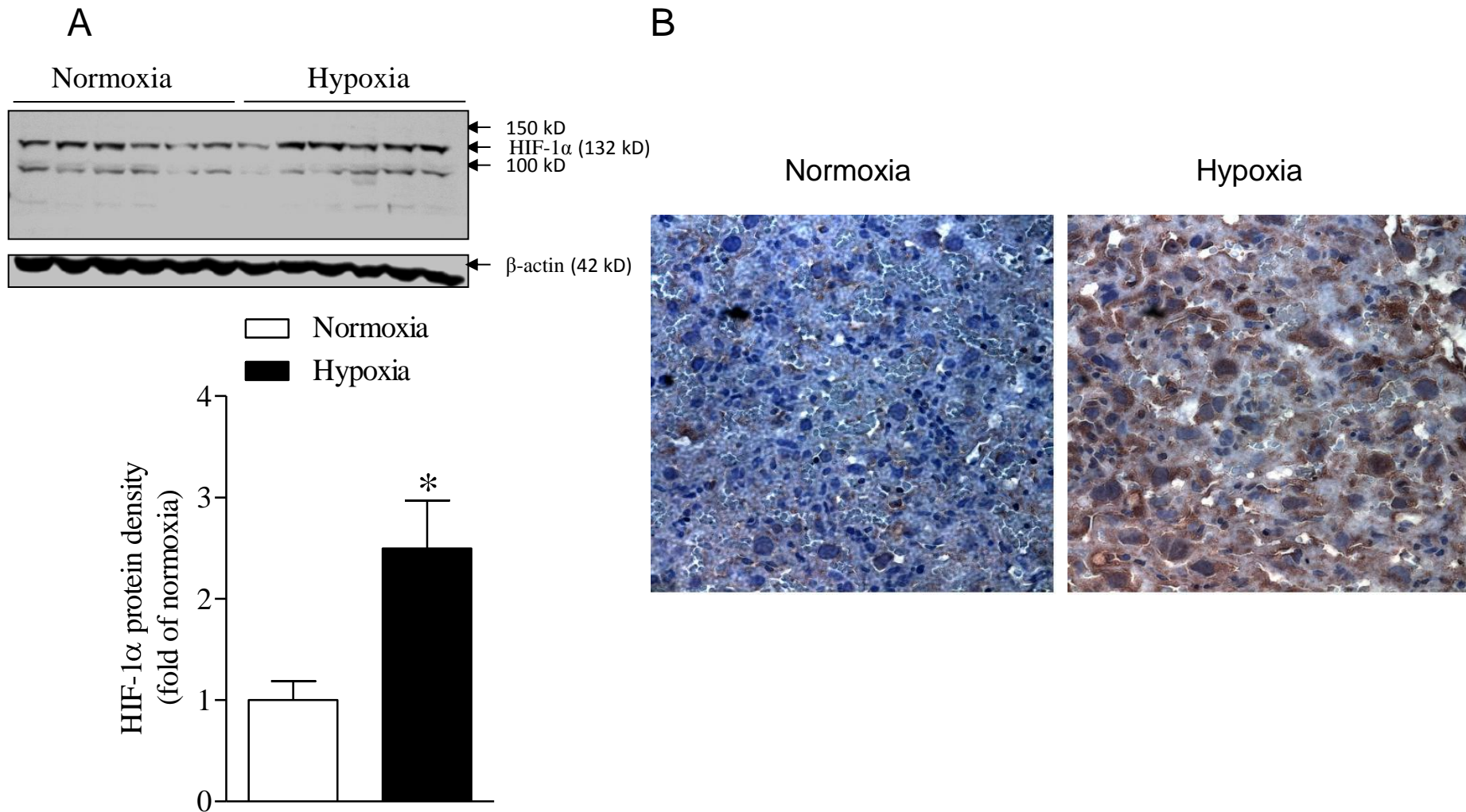
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**Online Figure S1. Expression of AT<sub>1</sub>R, AT<sub>2</sub>R, ET<sub>A</sub>R and ET<sub>B</sub>R in the placenta and kidney in normoxic pregnant rats at day 21 of gestation (×200).**

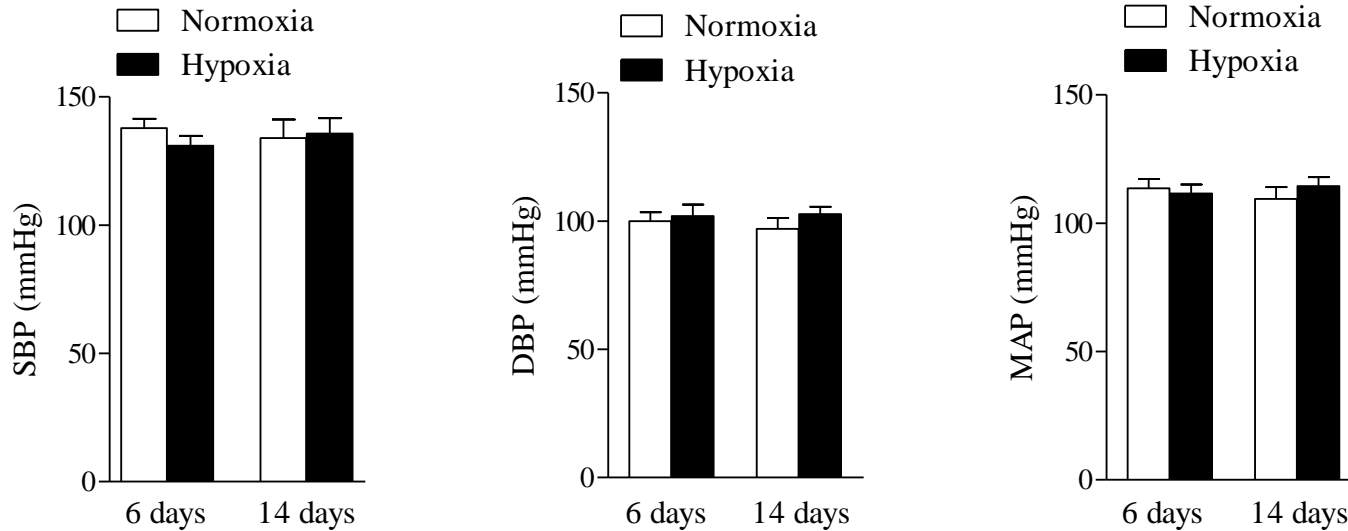


**Online Figure S2. Chronic hypoxia had no effect on plasma renin activity.** Plasma renin activity was measured in hypoxic and normoxic control pregnant rats at day 21 of gestation. Data are means  $\pm$  SEM, n = 6.

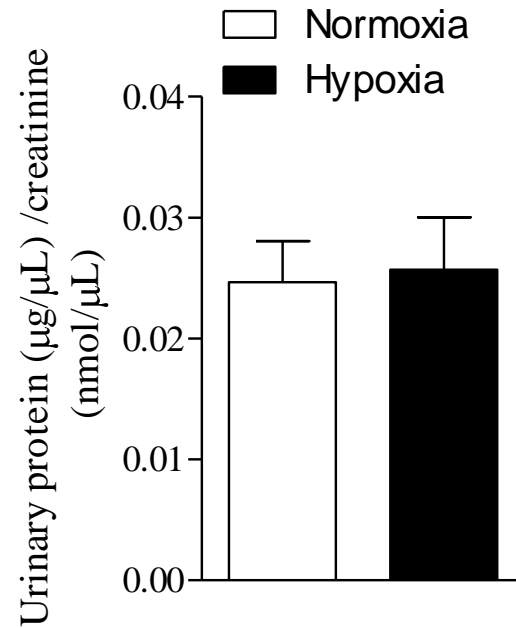


**Online Figure S3. Gestational hypoxia increased HIF-1 $\alpha$  expression in the placenta.** **A**, Protein abundance of HIF-1 $\alpha$  was determined in the placenta of hypoxic and normoxic control pregnant rats at day 21 of gestation. Data are means  $\pm$  SEM, n = 6. \* P < 0.05 versus normoxia. **B**, The placenta histology with HIF-1 $\alpha$  staining ( $\times 200$ ) was examined in hypoxic and normoxic control pregnant rats at day 21 of gestation.

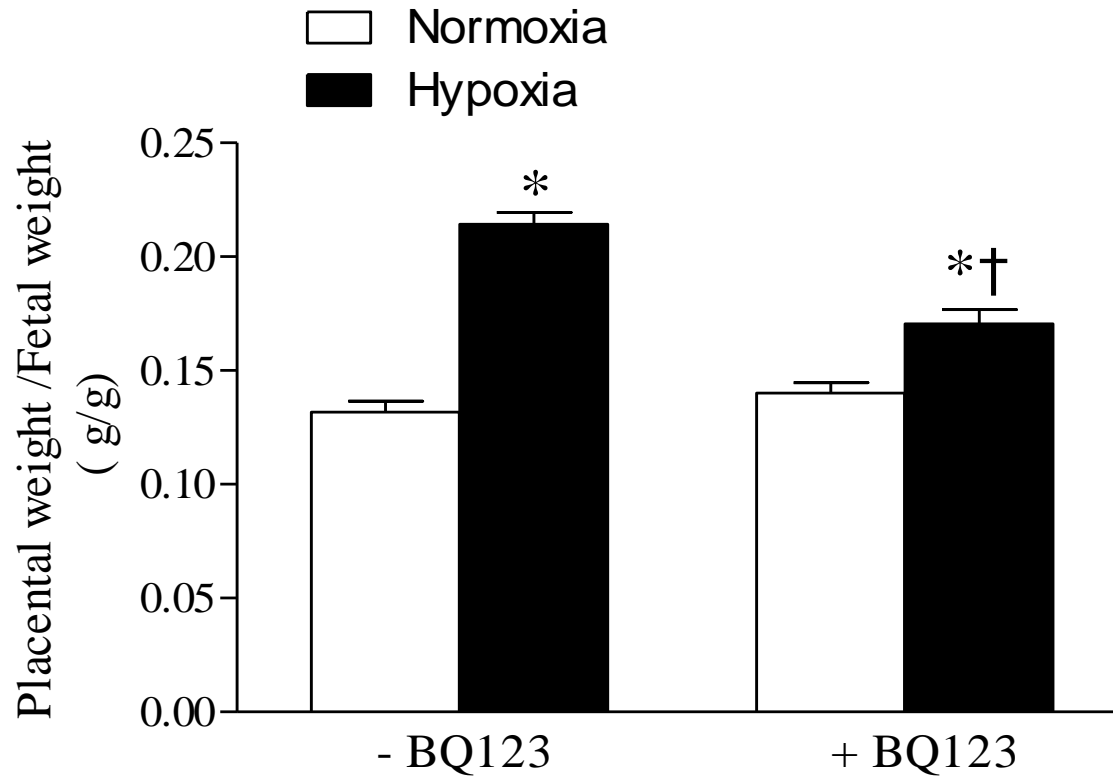




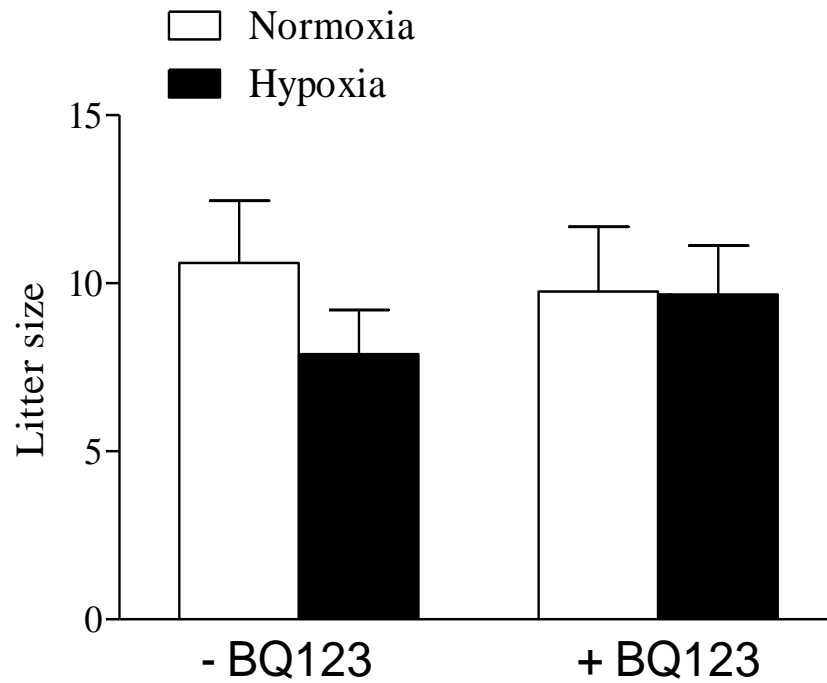
**Online Figure S4. Hypoxia treatment had no effect on blood pressure in non-pregnant rats.** Non-pregnant rats were treated with normoxic control or 10.5% O<sub>2</sub>, and systolic (SBP), diastolic (DBP) and mean (MBP) arterial blood pressure were measured at 6 days (equivalent to the duration of 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.



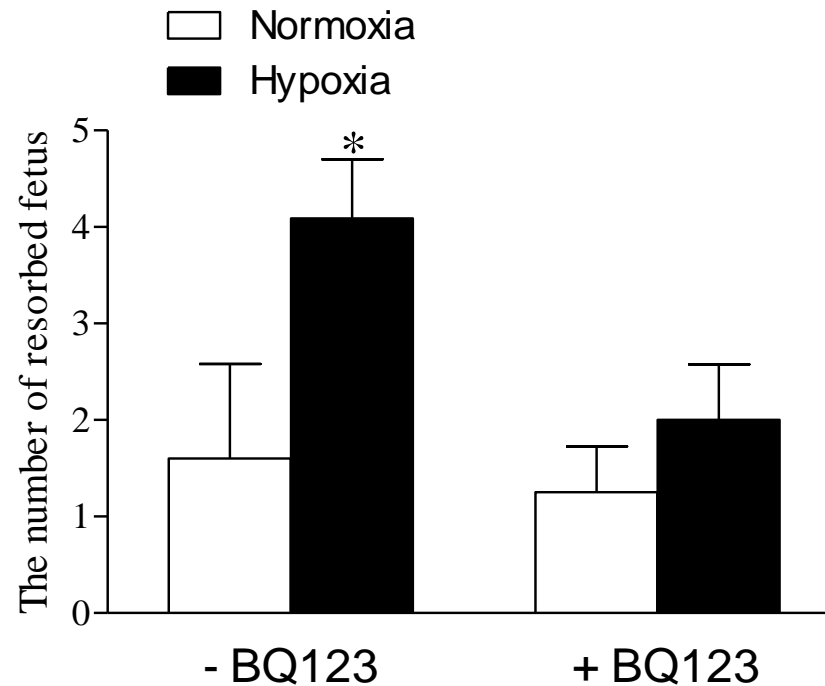
**Online Figure S5. Hypoxia treatment had no effect on proteinuria in non-pregnant rats.** Non-pregnant rats were treated with normoxic control or 10.5% O<sub>2</sub>, and 12-hour protein and creatinine levels were determined at 14 days (equivalent to the duration of hypoxic treatment of pregnant rats 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  $\pm$  SEM, n = 29-53. \* P < 0.05, versus normoxia; † P < 0.05, versus -BQ123.



**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  $\pm$  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  $\pm$  SEM, n = 5-6. \* P < 0.05, versus normoxia.