SUPPLEMENTAL MATERIALS

Sex-specific effect of endothelin in the blood pressure response to acute angiotensin II in growth-restricted rats.

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Running title: Endothelin, Sex, Ang II, and IUGR

MATERIALS AND METHODS

All experimental procedures were in accordance with National Institutes of Health guidelines with approval by the Animal Care and Use Committee at the University of Mississippi Medical Center. Offspring from 15 control pregnant and 17 reduced uterine perfusion pregnant litters were used to determine systemic and renal hemodynamics responses to acute Ang II plus and minus ABT 627 in male control (n=8) and growth-restricted (n=9) rats; and in female intact control (n=7) and female ovariectomized control (n=7), and female intact growth-restricted (n=9) and female ovariectomized growth-restricted (n=8) rats. A subgroup of males and female rats were analyzed simultaneously to measure concurrent actions of ET_A receptor blockade on blood pressure.

Animals. Rats were housed in a temperature-controlled room (23°C) with a 12:12-hour light/dark cycle with food and water available *ad libitum*. Timed pregnant Sprague Dawley (**SD**) rats were purchased from Harlan Inc (Indianapolis, IN). At day 14 of gestation rats destined for reduced uterine perfusion were clipped as described below. All dams were allowed to deliver at term with birth weight recorded within 12 hours of delivery. At this time the number of pups in the control and reduced uterine perfusion litter were culled to 8 pups per dam to ensure equal nutrient access for all offspring. Animals were weighed twice weekly. Pups were weaned at 3 weeks of age.

Reduced uterine perfusion in the pregnant rat. In brief and as previously described (1), at day 14 of gestation a silver clip (0.203-mm ID) was placed around the lower abdominal aorta above the iliac bifurcation and around each branch of the ovarian arteries (0.100-mm ID).

Ovariectomy in Female Offspring. Ovariectomy was performed at 10 weeks of age as previously described (2) with a sham operation involving visualization of the ovaries but no removal.

Drug administration. The ACE inhibitor, enalapril (40 mg/kg/day) (Sigma Aldrich, San Louis, Missouri, USA), was administered in the drinking water from 15 to 16 weeks of age at a dose previously shown to block endogenous production of angiotensin II (**Ang II**) in the rat (3). Water consumption was monitored daily for the duration of the treatment period and average daily water intake did not differ between same-sex animals during treatment with enalapril (data not shown). Prior to study all animals were pretreated with the angiotensin converter enzyme (ACE) inhibitor, enalapril, to investigate the blood pressure (**BP**) response to acute Ang II without participation of endogenous Ang II. Chronic enalapril resulted with all groups initiating with a similar baseline BP. At 16 weeks of age systemic and renal hemodynamics were measured in the conscious state during an acute infusion (30 minutes) of: a) 0.9% saline solution, b) Ang II (100ng/kg/min) in 0.9% saline solution, and c) Ang II (100ng/kg/min) plus or minus the ET_A receptor antagonist, ABT-627 (10 mg/kg/min in 0.9% saline solution) at a dose previously shown to reduce BP in the male (4) and pregnant female SD rat (5). The order of infusion of Ang II and Ang II + ABT-627 was interchanged in a subset of rats to ensure that pre-administration of Ang II did not alter the pressor response to co-administration of Ang II + ABT-627. Systemic and renal hemodynamic parameters were measured during each 30 minute infusion period. BP values were allowed to return to baseline between each infusion during 0.9% saline solution infusion. A subgroup of males and female rats were analyzed simultaneously to measure concurrent actions of ET_A receptor blockade on BP.

Measurement of systemic and renal hemodynamics. As previously described (1) animals were instrumented with flexible catheters (PE 50 tubing) in the right jugular vein for infusion and in the right carotid artery for measurement of BP and collection of blood; the bladder was also

instrumented with a flexible catheter (PE 90 tubing) for collection of urine. After a 24-hour recovery, renal function and arterial pressure measurements were performed in the conscious state with glomerular filtration rate (**GFR**) and effective renal plasma flow (**eRPF**) calculated from radioactivity of I^{125}-iothalamate and concentration of para-aminohippuric acid (PAH), respectively, in plasma and urine. Renal vascular resistance (**RVR**) and filtration fraction (**FF**) were calculated: RVR = (MAP / ERPF) X (1- hematocrit) and FF = (GFR / ERPF), respectively.

Measurement of PRA. PRA was measured by radioimmunoassay as previously described to confirm blockade of endogenous RAS (6).

Measurement of urinary endothelin. Urine was collected via 24 metabolism cages with quantitation of ET-1 determined using an Endothelin-1 QuantiGlo ELISA Kit (R&D Systems).

Western blot analysis for protein expression. As previously described (7) isolation of proteins and determination of protein expression was performed with ET_A and ET_B receptors antibodies purchased from Abcam, Cambridge, MA and the beta-Actin antibody was purchased from Sigma, St. Louis, MO. Secondary anti-rabbit and anti-mouse antibodies were purchased from GE Healthcare Bio-Science, PA. All ET receptor bands were normalized to beta-Actin control as described (8) and a molecular weight marker (BioRad, Precision Plus Protein ^{TM} Dual color Standards) was used to ensure identification of the correct band.

Determination of renal preproendothelin mRNA levels. Kidneys were snap frozen in liquid nitrogen and stored at −80°C. Total RNA was extracted using the ToTALLY RNA kit (Ambion). cDNA was synthesized from 1 μg of RNA with Bio-Rad Iscript cDNA reverse transcriptase and real-time PCR was performed using the Bio-Rad Sybre Green Supermix and iCycler. The following primer sequences were used for analysis of ppET: forward 1, ctaggtctaagcgatccttg, and reverse 1, tctttgtctgcttggc (Life Technologies). Level of mRNA expression was calculated using the mathematical formula for delta/delta CT recommended by Applied Biosystems (Applied Biosystems User Bulletin, No. 2, 1997).

Statistics. Statistical analysis was performed using unpaired Student's t test or 1 way ANOVA and Bonferoni's post hoc test was used to utilize for multiple comparisons. Graphpad PRISM version 5 was utilized for all statistical analysis. Statistical significance of interaction was set with $P<0.05$. All values are given as mean $\pm$ SEM.

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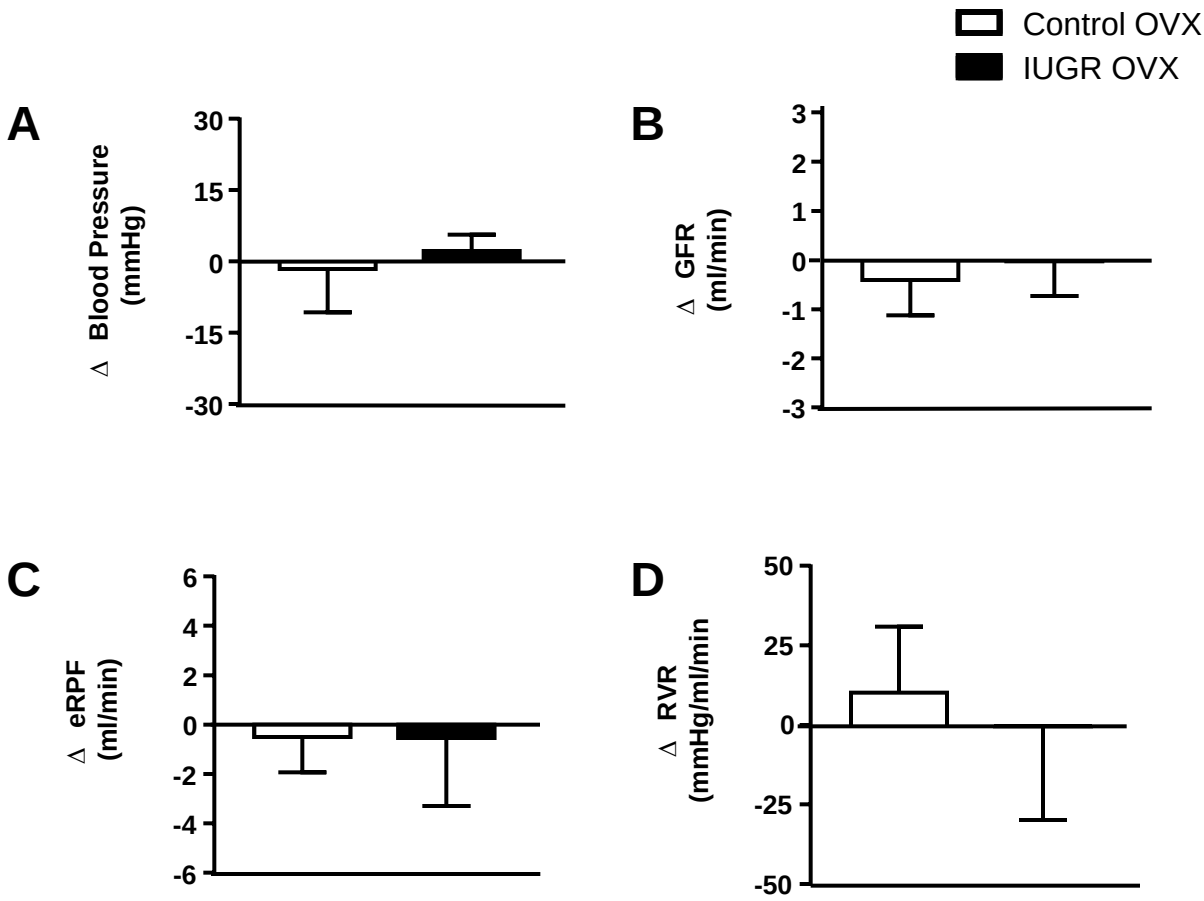

Figure S1. Difference in systemic and renal hemodynamic responses to acute Ang II versus Ang II plus ET_A receptor blockade in female ovariectomized offspring at 16 weeks of age. ***a)*** *Change in mean arterial pressure (**MAP**).* ***b)*** *Change in glomerular filtration rate (**GFR**).* ***c)*** *Change in effective renal plasma flow (**eRPF**).* ***d)*** *Change in renal vascular resistance (**RVR**).* Parameters were measured at 16 weeks of age in chronically instrumented, conscious animals pretreated with the angiotensin convertor enzyme inhibitor, enalapril (250mg/L for 1 week). MAP and renal hemodynamics were measured during an acute infusion of ANG II (100 ng/kg/min) for 30 min, and then during a 30 minute infusion of ANG II plus the ET_A receptor antagonist, ABT-627 (10 ng/kg/min for 30min). Values represent the difference between Ang II and Ang II plus ET_A receptor blockade in female control or growth-restricted (**IUGR**) ovariectomized rats. Data values represent mean±SEM.

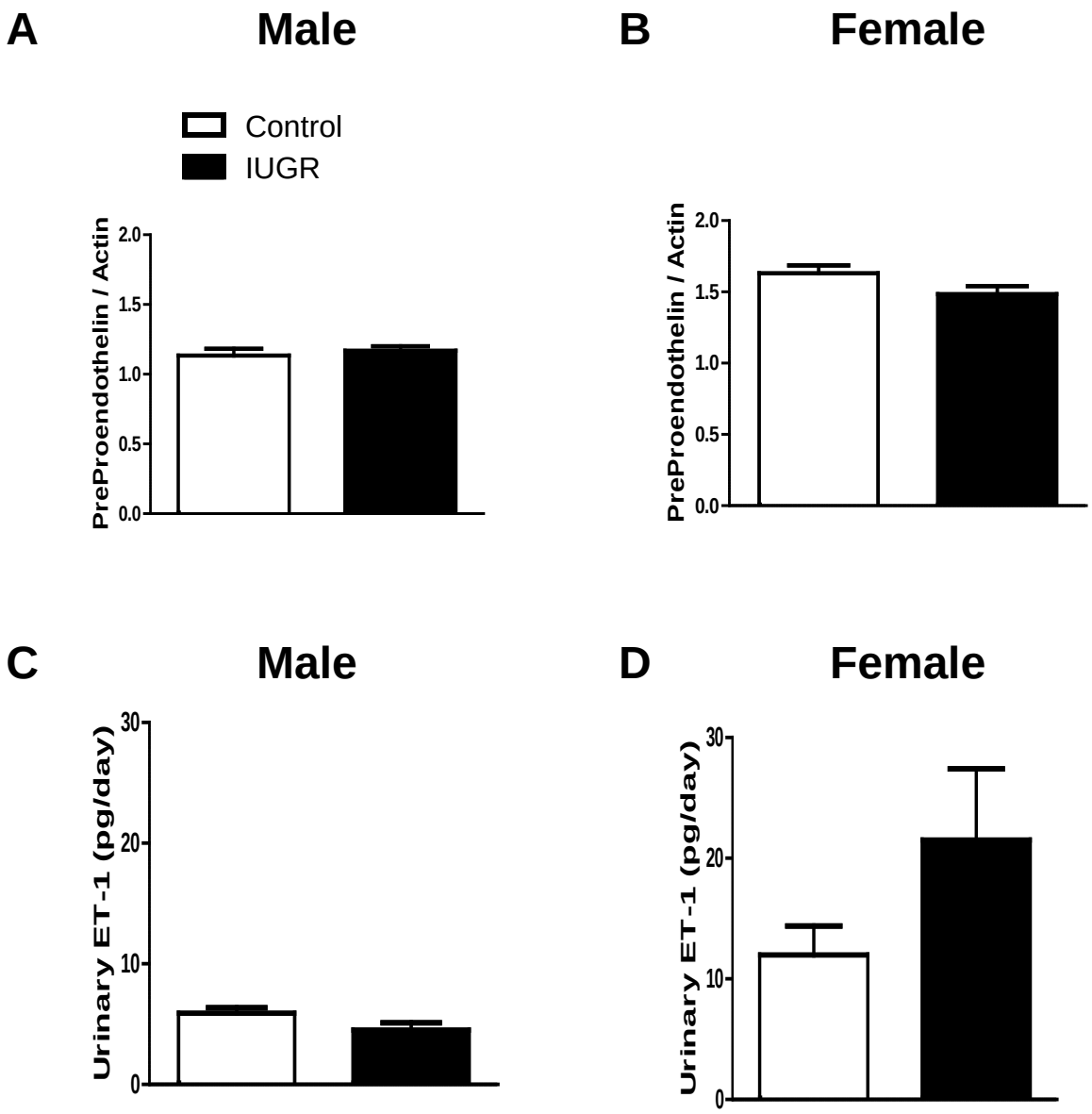

Figure S2. Renal preproendothelin (ppET) expression and urinary excretion of endothelin-1 (ET-1) in male and female control and intrauterine growth restricted (IUGR) offspring at 16 weeks of age. ***a)*** *Renal preproendothelin expression* ***b)*** *Urinary endothelin-1 excretion).* Values represent the difference between same-sex control and growth-restricted rats. Data values represent mean±SEM.

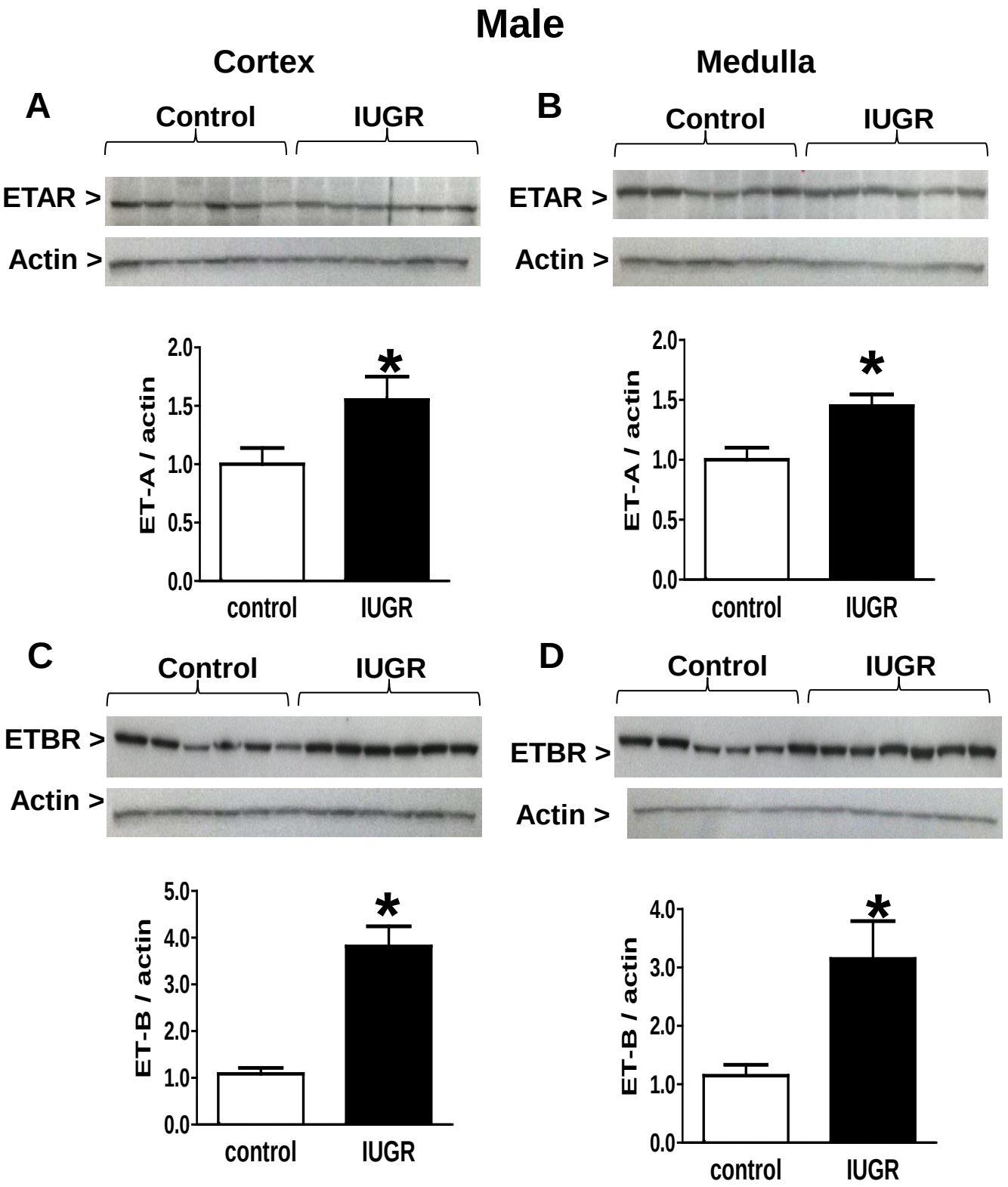

Figure S3. Renal protein expression of the endothelin receptor type A and B receptors (ET_A and ET_B) in male growth-restricted (IUGR) offspring at 16 weeks of age. Representative western blot for (a) quantification of renal cortex ET_A, (b) renal cortex ET_B, (c) renal medullary ET_A, (d) and renal medullary ET_B Values were normalized to beta-actin. **P*<0.05 versus control. Data values represent mean±SEM. n=4 in each group.

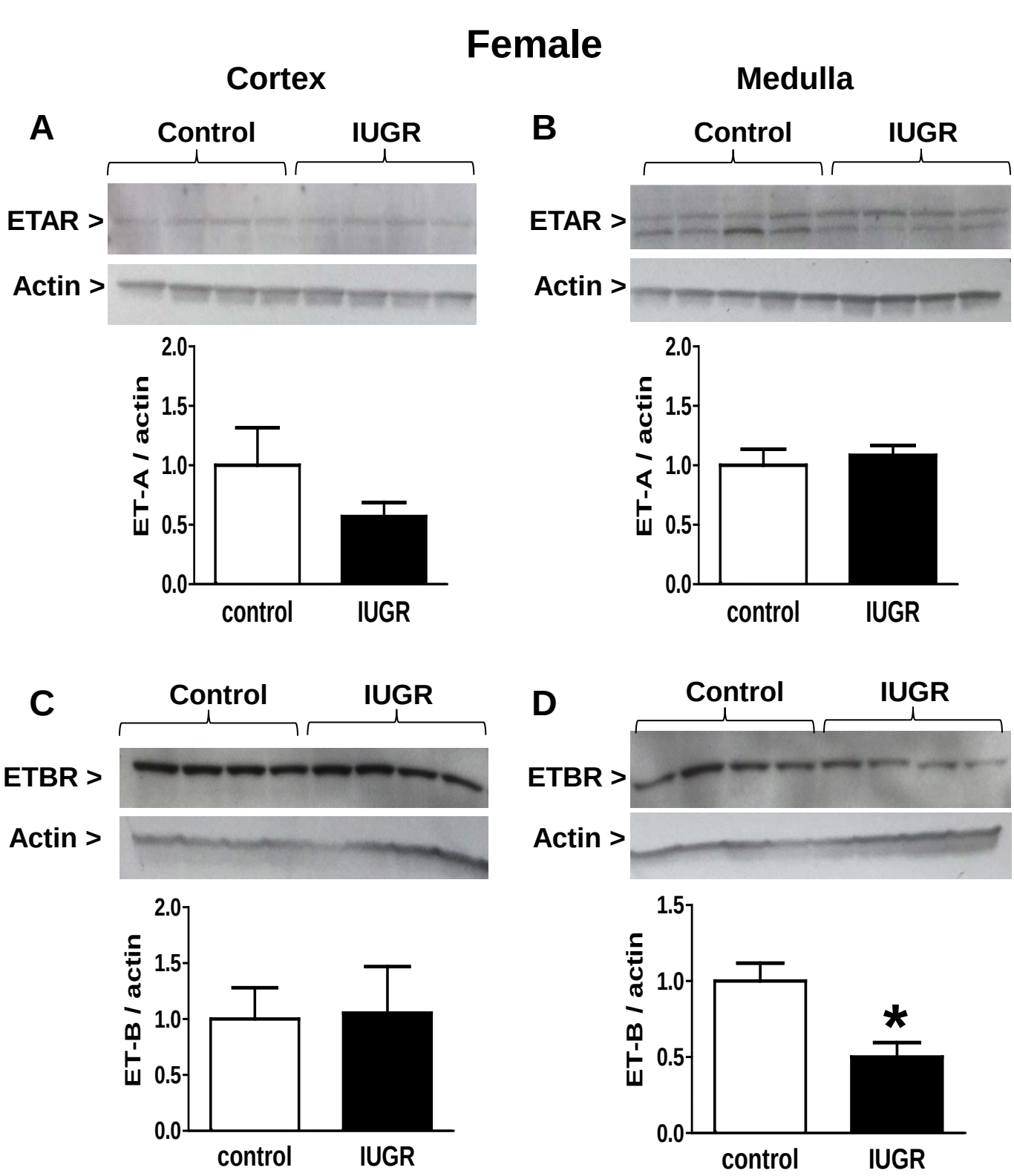

Figure S4. Renal protein expression of the endothelin receptor type A and B receptors (ET_A and ET_B) in female growth-restricted IUGR offspring at 16 weeks of age. Representative western blot for (a) quantification of renal cortex ET_A, (b) renal cortex ET_B, (c) renal medullary ET_A, (d) and renal medullary ET_B. Values were normalized to beta-actin. **P*<0.05 versus control. Data values represent mean±SEM. n=4 in each group.