Uteroplacental insufficiency alters nephrogenesis and downregulates cyclooxygenase-2 expression in a model of IUGR with adult-onset hypertension

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Baserga MA, Hale MA, Wang ZM, Yu X, Callaway CW, McKnight RA, Lane RH. Uteroplacental insufficiency alters nephrogenesis and downregulates cyclooxygenase-2 expression in a model of IUGR with adult-onset hypertension. Am J Physiol Regul Integr Comp Physiol 292: R1943–R1955, 2007. First published January 1, 2007; doi:10.1152/ajpregu.00558.2006.—Clinical and animal studies indicate that intrauterine growth restriction (IUGR) following uteroplacental insufficiency (UPI) reduces nephron number and predisposes toward renal insufficiency early in life and increased risk of adult-onset hypertension. In this study, we hypothesized that the inducible enzyme cyclooxygenase-2 (COX-2), a pivotal protein in nephrogenesis, constitutes a mechanism through which UPI and subsequent glucocorticoid overexposure can decrease nephron number. We further hypothesized that UPI downregulates the key enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), which converts corticosterone to inert 11-dehydrocorticosterone, thereby protecting both the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR) from the actions of corticosterone. Following bilateral uterine artery ligation on the pregnant rat, UPI significantly decreased renal COX-2, 11β-HSD2, and GR mRNA and protein levels, but upregulated expression of MR at birth. At day 21 of life, 11β-HSD2, GR, and also MR mRNA and protein levels were downregulated. UPI did not affect blood pressures (BP) at day 21 of life but significantly increased systolic BP in both genders at day 40. We conclude that in our animal model, UPI decreases fetal COX-2 expression during a period of active nephrogenesis in the IUGR rat, which is also characterized by decreased nephron number and adult-onset hypertension.

11β-hydroxysteroid dehydrogenase type 2; glucocorticoid receptor; mineralocorticoid receptor

UTEROPLACENTAL INSUFFICIENCY (UPI) follows many complications of pregnancy, such as hypertensive disorders and pre-eclampsia, and affects 3–10% of pregnancies in the Western society (60). Moreover, UPI causes an abnormal intrauterine environment exposing the fetus to stressors, such as hypoglycemia, hypoinsulinemia, acidosis, hypoxia, and decreased branched chain amino acids and results in intrauterine growth restriction (IUGR) (15).

In fetal hypoxia, peripheral blood flow and blood flow to the kidneys are reduced to maintain brain, heart, and adrenal perfusion. This leads to a condition termed “asymmetrical growth restriction.” This particular environment in the human fetus has been associated with increased risk to develop short-term morbidities, including hypertension (4, 54). Interestingly, both human and animal studies, including our animal model of UPI following bilateral uterine artery ligation in the pregnant rat, have shown that IUGR results in smaller kidneys with decreased nephron number (2, 9, 49, 56). Also, similar to IUGR infants, UPI in our animal model is associated with a significant increase in circulating corticosterone levels at birth that persist through day 21 of life (juvenile rat) (7, 16).

Several mechanisms regulate pathways that determine nephron number, and UPI affects multiple components of the fetal milieu; so a single mechanism responsible for abnormal kidney development is unlikely. For instance, our group has previously reported an increase in kidney apoptosis associated with decreased nephron number in IUGR rat pups (6, 49). In the present study, we focus upon another candidate mechanism that can disrupt nephrogenesis, the glucocorticoid pathway. Key participants of this pathway in the kidney are circulating corticosterone levels, the enzyme 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2), the glucocorticoid receptor (GR), and the mineralocorticoid receptor (MR). The enzyme 11β-HSD2 converts corticosterone to inert 11-dehydrocorticosterone in the kidney, thereby protecting both the GR and the MR from the actions of corticosterone (20). Interestingly, 11β-HSD2 deficiency or reduced activity causes hypertension in both humans and animal models, through overactivation of the MR by corticosterone with renal sodium retention, and salt-sensitive increase in blood pressure (18, 26).

In the human fetus, active nephrogenesis occurs at 15–24 wk gestational age and is completed by 32 wk (29). In the rat kidney, formation of nephrons continues for an additional 6–8 days after birth, which makes the newborn rat a good model to study active nephrogenesis (52). Of particular interest during this timeframe is the inducible enzyme cyclooxygenase-2 (COX-2), which is known to play a critical role in human nephrogenesis, as well as in rat kidney development (37). Fetal and adult kidneys are among the few organs that constitutively express COX-2, suggesting that its presence is key for normal renal architecture and function (30, 39). This enzyme’s expression is highly affected by glucocorticoid levels, which are, in turn, highly affected by the IUGR milieu (7, 16). Thus, corticosterone, through the GR, can also affect nephrogenesis by regulating the expression of the COX-2 mRNA levels.

We therefore hypothesized that IUGR, and subsequent glucocorticoid overexposure of the fetus, leads to decreased kid-
Animals preformed on bilateral uterine artery ligation (IUGR) and control surgery are number predisposes toward adult-onset hypertension.

Changes in the glucocorticoid pathway and reduced nephron -HSD2, GR, and MR expression levels. Furthermore, we 11/H9252 kidney COX-2 expression and protein levels, as well as affects 11β-HSD2, GR, and MR expression levels. Furthermore, we hypothesized that the previous association of developmental changes in the glucocorticoid pathway and reduced nephron number predisposes toward adult-onset hypertension.

To test these hypotheses, we induced IUGR using a well-established rat model of UPI (5, 32, 33, 36). In this model, bilateral uterine artery ligation (IUGR) and control surgery are preformed on day 19 of gestation in Sprague-Dawley rats (term, 21.5 days). For this study, we quantified the effects of IUGR upon kidney COX-2, 11β-HSD2, GR, and MR mRNA expression and protein levels at day 0, day 8 (completion of rat nephrogenesis) and day 21 of life (juvenile rat). Localization of these gene products was investigated by immunohistochemistry. We then measured systolic (SBP), diastolic (DBP), and mean (MBP) blood pressure using the tail cuff method at day 21 and day 140 of life.

METHODS

Animals

All procedures were approved by the University of Utah Animal Care Committee and are in accordance with the American Psychological Society’s Guiding Principles (1). These surgical methods have been previously described (49, 55). In brief, on day 19 of gestation, the maternal rats (Sprague-Dawley) were anesthetized with intraperitoneal xylazine (8 mg/kg) and ketamine (40 mg/kg), and both inferior uterine arteries were ligated (IUGR) (n = 12 litters). Sham surgery was performed in control animals that underwent identical anesthetic and surgical procedures except for the uterine artery ligation (control) (n = 12 litters). Rats recovered within a few hours and had ad libitum access to food and water. At term (21.5 days gestation), day 0 (P0) pups were delivered by caesarian section, weighed, and decapitated (n = 6 litters IUGR and control, respectively). One pup from each litter was used for all day 0 studies. The selection of the pups was done randomly, without knowledge of size or position in the uterine horn. To study 8-day-old (P8) and 21-day-old (P21) rats, the remaining maternal rats were allowed to deliver spontaneously at term (n = 6 litters IUGR and control, respectively), and litters were randomly culled to six pups. Based on our previous studies where we observed that operated dams are able to provide normal lactation, we did not cross foster to nonoperated female rats. Furthermore, our group has recently reported the breast milk content of operated dams compared with control nonoperated dams measured after dams were separated (6 litters IUGR and control, respectively). One pup from each litter was used for all day 0 studies. The selection of the pups was done randomly, without knowledge of size or position in the uterine horn. To study 8-day-old (P8) and 21-day-old (P21) rats, the remaining maternal rats were allowed to deliver spontaneously at term (n = 6 litters IUGR and control, respectively), and litters were randomly culled to six pups. Based on our previous studies where we observed that operated dams are able to provide normal lactation, we did not cross foster to nonoperated female rats. Furthermore, our group has recently reported the breast milk content of operated dams compared with control nonoperated dams measured after dams were separated from day 21 pups. Breast milk from dams that underwent the IUGR surgery did not significantly differ from control breast milk in terms of caloric, fat, protein, zinc, and sodium content (27).

At P8 and P21, one male and one female pup from each litter was selected randomly and separated from their dams, anesthetized, and killed. For P8 and P21, both male and female rats were included in the study in equal numbers (n = 6 male and 6 female animals, for IUGR and control groups, respectively). Pup gender was determined by dissection and visualization. For all dates, kidneys were quickly harvested and frozen in liquid nitrogen or placed in 10% formalin.

RNA Isolation

DNase I-treated total RNA (Ambion, Austin TX) was extracted from 30–100 mg of P0, P8, and P21 IUGR and control rat pup kidneys using the NucleoSpin RNA and Virus Purification Kit (BD Biosciences Clontech, Palo Alto, CA). Total RNA was quantified using the NanoDrop Spectrometer ND-1000 (NanoDrop Technologies, Wilmington, DE). RNA integrity was confirmed by gel electrophoresis.

Real-Time RT-PCR

Kidney mRNA levels of 11β-HSD2 and COX-2 were measured on P0, P8, and P21, and kidney mRNA levels of GR and MR on P0 and P21 using the real-time RT-PCR method as previously described (36). cDNA was synthesized using random hexamers and SUPERSPSCRIPT III RT (Life Technologies, Gaithersburg, MD) from 1 to 4.0 μg of DNase-treated total RNA as described above. Primers and probes for 11β-HSD2, GR, MR, COX-2, and GAPDH were designed using Primer Express Software (Applied Biosystems, Foster, CA). The sequences for GAPDH were forward: 5′-CAAGATGGTGAGGTCCGGTGT-3′; reverse: 5′-CAAAGAAGGAGGCCAGCTGTG-3′; and probe: 5′-CCGTCGGATACGGCAAAATCCG-3′. The sequences for GR were forward: 5′-GGACAGCCTGACTCTTGGG-3′; reverse: 5′-TCAAGGGCTTGAAGTACCAT-3′; and probe: 5′-TGAATTCG-GAGCCTGCTAGGTG-3′. Assay-on-Demand no. for 11β-HSD2 was Rh00492539_m1, for COX-2 was Rh00565825_m1, and for MR was Rh00565626_m1. Target probes were labeled with fluorescent reporter dye FAM. Reporter dye emission is detected by an automated sequence detector combined with ABI Prism 7900 Sequence Detection System software (Applied Biosystems). An algorithm normalizes the reporter signal (Rn) to a passive reference and multiplies the standard deviation of the background Rn in the first cycles by a default factor of 10 to determine threshold (Ct). Ct has a linear relation with the logarithm of the initial template copy number. Rat GAPDH was used as an internal control to correct for differences in cDNA loading. Relative quantification of PCR products is then based upon value differences between the target and GAPDH control using the comparative Ct method (38). Cycle parameters were 50°C × 2 min, 95°C × 10 min, and then 40 cycles of 95°C × 15 s and 60°C × 60 s. Each sample was run in quadruplicate.

Immunoblotting and Antibodies

Whole kidneys were obtained from P0, P8, and P21 IUGR and control rat pups. Total protein was isolated by homogenizing 30–100 μg of tissue in RIPA buffer (150 mM NaCl, 50 mM Tris pH 7.4, 1 mM EDTA, 0.25% Na-deoxycholate, 1% Igepal CA-630) with EDTA protease inhibitor (400 μl) (Roche, Mannheim, Germany), centrifuged at 10,000 g for 15 min at 4°C. The supernatants were collected and stored at –80°C until use. Total protein concentration was determined using the BCA Protein Assay Kit method (Pierce, Rockford, IL). Total protein (20–50 μg) and molecular weight markers were loaded and separated by XT Criterion gels (Bio-Rad Laboratories, Hercules, CA) at 200 V for 60 min. After gel electrophoresis, the proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA) for 1 h at 100 V or 4°C overnight. Posttransfer, the membranes were blocked in either 5% milk Tris-buffered saline-Tween (TBS-T) or 5% BSA TBS-T for 1 h and then washed multiple times in 1× TBS-T. After blocking, bound proteins were exposed to antibodies against 11β-HSD2 (Alpha Diagnostics International, San Antonio, TX) 1:100 in 5% milk; COX-2 (Abcam, Cambridge, MA) 1:1000 in 5% BSA; GR (Abcam), 1:500 in 5% milk; MR (Santa Cruz Biotechnology, Santa Cruz, CA) 1:200 in 5% milk; and GAPDH (Abcam) 1:2000 in 5% milk. Blots were incubated overnight at 4°C or at room temperature for 1 h. After multiple wash steps in TBS-T, membranes were probed with secondary antibody; horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, Beverly, MA) 1:2000 (1:5000 GAPDH; anti-mouse (Cell Signaling Technology) 1:2000; bovine anti-goat (Santa Cruz Biotechnology) 1:2000 in either 5% milk or 5% BSA for 1 h at room temperature. After multiple wash steps in TBS-T, antibody signals were detected with Western Lighting enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA) and quantitated using a Kodak Image Station 2000R (Eastman Kodak/SIS, Rochester, NY). GAPDH signal was used to normalize samples.
**Immunohistochemistry**

P0 and P21 IUGR and control rat kidney sections were deparaffinized and dehydrated in a graded series of ethanol and water. The slides were then incubated in a preheated, high pH, antigen retrieval buffer (DakoCytomation, Carpinteria, CA), in a Coplin jar in a 95°C water bath and then washed with water. Sections were incubated in a 3% H₂O₂ solution for 15 min at room temperature (20–22°C) to quench endogenous peroxidase activity. After being rinsed with tap water, sections were washed in 0.1% PBS (Sigma, St. Louis, MO) for 5 min. Next, the slide sections were blocked for endogenous avidin and biotin by incubating with avidin and biotin solutions (Vector Laboratories, Burlingame, CA) for 15 min at room temperature and then washed in PBS three times. Sections were then blocked using a blocking buffer (0.5% Casein in PBS, Sigma) at room temperature for 30–60 min. Control and IUGR sections were then treated with sheep polyclonal antibody 11β-HSD2 (Chemicon International, Temecula, CA) 1:2,000 in PBS; rabbit polyclonal antibody GR (Abcam), 1:500 in PBS; rabbit polyclonal antibody MR (Santa Cruz Biotechnology) 1:200 in PBS; rabbit polyclonal antibody COX-2 (Abcam) 1:500 in PBS. Antibody specificity was tested by treating negative control sections with appropriate secondary antibodies (Vector Laboratories, Burlingame, CA) 1:200 in PBS, normal serum (Rabbit IgG/FITC DakoCytomation) 1:200 in PBS, and avidin and biotin blocking buffer (Vector Laboratories) straight. Positive control sections were treated with anti-pan cytokeratin (Sigma Aldrich, St. Louis, MO) 1:1:000 in PBS. Each slide was incubated with the primary antibody overnight at 4°C in a humidified chamber. The next day, sections were washed in PBS and were exposed to the appropriate biotinylated secondary antibody in PBS containing 10% Tween-20 for 1 h at room temperature (Vector Laboratories) and washed in PBS. Following exposure to a TSA Biotin System (PerkinElmer), slides were washed in PBS for 15 min, stained with DAB (Sigma), counterstained with hematoxylin (Sigma), dehydrated in a graded series of ethanol and coverslipped with Cytoseal 60 (Stephens Scientific, Kalamazo, MI).

The slides were observed with an Axiosplan microscope (Zeiss, Germany) and photographed with an Olympus DP70 camera. Photos were captured with computer software (DP controller and DP Manager) and processed with Adobe Photoshop.

**Blood Pressure Measurements**

SBP, DBP, and MBP were measured in unanesthetized male and female offspring (12 litters: 6 IUGR and 6 control) at day 21 and day 140 of life by the indirect tail cuff method (blood pressure analysis system model MC4000; Hatteras Instruments, Cary, NC). The rats received careful training by one operator and were periodically acclimated to restraint and tail cuff inflation starting at day 10 of life, as previously described (58).

**Statistics**

All data presented are expressed as means ± SE. Western blot analysis and real-time RT-PCR were analyzed using ANOVA (Fisher’s protected least significance difference test), and Student’s unpaired t-test as applicable. We accepted P < 0.05 for statistical significance.

**RESULTS**

In our animal model of UPI, IUGR pups weigh 20–25% less than the control-operated animals (IUGR: 4.00 ± 25 vs. sham: 5.25 ± 22, P < 0.05), with birth weights normally distributed within and among litters. Litter size does not differ significantly between control and IUGR groups (5).

**Real-time RT-PCR and Western Blot Analysis Results**

UPI decreases kidney COX-2 mRNA and protein levels in newborn IUGR rats. UPI significantly decreased kidney COX-2 mRNA expression in IUGR pups at P0 to 79 ± 7% of control values (P < 0.05). However, at P8 and P21, there was no significant difference in COX-2 mRNA expression between IUGR and control kidneys, both in male and female rats (P8 males: 92 ± 5 and P8 females: 102 ± 2% of control values; P21 males: 93 ± 6 and P21 females: 117 ± 3% of control values). Consistent with the real-time RT-PCR results, UPI significantly decreased IUGR kidney protein levels of COX-2 at P0 in IUGR rats to 69 ± 1% of control values (P < 0.001). Interestingly, the decrease in COX-2 protein levels was still significant in P8 IUGR males (81 ± 5% of control values (P < 0.05) but not in P8 IUGR females compared with control (108 ± 7% of control)). COX-2 protein levels were not statistically different in P21 male and female IUGR rats compared with control animals (77 ± 16 and 125 ± 31% of control values, respectively). UPI decreases kidney 11β-HSD2 mRNA and protein levels in IUGR rats. UPI significantly decreased kidney 11β-HSD2 mRNA expression in IUGR pups at P0 to 78 ± 6% of control values (P < 0.05). The decrease in kidney 11β-HSD2 persisted both in male and female IUGR rats at P8 (79.7 ± 7, P < 0.05, and 72 ± 6, P < 0.01, % of control values, respectively) and
in male and female IUGR rats at P21 (73 ± 1, P < 0.05, and 69 ± 7, P < 0.05, % of control values, respectively) (Fig. 1B). Concordant with the real-time RT-PCR results, UPI significantly decreased IUGR kidney protein levels of 11β-HSD2 at P0, P8, and P21, both in male and female IUGR rats (P0: 73 ± 5, P < 0.01, % of control values; P8 males: 87 ± 4, P < 0.05, and P8 females: 81 ± 5, P < 0.05, % of control values; P21 males: 65 ± 10, P < 0.05, and P21 females: 52 ± 9, P < 0.01 % of control values; Fig. 2B).

UPI decreases kidney GR mRNA and protein levels in IUGR rats. UPI significantly decreased kidney GR mRNA expression in IUGR pups at P0 to 72 ± 9% of control values (P < 0.05). Interestingly, the decrease in GR mRNA expression persisted through P21 in the IUGR kidney, both in male and female rats (P21 males: 60 ± 8, P < 0.01, and P21 females: 74 ± 11, P < 0.05, % of control values; Fig. 1C). Following UPI, kidney GR protein levels were significantly decreased at P0 in IUGR rats to 76 ± 5, P < 0.01, % of control values, and at P21 in both males and females (30 ± 5, P < 0.001, and 66 ± 10, P < 0.05, % of control, respectively; Fig. 2C).

UPI affects kidney MR mRNA and protein levels in IUGR rats. UPI significantly increased kidney MR mRNA expression in IUGR pups at P0 to 119 ± 8% of control values (P < 0.05). However, MR mRNA expression was significantly decreased in the IUGR kidney at P21 both in male and female rats (P21 males: 58 ± 7, P < 0.01, and P21 females: 65 ± 7, P < 0.05, % of control values; Fig. 1D). UPI significantly increased IUGR kidney protein levels of MR at P0 to 122 ± 2% of control values (P < 0.001). However, MR protein levels were not statistically different in P21 male and female IUGR rats compared with control animals (P21 males: 110 ± 12 and P21 females: 104 ± 20% of control values, respectively; Fig. 2D).

Effect of Sex Upon COX-2 and 11β-HSD2 Protein Expression in Control Animals at P8 and P21

Control males had decreased kidney COX-2 protein levels at P8 compared with control females (78 ± 6, P < 0.05, % of female control values). However, at P21 this difference was not statistically significant (81 ± 8% of female control values). Regarding 11β-HSD2 protein levels, at P8 we observed no significant difference between sexes (103 ± 6% of female control values). However, by P21 kidney 11β-HSD2 protein levels were increased in male vs. female control rats (141 ± 8, P < 0.05, % of control values; Fig. 3).

Immunohistochemistry for Localization of Kidney COX-2, 11β-HSD2, GR, and MR

P0 findings. The immunohistochemistry findings in the present study show that COX-2 and 11β-HSD2 are expressed at high levels in the rat kidney during nephrogenesis. At P0, COX-2 was found to be expressed in the macula densa and
ascending limb of loops of Henle in the cortex of control and IUGR kidneys (Fig. 4, A and B, respectively) and in the collecting ducts and vasculature in the inner medulla (Fig. 5, A and B). At this point of nephrogenesis, 11β-HSD2 immunolabeling was associated with epithelial cells of cortical proximal and distal convoluted tubules (Fig. 4, C and D) and in the inner medulla collecting tubules (Fig. 5, C and D). Labeling for both COX-2 and 11β-HSD2 was significantly decreased in the IUGR kidney, both in the cortical and medullar regions. Regarding GR expression, at P0, immunoreactivity was mainly associated with nuclei in scattered cortical glomeruli and cytoplasmatic distribution in proximal tubules and macula densa of distal convoluted tubules (Fig. 4, E and F). Expression of GR in the medulla was positive in the collecting ducts and thin limbs, as well as nuclei of interstitial cells (Fig. 5, E and F). Again, overall GR immunolabeling was less pronounced in the IUGR rat kidney. The pattern of MR expression was significant for marked expression within epithelial cells of collecting ducts with very little expression in extratubular sites (Fig. 4, G and H; and Fig. 5, G and H). In contrast to GR, MR expression was more prominent in the IUGR rat kidney cortex and medulla.

**p21 findings.** The pattern of distribution of COX-2, 11β-HSD2, GR, and MR was not different between males and females when comparing IUGR vs. control kidneys; thus we are only showing male data. In general, immunolabeling of COX-2 was more prominent in the medullar interstitial cells and was almost absent in the cortical area. There was no significant difference between control and IUGR COX-2 expression (Fig. 6, A and B and Fig. 7, A and B).

The 11β-HSD2 pattern of expression also differed from P0 kidneys in that there was an overall decrease in immunolabeling with a redistribution mainly within epithelial cells of collecting ducts and less so in the cortical tubules. This labeling was less pronounced in the IUGR medulla (Fig. 6, C and D and Fig. 7, C and D). GR expression was associated with nuclei and cytoplasmatic labeling along the cortical tubules and medullary loops of Henle, as well as some collecting ducts. Again, IUGR kidneys displayed a significant reduction in GR immunolabeling (Fig. 6, E and F and Fig. 7, E and F). Finally, MR localization at P21 was similar to the areas of expression in P0 kidneys, i.e., principally observed within epithelial cells of collecting ducts. However, in contrast to P0, MR expression at P21 was significantly decreased in the IUGR kidney (Fig. 6, G and H and Fig. 7, G and H).

**Effect of IUGR on SBP, DBP, and MBP**

Blood pressure was not significantly different at P21 in male and female IUGR rats compared with control (males: SBP = 107 ± 25 vs. 102 ± 12, DBP = 75 ± 21 vs. 76 ± 13, MBP = 91 ± 15 vs. 89 ± 12; females: SBP = 112 ± 18 vs. 108 ± 14, DBP = 85 ± 15 vs. 76 ± 18, MBP = 93 ± 12 vs. 83 ± 11, IUGR vs. control, respectively). However, by day 140 of life, a significant increase in arterial blood pressure was evident in growth-restricted male and female offspring compared with control. A marked elevation in SBP and MBP was present in both IUGR males and females (males: SBP = 176 ± 7 vs. 159 ± 4, P < 0.001; MBP = 145 ± 15 vs. 131 ± 8, P < 0.05; females: SBP = 149 ± 9 vs. 128 ± 7, P < 0.01; MBP = 113 ± 12 vs. 103 ± 10, P < 0.05; IUGR vs. control, respectively). However, DBP was only significantly elevated in IUGR females at day 140 of life [males: DBP = 132 ± 21 vs. 126 ± 20; females: DBP = 129 ± 15 vs. 91 ± 18 (P < 0.05), IUGR vs. control, respectively] (Fig. 8).

**DISCUSSION**

UPI complicates ~6% of all pregnancies and is the second leading cause of perinatal death after prematurity. Among renal morbidities, IUGR can lead to fetal renal failure and oligohydramnios (46, 56). As adults, asymmetrically growth-restricted humans are at risk for developing hypertension (4, 23).

Although several studies in humans and in different animal models have reported the short- and long-term renal morbidities associated with an abnormal intrauterine milieu and IUGR, the responsible mechanisms underlying altered nephrogenesis, abnormal renal function, and adult-onset hypertension have not been fully investigated (2, 8, 61).

Importantly, we have recently demonstrated that the compromised in utero environment (acidosis, hypoxia) is also associated with increased serum levels of corticosterone in the IUGR rat pup that persists through day 21 in the juvenile rat (7). Therefore, a single mechanism responsible for abnormal kidney development is unlikely (15, 43). With this in mind, we have investigated different mechanisms that can be responsible for permanent changes in cellular biology and systemic physiology in the IUGR rat kidney. For example, we have recently demonstrated that UPI induces an increase in apoptosis as evidenced by increased p53 phosphorylation without a corresponding increase in murine double-minute 2 expression in the IUGR kidney (6). These novel findings suggest a molecular mechanism through which UPI induces fetal renal apoptosis and a permanent loss in glomeruli.

Using this animal model of IUGR, the present study focuses upon the glucocorticoid pathway and its possible role underlying abnormal nephrogenesis and the adult development of hypertension through downregulation of COX-2.

Other animal models of fetal exposure to glucocorticoids include the antenatal maternal administration of dexamethasone in sheep and rats. These studies have shown reduced
nephron number and decreased glomerular filtration rate in the dexamethasone offspring, as well as adult-onset hypertension (17, 51, 59). However, these reports did not investigate molecular mechanisms involving the glucocorticoid pathway.

The most important novel molecular findings from the present study are that UPI induces a decrease in renal COX-2 mRNA and protein levels at birth, as well as a decrease in kidney 11β-HSD2 mRNA and protein levels at P0, which

Fig. 4. Histological sections of kidney cortex from IUGR and control rats at day 0 of life. Top: I, positive control section (anti-Pan Cytokeratin); II, negative control section. Localization of COX-2 positive cells (brown staining) associated with the macula densa of distal convoluted tubes and ascending limb of loops of Henle in the cortex in control (A) and IUGR (B) kidneys; 11β-HSD2 positive cells (brown staining) observed in epithelial cells of cortical proximal and distal convoluted tubules in control (C) and IUGR (D) kidneys; GR positive cells (brown staining) mainly associated with nuclei in cortical glomeruli and cytoplasmatic distribution in proximal tubules and macula densa in control (E) and IUGR (F) kidneys; and MR positive cells (brown staining) with marked expression within epithelial cells of proximal convoluted tubules and collecting ducts in control (G) and IUGR (H) kidneys. Following UPI, COX-2, 11β-HSD2, and GR, immunoreactivity was less widely distributed and decreased in intensity in IUGR kidney cortex. MR immunoreactivity was more prominent in the control cortex. Arrows denote representative positive cells. Scale bar = 500 μm.
persists through postnatal day 21 in the juvenile male and female IUGR rats. Further findings include 1) a decrease in kidney GR expression and protein levels at P0 and P21, and 2) a significant increase in kidney MR mRNA and protein levels at birth but decreased levels of MR mRNA in the juvenile rat at P21.

The mechanism by which the upregulation of the glucocorticoid pathway can affect nephrogenesis is intriguing. For instance, rat renal cell cultures treated with GR-specific dexamethasone showed a significant decrease in COX-2 protein levels (37).

Based on previous studies that highlight the importance of COX-2 during renal development, we suggest that downregulation of this enzyme may play a significant role in the IUGR kidney pathology. Interestingly, COX-2 knockout mice have marked renal pathology including hypoplasia and tubular atrophy (14). These results were reproduced by Norwood et al.

Fig. 5. Histological sections of kidney medulla from IUGR and control rats at day 0 of life. Top: I, positive control section (anti-Pan Cytokeratin); II, negative control section. Localization of COX-2 positive cells (brown staining) associated with collecting ducts and vasculature in the inner medulla in control (A) and IUGR (B) kidneys; 11β-HSD2 positive cells (brown staining) expressed in the inner medulla collecting tubules in control (C) and IUGR (D) kidneys; GR positive cells (brown staining) in the medulla was positive in the collecting ducts and thin limbs and nuclei of interstitial cells in control (E) and IUGR (F) kidneys; and MR positive cells (brown staining) observed expression within collecting ducts in control (G) and IUGR (H) kidneys. Following UPI, COX-2, 11β-HSD2, and GR immunoreactivity was decreased in intensity in IUGR kidney medulla. MR immunoreactivity was more prominent in the control medulla. Arrows denote representative positive cells. Scale bar = 500 μm.
who showed that deficiency of COX-2 resulted in progressive and specific renal architectural disruption and functional deterioration. The authors suggested that time-dependent and tissue-specific expression of COX-2 is necessary for normal renal development and the maintenance of normal renal architecture and function. Similarly, the use of COX-2-selective inhibitor (SC58236) from postnatal day 0 to day 21 severely reduced glomerular diameter in both mice and rats, confirming the importance of an intact COX-2 system in renal development. Although it is always necessary to be cautious when applying data from animal models to human pathophysiology, the above experimental findings are concordant with reports of renal dysgenesis in newborns of mothers who received treatment with NSAIDs, such as indomethacin, as well as with nimesulide (a COX-2 preferential inhibitor). This is of particular relevance during the 24th to 32nd gestational week in humans when COX-2 inhibition has been shown to be involved in the development of renal damage.

In the mammalian kidney, glucocorticoids and mineralocorticoids bind to MR with equal affinity. However, the localization of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) was observed differently. GR immunolabeling was associated with nucleic and cytoplasmatic labeling along the cortical tubules and was significantly reduced in IUGR kidney. MR localization was principally observed within loops of Henle and not significantly different in the IUGR kidney cortex.
corticoid target tissues, such as the kidney, the receptors are selective for aldosterone in vivo because of the presence of the enzyme 11β-HSD2, which converts cortisol and corticosterone to their inactive 11-keto analogs. These analogs cannot bind to MR, thus conferring specificity to MR renal action (18). The clinical relevance of 11β-HSD2 can be observed in patients with apparent mineralocorticoid excess (AME), which results from loss-of-function mutations in the gene encoding for 11β-HSD2 (57). In patients with AME, peripheral inactivation of cortisol is impaired most notably in key MR-expressing tissues, such as the kidney and colon. This, in turn, leads to inappropriate MR-mediated responses, including increased sodium retention, hypokalemia, and hypertension. Licorice derivatives, such as glycyrrhetinic acid, also have potent effects on 11β-HSD2 in classical tissues, such as the kidney (35). As such, they are able to reproduce the symptoms of AME, leading, in turn, to abnormal sodium retention and increased blood pressure.

Alternatively, 11β-HSD2 abundance and activity can be affected by corticosterone hormones, as well as growth factors, shear stress, and hypoxia (12, 24). For instance hypoxia has been shown to downregulate the expression of 11β-HSD2 by induction of early growth response gene Egr-1 in renal cell culture (24). In the case of corticosterone regulation of 11β-HSD2, a glucocorticoid responsive element has not yet been found in the 11β-HSD2 promoter region, suggesting a probable posttranscriptional change as a possible indirect nongenomic role for glucocorticoids on 11β-HSD2. It is also likely that glucocorticoids could affect 11β-HSD2 gene expression by epigenetic regulation. Interestingly, CpG islands covering the promoter and exon 1 regions of this gene have been found to be differentially methylated in diverse tissues and cell lines (3).

In the present study, we described an overall sustained decrease in kidney 11β-HSD2 expression in the IUGR rat that, in association with previously reported increased persistent corticosterone levels, would chronically expose both the GR
and MR to supraphysiological glucocorticoid levels. In response to this abnormal milieu following UPI, both GR and MR mRNA and protein expression were found to be affected in the IUGR offspring. Total GR was found to be decreased both at birth (P0), as well as at P21. MR expression was initially upregulated at birth but was significantly decreased by P21 in the IUGR kidney.

GR levels are programmed to a “set point” by environmental manipulations, including neonatal stress and in utero treatment with dexamethasone during the perinatal time (13, 42). In the juvenile rat, persistent downregulation of GR in the kidney could be, in part, explained by a negative feedback secondary to the persistent elevated corticosterone levels (19). However, regulation of both GR and MR has been shown to be a complex process that involves transcriptional, as well as posttranscriptional mechanisms (44, 47).

The effect of sex and age upon COX-2 and 11β-HSD2 protein levels was also analyzed, comparing control males with control females at P8 and P21. Interestingly, we found that COX-2 levels were higher in the female kidney at P8. This trend persisted at P21, although it did not reach statistical significance. In contrast, 11β-HSD2 levels were higher in the male control kidney at P21. These sex-specific differences in young and adult gene expression or phenotype could eventually affect the response to environmental stresses, as our laboratory has observed in previous reports following UPI (21).

Our IHC findings are concordant with mRNA and protein levels in IUGR and control kidneys and further delineate the pattern of expression of COX-2, 11β-HSD2, GR, and MR during normal development and under the effect of UPI and elevated glucocorticoids. Interestingly, in the more immature IUGR kidney (P0), increased levels of corticosterone seem to have a more pronounced effect on COX-2 and 11β-HSD2 expression compared with the IUGR juvenile kidney at P21. We also confirmed that localization of COX-2 and 11β-HSD2 follows a developmental pattern. Under normal circumstances, following a marked birth peak, there is a developmental decrease in plasma corticosterone levels in the first postnatal weeks (25), which coincides in the juvenile rat with localization of both 11β-HSD2 and MR mainly in the collecting ducts. Conversely, we found GR to be mainly expressed in the developing loops of Henle at all stages of postnatal renal development where 11β-HSD2 is much less abundant after birth, suggesting that 11β-HSD2 activity is insufficient for protection against GR activation (37). Our findings are consistent with a previous study that showed that earlier in human gestation, 11β-HSD2 colocalized with GR in metanephros in the developing glomeruli, as they migrated from the surface of the kidney to the inner cortex. Conversely, after 16 wk of gestation, 11β-HSD2 colocalized principally with MR within epithelial cells of collecting ducts, a pattern more adult in nature. These findings suggest that 11β-HSD2 earlier in fetal

Fig. 8. A: measure of systolic blood pressure in IUGR vs. control rats. Data shown are for both males and females at postnatal day 21 and 140 of life. All data are means ± SE. (n = 6; **P < 0.01, ***P < 0.001). B: measure of diastolic blood pressure in IUGR vs. control rats. Data shown are for both males and females at postnatal day 21 and 140 of life. All data are means ± SE. (n = 6; *P < 0.05). C: measure of mean blood pressure in IUGR vs. control rats. Data shown are for both males and females at postnatal day 21 and 140 of life. All data are means ± SE. (n = 6; *P < 0.05).
life principally modulates ligand access to the GR in the glomeruli and tubules of the developing kidney (11).

Of interest, Yao et al. (62) observed that inhibition of 11β-HSD2 by glycyrrhizic acid had similar effects in weaning rats (P21 to P28) and in adult rats, leading to increased medullary COX-2 and decreased cortical COX-2 expression. In contrast, the authors found no effect on COX-2 expression in suckling rats after 11β-HSD2 inhibition and suggested this was likely secondary to low levels of corticosterone in plasma in these animals at that developmental stage. These results are in contrast to our observations, since decreased 11β-HSD2 was associated with decreased medullary and cortical COX-2 at P0 in the IUGR kidney. These dissimilar findings could be in part explained by the fact that following UPI, the IUGR fetus is exposed to increased levels of corticosterone that, associated with decreased expression of 11β-HSD2, would preclude the developing kidney from normal expression of “glucocorticoid-suppressed” pathways, such as renal COX-2.

An important goal of the present study was to determine whether our model of placental insufficiency by bilateral uterine artery ligation late in gestation in the pregnant rat would result in the development of hypertension in the IUGR offspring. SBP, MBP, and DBP were not affected by UPI on P21 in IUGR rats. However, arterial blood pressure as measured by the indirect tail cuff method was significantly increased in both IUGR males and females at P140 compared with control. Noninvasive measurements of blood pressure have long been accepted for large rodents. Recently, Whitesall et al. (58) compared simultaneous measurement of SBP in unanesthetized mouse by radiotelemetry and tail cuff methods and found the pulse-based tail cuff instrument to be in agreement with radiotelemetry. A limitation of our study is that with the tail cuff method we cannot differentiate whether the variation in blood pressures found between controls and IUGR animals could be due to an exaggerated stress response vs. baseline blood pressures. Moreover, in this study, we cannot establish whether the elevated blood pressures observed in the IUGR group are secondary to a renal or a hypothalamic-pituitary-adrenal axis effect. We speculate that both systems are, to a different extent, involved in our animal model.

Our results are concordant with several other animal models of placental insufficiency and fetal malnutrition that also observed development of hypertension in growth-restricted offspring. These include nutritional manipulations (global food vs. protein restriction) in the rat and sheep animal models (34, 61), impairment of placental perfusion or function by placement of clips around the aorta in the rat (2), and umbilicoplacental embolization in the sheep (10). Interestingly, antenatal administration of dexamethasone to sheep and rats has also been shown to contribute to the development of hypertension in the offspring (59). Although the initial insult underlying the intrauterine programming of hypertension is different in all of the above studies, most of these animal models share in common a decrease in nephron number and, therefore, evidence for impaired fetal renal development. The long-term control of arterial pressure is very complex, and underlying renal mechanisms can be responsible for mediating hypertension in the IUGR offspring. For instance, a reduction in nephron endowment followed by compensatory glomerular enlargement and development of hypertension later in life has been demonstrated in IUGR offspring (hyperfiltration theory) (53). Interestingly, a relationship between low nephron number and increased blood pressure has also been demonstrated in patients with “essential” hypertension when compared with individuals with normal blood pressures at autopsy (28). Since no new nephrons are formed after nephrogenesis is completed by about postnatal day 8 in the rat, and there is an expected loss of glomeruli during aging, it is conceivable that our adult IUGR rats develop compensatory glomerular enlargement as part of the expected morphological changes.

In the present study, we have further identified important molecular mechanisms that could underlie hypertension in our animal model, including a persistent decrease of renal 11β-HSD2 associated with elevated corticosterone levels in the IUGR offspring. Furthermore, we described a decrease in renal COX-2 expression during nephrogenesis in the IUGR pup that could both affect nephron development, as well as renin secretion and renal hemodynamics (50), therefore altering fetal programming of normal regulatory systems that control sodium balance and blood pressure.

In summary, we have identified in the IUGR rat kidney altered expression of the key genes COX-2 and 11β-HSD2 during a “window” of nephrogenesis that in our animal model is associated with increased serum corticosterone levels. We speculate that these alterations could have a role in renal development and in the perinatal programming of adult hypertension observed in the IUGR offspring.

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REFERENCES


