Uteroplacental insufficiency increases apoptosis and alters p53 gene methylation in the full-term IUGR rat kidney

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Pham, Tho D., Nicole K. MacLennan, Christina T. Chiu, Gisella S. Laksana, Jennifer L. Hsu, and Robert H. Lane. Uteroplacental insufficiency increases apoptosis and alters p53 gene methylation in the full-term IUGR rat kidney. *Am J Physiol Regul Integr Comp Physiol* 285: R962–R970, 2003. First published July 17, 2003; 10.1152/ajpregu.00201.2003.—Uteroplacental insufficiency causes intrauterine growth retardation (IUGR), which is associated with adult onset diseases such as hypertension. Previous studies demonstrate that growth retardation in humans and rats decreases glomerular number; however, the molecular mechanisms responsible for this reduction are unknown. Apoptosis plays a key role in renal organogenesis. We therefore hypothesized that the in utero deprivation associated with uteroplacental insufficiency decreases glomeruli, increases apoptosis, and alters the mRNA levels of key apoptosis-related proteins in full-term IUGR kidneys. To prove this hypothesis, we induced asymmetric IUGR through bilateral uterine artery ligation of the pregnant rat. We found that uteroplacental insufficiency significantly reduced glomeruli number while increasing TUNEL staining and caspase-3 activity in the IUGR kidney. A significant decrease in Bcl-2 mRNA and a significant increase in Bax and p53 mRNA further characterized the IUGR kidney. Because altered p53 CpG methylation affects p53 expression, we analyzed p53 promoter CpG methylation using methylation-sensitive restriction enzymes and real-time PCR. Uteroplacental insufficiency specifically decreased CpG methylation of the renal p53 BstU I site promoter without affecting the Hha I or the Aci I sites. Uteroplacental insufficiency also induced a relative hypomethylation from exon 5 to exon 8, which was associated with decreased mRNA levels of DNMT1. We conclude that uteroplacental insufficiency alters p53 DNA CpG methylation, affects mRNA levels of key apoptosis-related proteins, increases renal apoptosis, and reduces glomeruli number in the IUGR kidney. We speculate that these changes represent mechanisms that contribute to the fetal origins of adult disease.

intrauterine growth retardation; Bcl-2; Bax; DNA methylation; epigenetics

BARKER’S FETAL ORIGINS OF ADULT DISEASE hypothesis proposes that a deprived intrauterine milieu leads to permanent changes in cellular biology and systemic physiology. Early stigmata of this phenotype include intra-uterine growth retardation (IUGR), and several epidemiological studies closely link IUGR to adult onset diseases such as hypertension (2, 71, 78). Interestingly, both human and animal model studies demonstrate that IUGR leads to a decrease in glomeruli; however, these studies do not reveal a specific molecular mechanism (3, 4, 25, 45, 50, 73).

Nephrogenesis involves the rapid remodeling of structures, which requires massive apoptosis (12, 34). Moreover, maternal protein restriction reduces glomeruli number in association with increased metanephric apoptosis on day 13 of gestation (73). The gene expression of apoptosis-related molecules Bcl-2 and Bax responds to the hypoxia, acidosis, and hypoglycemia that characterize the IUGR in utero environment, which is induced by uteroplacental insufficiency. Bcl-2 is an antiapoptosis protein that attenuates the effects of cytochrome c release from the mitochondria and counters the effects of the proapoptosis protein Bax (29, 33). Bcl-2 and Bax contribute to the signaling pathways that activate caspase-3 (casp-3), which is necessary for the chromatin condensation and DNA fragmentation that characterize apoptosis (28, 60). We hypothesized that induction of IUGR in the rat would reduce glomeruli number, increase renal apoptosis, and alter mRNA levels of apoptosis-related proteins.

To test this hypothesis, we induced IUGR using a rat model of uteroplacental insufficiency. Uteroplacental insufficiency causes asymmetric IUGR, in which brain growth is spared relative to body weight and is associated with the hypertensive disorders of pregnancy. These disorders are the most common medical complications of pregnancy and lead to serious perinatal mortality and morbidity (74). The IUGR human fetus endures in utero hypoxia, acidosis, hypoglycemia, altered levels of growth factors, and hypoinsulinemia (15–17). Similarly, when induced via bilateral uterine artery ligation of the pregnant rat, IUGR induces an identical response in the late gestation rat fetus (52, 54, 70). In this well-characterized and widely published rat model of asymmetrical growth retardation, IUGR pups are 20–25% lighter than the sham-operated control animals, and birth weights are normally...
distributed within and among litters (9, 36–41, 53, 54, 64). Litter size does not significantly differ between control and IUGR groups (36).

We quantified glomeruli number in sham-operated control (Con) and IUGR term rat pups (N0), as well as 21-day-old juvenile rat pups (J21). Apoptosis and the mRNA levels of the Bcl-2 and Bax were quantified in the N0 rat pups. Interestingly, Gobe et al. (21) found that renal Bcl-2 expression was associated with IGF-1 expression. The authors of this study speculated that this phenomenon was due to the promotion of distal tubule survival allowing the expression of growth factors. We therefore similarly followed up our findings of altered Bcl-2 expression by measuring renal IGF-1 mRNA levels.

p53 negatively regulates Bcl-2 expression and positively regulates Bax expression (63, 76). To investigate a mechanism through which the altered intrauterine milieu may affect Bcl-2 and Bax mRNA levels, we quantified mRNA levels of the transcription factor p53 and found significant differences between control and IUGR animals. Because promoter and exon 5–8 CpG methylation affects p53 mRNA levels, we determined CpG methylation of these regions using methylation-sensitive restriction enzymes and real-time PCR (57–59). Moreover, because p53-dependent apoptosis occurs in the absence of DNA methyltransferase 1 (DNMT1), we quantified mRNA levels of this enzyme (27).

METHODS

Animals. All procedures were approved by the University of California-Los Angeles (UCLA) Chancellor’s Animal Research Committee and are in accordance with the American Physiological Society’s guiding principles (1). These surgical methods have been described previously (32, 36, 40, 69). On day 19 of gestation, the maternal rats were anesthetized with intraperitoneal xylazine (8 mg/kg) and ketamine (40 mg/kg), and both uterine arteries were ligated (IUGR) (n = 12 litters). Term gestation in the rat is 21.5 days. Sham surgery was performed on control animals who underwent identical anesthetic and surgical procedures except for the uterine artery ligation (Con) (n = 12 litters). N0 pups were delivered by caesarian section (n = 6 litters Con and IUGR, respectively) at term, 2.5 days after the bilateral uterine artery ligation. J21 animals were separated from their dams for 4 h (to minimize individual hormonal variations associated with feeding), anesthetized, and killed (n = 6 litters Con and IUGR, respectively). At both ages, kidneys were quickly dehydrated and dewaxed tissue samples. Apoptotic nuclei were labeled using the terminal deoxynucleotidyl transferase uridine nick end-label technique (TUNEL) via the In Situ Death Detection Kit, POD (Roche, Nutley, NJ). A light hematoxylin stain was used for tissue orientation after the DAB rinses.

A quantification of apoptosis using the TUNEL assay was performed using a modification of the technique described by Malik et al. (46). In brief, an observer blinded to the study group counted the total number of TUNEL-positive nuclei per high-power field (HPF) in six nonoverlapping regions per section (×400). Previous reports demonstrate that the TUNEL technique correlates well with electron microscopy and accurately represents apoptosis in the developing rat kidney (30).

Casp-3 activity. We used the Apoalert CPP32/casp-3 colorimetric assay kit to quantitate N0 renal casp-3 activity as previously described (42). This kit measures the proteolytic cleavage of the chromophore ρ-nitroanilide from a DEVD tetrapeptide sequence. In brief, kidneys were homogenized in chilled lysis buffer and subsequently centrifuged to precipitate cellular debris. The supernatant was then transferred to a new microcentrifuge tube. Fifty microliters of reaction buffer containing DTT was added to each reaction, and then 1 mM of conjugated substrate was added to each tube. The reaction was incubated at 37°C for 1 h. Absorbencies were read at 405 nm, and a standard curve was generated using a positive control of CPP32 chromogenic substrate. Negative controls were generated by running a reaction without conjugated substrate and with the casp-3 inhibitor DEVD-fmk, respectively.

RNA isolation. Total RNA was extracted from N0 kidneys and quantified in triplicate using ultraviolet absorbance (11). Gel electrophoresis confirmed the integrity of the samples. RNA was treated to DNase (Ambion, Austin, TX).

Real-time PCR. Kidney mRNA levels of Bcl-2, IGF-1, Bax, and p53 were measured using real-time PCR as previously described (41). cDNA was synthesized from 0.5 μg of DNase-treated total RNA as described above. Target (Bcl-2, IGF-1, Bax, p53, DNMT1) primers and probes were designed using Primer Express Software (Applied Biosystems, Foster, CA) (Table 1); target probes were labeled with fluorescent reporter dye FAM. Before the performance of real-time PCR, all primer pairs were tested with serial Mg2+ and primer concentrations to determine the optimal reaction conditions and to demonstrate the specificity of each primer pair. Reporter dye emission is detected by an automated sequence detector combined with ABI Prism 7700 Sequence Detection System software (Applied Biosystem). An algorithm normalizes the reporter signal (Rf) to a passive reference and multiplies the standard deviation of the background Rf in the first cycles by a default factor of 10 to determine the threshold Cr. Cr has a linear relation with the logarithm of the initial template copy number (24). Real-time PCR quantification is then performed using the Taqman glyceraldehyde-3-phosphate dehydrogenase controls. Before the use of GAPDH as a control, serial
dilutions of cDNA are quantified to prove the validity of using GAPDH as an internal control. Relative quantification of PCR products are then based on value differences between the target and GAPDH control using the comparative C\(_T\) method (49). Cycle parameters were 55°C × 5 min, 95°C × 10 min, and then 40 cycles of 95°C × 15 s → 58°C × 60 s. For every sample, each PCR reaction was performed on three separate occasions; in each set of reactions, every sample was present in triplicate.

p53 promoter CpG methylation. The methylation status of the p53 promoter region was determined using the technique described by Pogrribny and James (58). We modified the technique by using real-time PCR vs. conventional PCR and added an internal control. The internal control was a segment of DNA from the hexokinase I promoter that contains no CpGs. In brief, 10 µg of DNA with the methylation-sensitive restriction enzymes Hpa II, Msp I, Aci I, and Hha I. Msp I cleavage is not affected by CpG methylation and therefore acts as a control for the Hpa II. Real-time PCR was subsequently performed using a total volume of 20 µl and the Sybr Green PCR Master Mix per the manufacturer's instructions. Table 1 contains the primer sequences. Cycle parameters were 55°C × 5 min, 95°C × 10 min, and then 40 cycles of 95°C × 30 s → 60°C × 30 s → 72°C × 60 s. Each sample was analyzed as described for the p53 promoter. The targeted region of DNA for exon 5–6 contains two Hpa II sites and one Aci site. The targeted region of DNA for exon 7–8 contains one restriction site for each methylation-sensitive restriction enzyme.

Statistics. All data presented are expressed as mean percent of control ± SE. ANOVA (Fisher’s protected least significant difference) and the Student’s unpaired t-test determined statistical significance.

RESULTS

Glomeruli number. In N0 IUGR pups, uteroplacental insufficiency significantly reduced the glomeruli per kidney section to 111 ± 5.9 (P < 0.05). In contrast, N0 sham-operated control sections contained 144 ± 9.6 glomeruli (Fig. 2A). For J21 animals, IUGR kidney

![Fig. 1. Diagram of the p53 promoter.](http://ajpregu.physiology.org/)

**Table 1. Sequences of PCR Primers**

<table>
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<tr>
<th>Gene</th>
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</table>

*Primers from Progrribny and James (56). NA, not applicable.

modified the technique by using real-time PCR, adding the same internal control as described above. In brief, we treated 10 µg of DNA with the methylation-sensitive restriction enzymes Hpa II, Msp I, Aci I, and Hha I. Msp I cleavage is not affected by CpG methylation and therefore acts as a control for the Hpa II. Real-time PCR was subsequently performed using a total volume of 20 µl and the Sybr Green PCR Master Mix per the manufacturer's instructions. Table 1 contains the primer sequences. Cycle parameters were 55°C × 5 min, 95°C × 10 min, and then 40 cycles of 95°C × 30 s → 60°C × 30 s → 72°C × 60 s. Each sample was analyzed as described for the p53 promoter. The targeted region of DNA for exon 5–6 contains two Hpa II sites and one Aci site. The targeted region of DNA for exon 7–8 contains one restriction site for each methylation-sensitive restriction enzyme.
sections contained 112 ± 6.4 glomeruli per section, which was significantly reduced vs. the control value of 151 ± 10.7 (P < 0.01) (Fig. 2A). These J21 values retained statistical significance when evaluated per gram of animal weight. Uteroplacental insufficiency decreased glomeruli per section per gram to 2.15 ± 0.08 from J21 IUGR kidneys vs. 1.72 ± 0.05 in J21 control kidneys (P < 0.01).

TUNEL assay. Apoptotic nuclei in N0 kidneys were quantified as described by Malik et al. (47) (n = 6 litters). Wide-spread mesenchymal apoptosis, and to a lesser extent tubular apoptosis, characterized both control and IUGR animals. Uteroplacental insufficiency significantly increased the number of apoptotic nuclei in IUGR N0 kidneys to ~180% of control values [control 5.7 ± 0.5 TUNEL-positive cells/HPF; IUGR 10.5 ± 0.5 TUNEL-positive cells/HPF (P < 0.01); quantification, Fig. 2B; representative photograph, Fig. 3]. No difference in the relative histological distribution of the apoptotic nuclei was noted.

Casp-3 activity. The nuclear morphological changes that occur with apoptosis, as well as DNA fragmentation, require casp-3 (28). Uteroplacental insufficiency significantly increased casp-3 activity in IUGR kidneys to 201 ± 28% (P < 0.01) of control values (Fig. 4A).

mRNA levels of Bcl-2, IGF-1, Bax, and p53. Uteroplacental insufficiency significantly reduced N0 IUGR renal Bcl-2 and IGF-1 mRNA levels to 49 ± 11.5 (P < 0.01) and 38 ± 7.0 (P < 0.01) percent of control values, respectively (Fig. 4B). Control value SEs were 8.4 and 9.2% for Bcl-2 and IGF-1, respectively. In contrast, real-time RT-PCR revealed that Bax and p53 mRNA levels were significantly increased to 122 ± 9.1 (P < 0.05) and 195 ± 23.6% (P < 0.01) of control values, respectively (Fig. 4B). For Bax and p53, control value SEs were 3.5 and 11.6%, respectively.

CpG methylation of the p53 promoter. Hha I, Aci I, and BstU I methylation-sensitive restriction enzyme sites characterize the p53 promoter (6, 58). Digestion with these enzymes followed by PCR determined the percent of methylation at these sites as described by James and colleagues (58). Uteroplacental insufficiency resulting in IUGR significantly reduced CpG methylation at the BstU I site (~179) to 43.7 ± 3% (P < 0.001) of control values (Fig. 5). This site is located in the rat p53 85-bp basal promoter region. No differences in DNA methylation were noted for either the Aci I sites (~410, ~364, ~304, ~180) or the Hha I site (~450; Fig. 5; Ref. 6).
**CpG methylation of exon 5 through exon 8 and DNMT1 mRNA levels.** Digestion with the methylation-sensitive restriction enzymes Hha I, Aci I, and Hpa II and real-time PCR determined relative CpG methylation within exons 5–6 and exons 7–8 (Fig. 6A). For exons 5–6, uteroplacental insufficiency significantly reduced methylation at the Aci I site without affecting the Hpa II sites. No product was amplified from the Msp I digest and no difference between control and IUGR was noted when exon 5–6 primers amplified an Hha I digest. The latter is expected because this region of DNA does not contain any Hha I sites. For exons 7–8, uteroplacental insufficiency significantly reduced methylation by 30–40% for all three restriction sites. In association with this relative hypomethylation, DNMT1 mRNA levels were also significantly decreased to 70±5.2% (P<0.05) of control values (Fig. 6B). The SE for DNMT1 control mRNA levels was 7.0%.

**DISCUSSION**

Uteroplacental insufficiency and the subsequent IUGR response caused renal p53 hypomethylation in association with increased p53 and Bax mRNA, as well as decreased Bcl-2 mRNA. These novel findings suggest a molecular mechanism through which IUGR induces fetal renal apoptosis and a resultant permanent loss in glomeruli.

In this study, uteroplacental insufficiency reduced glomeruli numbers by ~25% in the asymptomatically growth-retarded juvenile rat. Similarly, uteroplacental insufficiency and asymmetrical IUGR in rabbits induced a 25–30% decrease in nephron number, and asymmetrical IUGR in piglets reduced the nephron number index (total nephron number/body weight) by 20–30%; moreover, the change in nephron number caused a parallel drop in glomerular filtration rate (3,4). Merlet-Benichou et al. (50) used partial unilateral artery ligation of the pregnant rat to induce IUGR, which reduced nephron number by 37% and glomerular filtration rate by 50%, respectively. Although the above studies clearly describe a clear relationship between IUGR and reduced nephron number, they do not demonstrate by what mechanism it occurs.

**Fig. 4.** Caspase-3 activity in N0 control and IUGR kidneys. Data are expressed as the IUGR percent of control ± SE. Uteroplacental insufficiency significantly increased IUGR caspase-3 activity (**P<0.01). B: mRNA levels of Bel-2, IGF-I, Bax, and p53. Data are expressed as the IUGR percent of control ± SE. Uteroplacental insufficiency significantly decreased Bel-2 and IGF-I mRNA levels, whereas it increased both Bax and p53 mRNA levels (**P < 0.01; *P < 0.05).

**Fig. 5.** Relative methylation of the p53 promoter at the Hha I, Aci I, and BstU I sites. Data are expressed as IUGR percent of control methylation ± SE. Uteroplacental insufficiency significantly reduced CpG methylation at the BstU I sites, without affecting either the Hha I site or the Aci I sites (*P < 0.001).
In contrast, both Welham et al. (73) and Vehaskari et al. (72) used low-protein diets to induce IUGR and subsequently demonstrated increased renal apoptosis. The former study found that increased metanephric apoptosis and decreased glomeruli characterized the IUGR rat. The latter Vehaskari study found a 28–29% reduction in glomeruli number in association with increased TUNEL staining in IUGR kidneys. Both of these studies are important because they established the initial link between renal apoptosis and IUGR; however, neither study determined whether protein deprivation affects expression of Bcl-2 and Bax.

The Welham and Vehaskari studies are also important because severe malnutrition during pregnancy is a concern in developing countries (5). In Western societies, however, uteroplacental insufficiency causes a significant majority of IUGR infants (5). Nephrogenesis depends heavily on apoptosis (12, 34). In the rat, renal apoptosis peaks late in gestation at approximately the same time that the present study induces IUGR (46).

Bcl-2 and Bax are important because the molecular ratio of Bax/Bcl-2 acts as a cellular “rheostat” determining cellular flux toward or away from apoptosis (33, 55). The combination of decreased Bcl-2 mRNA, increased Bax mRNA, and increased casp-3 activity characterize other experimental models of increased renal apoptosis such as urinary outflow obstruction and experimental glomerulonephritis (63, 67, 77). Bcl-2 renal expression may be particularly important because it plays a role in mediating the formation of condensations of cells that are “committed” to differentiated structures (43). Moreover, a decrease in renal Bcl-2 mRNA promotes tubular apoptosis in hypokalemic nephropathy in rats and appears to threaten tubular production of key growth factors such as IGF-1, which is consistent with our findings of decreased renal IGF-1 mRNA levels (20, 31). Uteroplacental insufficiency and IUGR in the rat also cause a significant decrease in cerebral Bcl-2 mRNA levels (42). Bcl-2 may be a pivotal gene in the systemic IUGR response to uteroplacental insufficiency and may contribute to the close link between cerebral and renal injury in infants suffering asphyxia.


Multiple factors affect p53 gene expression, and acute insults stimulate posttranslational changes in accordance with the need to respond rapidly; however, p53 mRNA levels appear to be meaningful under the following three conditions (35). First, p53 mRNA levels regulate its protein expression during development (19). Second, chronic or moderate insults upregulate renal p53 mRNA and induce apoptosis (13, 51, 63). Third, altered p53 mRNA levels are linked to tumor progression and are associated with altered CpG methylation of the p53 gene (56, 57, 75).

James and colleagues (57) used bisulfite mapping to demonstrate that hypomethylation of exons 6–7 increases p53 mRNA levels. With the use of a technique that has been previously validated for studying p53 methylation, the current study found a relative hypomethylation at the exon 6 Aci I site on the sense strand with the first set of p53 downstream primers, without detecting any difference in the Hpa II site (65). Similarly, with the use of a second set of downstream primers, the current study also found that uteroplacental insufficiency decreased methylation in the Hpa II sites (intron 6 and exon 7), the Aci I site (exon 7), and the Hpa I site (intron 7). Both this study and the James study note that loss of the cytosine methyl group was never 100% (59).

The James group is also responsible for the seminal papers that characterize the affects of CpG methylation on the rat p53 promoter (56, 58). In general,
hypermethylation of CpG p53 promoter sites between -100 and -442 decreases p53 mRNA levels (56). In vitro methylation and transient transfection CAT assays demonstrate that CpG methylation affects p53 transcription, particularly at the -450 Hha I restriction sites (58). Isolated methylation of the BstU I site decreased p53 transcription by ~20% (58). This latter site is significant because it is within the p53 85-bp basal promoter region, and we speculate that hypomethylation of this site and of exons 6–7 contributes significantly to the increased levels of p53 mRNA and increased apoptosis observed in this study (6). Our findings of decreased DNMT1 mRNA support this speculation, because Cre-mediated deletion of DNMT1 induces p53-dependent apoptosis (27). Moreover, because the BstU I site is methylated in normal tissue and becomes demethylated in preneoplastic tissue, it is intriguing that IUGR significantly increases the risk for nephroblastoma (58, 66).

Recent investigations demonstrate that promoter demethylation contributes to transcriptional regulation of specific genes (7, 22). It is now also clear that an active DNA demethylase exists (7, 14). The factors that regulate the expression of this demethylase are unknown; however, glutathione depletion causes genomic-wide DNA hypomethylation (44). The intrauterine environment associated with IUGR and uteroplacental insufficiency leads to oxidative stress, which is known to reduce renal glutathione levels (54, 62).

Caution is necessary when attempting to apply data from a rat model to human pathophysiology. The timing and impact of uteroplacental insufficiency experienced by humans range across a continuum, and the human life experience is confounded by both genetic and environmental variables. In contrast, the laboratory rat in this study is inbred and experiences a homogeneous diet and environment. The insult imposed on the fetal rat in this model of uteroplacental insufficiency is severe and occurs relatively late in gestation. However, when Hinchliffe et al. (25) assessed kidneys from IUGR and appropriately sized infants, they found that IUGR reduced the mean glomerular count by ~35%. IUGR also increases the risk of the arterial hypertension and glomerulosclerosis in children with IgA glomerulonephritis (79).

In summary, uteroplacental insufficiency reduces nephron number and increases renal apoptosis in the IUGR newborn rat. The molecular mechanisms behind these findings include increased Bax and decreased Bcl-2 expression, which leads to increased casp-3 activity. Uteroplacental insufficiency also increases p53 expression in association with hypomethylation of its promoter and exon 6–7. Changes in CpG methylation may represent a mechanism through which an altered intrauterine environment predisposes the affected individual to adult onset diseases.

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DISCLOSURES
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