Uteroplacental insufficiency alters hepatic expression, phosphorylation, and activity of the glucocorticoid receptor in fetal IUGR rats

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Uteroplacental insufficiency alters hepatic expression, phosphorylation, and activity of the glucocorticoid receptor in fetal IUGR rats. The glucocorticoid receptor (GR) is a transcription factor that when activated can induce changes in chromatin structure. To begin the process of identifying pathways by which IUGR affects chromatin structure, we hypothesized that UPI in the rat induces a significant increase in endogenous glucocorticoids (corticosterone) and increases GR expression and activation. To prove our hypothesis, we induced IUGR through bilateral uterine artery ligation of the pregnant rat. At day 1, UPI significantly increased corticosterone levels and was associated with increased total GR mRNA and protein levels in the liver, as well as increased hepatic phosphorylation of GR serine 211. Moreover, cyclin-dependent kinase 2 (CDK2) cyclinA/CDK2 protein levels, which selectively phosphorylate GR serine 211, were also significantly increased. To assess activity of the GR, we measured protein levels of the transcription factor p53 whose levels are downregulated, at least in part, by active GR. In this study, UPI decreased p53 protein and its downstream target Bax mRNA levels. We conclude that UPI in rats affects GR expression and activity in the liver. We speculate that these alterations early in life may contribute to the changes in chromatin structure and gene expression previously described in the IUGR liver.

11β-hydroxysteroid dehydrogenase type 1; cyclin A; cyclin-dependent kinase 2; phosphoglucocorticoid receptor (serine 211)

A SERIES OF EPIDEMIOLOGICAL and animal studies conducted in the past closely link intrauterine growth restriction (IUGR) to adult onset diseases such as diabetes and hypertension (3, 28, 39, 40). IUGR caused by uteroplacental insufficiency (UPI) is a morbidity associated with several common complications of pregnancy, such as preeclampsia and maternal diabetes (55). This condition can be induced in the pregnant rat through bilateral uterine artery ligation, and, similar to the human, results in offspring with low birth weight and asymmetrical IUGR (48). Also similar to the human, IUGR fetal rats suffer from hypoxia, acidosis, hypoinsulinemia, and hypoglycemia (9, 10, 35, 36). Juvenile IUGR rats develop insulin resistance and by adulthood suffer overt diabetes and hypertriglyceridemia (26, 48).

To explain the fetal origins of adult disease, Barker (2) proposed that the fetus adapts to a deprived intrauterine environment, such as that seen in UPI, by making permanent changes in cell biology. Our group has previously demonstrated (30) that IUGR rats have persistent changes in hepatic gene expression secondary to modifications in histone acetylation and DNA methylation. The pathways that signal the alterations in the environment that lead to changes in chromatin structure are unknown. One potential candidate underlying these epigenetic changes is the glucocorticoid receptor (GR). GR is a nuclear receptor that functions as a hormone-dependent transcription factor and is known to induce changes in chromatin structure (8, 52). It is a member of the steroid receptor superfamily involved in survival responses (5, 41) mainly through the actions of glucocorticoids. Interestingly, mice that have increased GR expression are more resistant to endotoxic shock and stress (41).

There are three key components involved in the GR signaling pathway: 1) circulating concentration of active glucocorticoids (corticosterone), 2) GR expression, and 3) posttranslational phosphorylation. The ligand-free form of GR is inactive and is held in the cytoplasm. Upon hormone binding, the GR gets translocated into the nucleus where it binds as a homodimer to specific DNA motifs [glucocorticoid response elements (GRE)] and activates or represses the transcription of GRE-containing genes (20, 51).

It has been demonstrated that GR-mediated transcriptional activation is also modulated by phosphorylation (6, 17). The rat and human GR have phosphorylated serine residues that have been shown to be conserved among species. Phosphorylation at serine 232 in rat GR (serine 211 in human GR) is dependent on the presence of corticosterone and is a biomarker for activated GR in vivo (54). Furthermore, there is mounting evidence that cortisol-dependent phosphorylation of the GR may determine the strength and duration of GR signaling, as well as determine target promoter specificity (17). The cyclin-dependent kinase 1 (CDK1) cyclinA/CDK2 complex of protein kinas es efficiently phosphorylates the GR at serine 211 (24).

Variation in tissue sensitivity to glucocorticoids is not only determined by circulating steroid concentrations and cellular GR levels but also by prereceptor metabolism of ligands by tissue-specific enzymes. For steroid metabolism, the key enzymes are 11β-hydroxysteroid dehydrogenases (11β-HSD). In liver, the enzyme 11β-HSD type 1 (11β-HSD1) acts mainly as a reductase that activates cortisone to cortisol in the human, and 11-dehydrocorticosterone to corticosterone in mice and rats (23, 45).

GR also interacts with other cell-signaling molecules to affect cell survival. The p53, an important transcription factor...
as well, also has a key role during physiological stress through regulation of genes involved in apoptosis (Bax family) (22, 43). Under certain physiological stress circumstances, such as hypoxia, GR can contribute to the sequestration of p53 in the cytoplasm where it undergoes proteosomal degradation and thus can suppress p53 function (14, 33, 46, 47).

Based on this background, we hypothesized that UPI induces an increase in glucocorticoid levels and, at the molecular level, affects hepatic GR expression and phosphorylation. Furthermore, we also hypothesized that IUGR is associated with increased GR activity in the newborn rat, and that this is reflected in decreased levels of hepatic p53 protein and mRNA levels of its downstream target Bax.

To prove our hypothesis, bilateral uterine artery ligation (IUGR) and sham surgery were performed on day 19 of gestation in Sprague-Dawley rats (term, 21.5 days). This well-established model of asymmetrical growth restriction is characterized by alterations in hepatic epigenetic determinants, gene expression, and adult onset of insulin resistance (13, 27, 48). At birth, IUGR pups are 20–25% lighter than the sham-operated control animals (IUGR: 4.00 ± 25 vs. sham: 5.25 ± 22, P < 0.05) with normal distribution of birth weight within and among litters and no difference in litter size between control and IUGR groups (4, 25, 27, 35, 37).

For this study, we quantified the effects of IUGR on circulating glucocorticoids, hepatic GR mRNA expression, and protein levels, as well as GR phosphorylation (phospho-GR (serine 211)). We then studied UPI effects on GR modulators: cyclinA/CDK2 complex protein levels and 11β-HSD1 mRNA levels. Finally, we measured the effects of UPI on p53 protein levels and mRNA levels of its downstream target, Bax.

MATERIALS AND METHODS

Animals

All procedures were approved by the University of Utah Animal Care Committee and are in accordance with the American Physiological Society’s guiding principles (1). These surgical methods have been previously described (21, 38, 48). 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 = 6 litters). Sham surgery was performed in control animals who underwent identical anesthetic and surgical procedures except for the uterine artery ligation (control) (n = 6 litters). Rats recovered within a few hours and had ad libitum access to food and water. At term (21.5 days gestation), pups were delivered by caesarian section, weighed, and decapitated. Blood was collected to quantify plasma corticosterone levels. Livers were delivered by caesarian section, weighed, and decapitated. Blood was washed with a buffered saline solution, treated with tetramethylbenzidine (TM) chromogen solution, and incubated. A stopping solution was then added to each well, absorbance was read at the same wavelength (ng/ml), and quantitation was performed using KC Junior software and EL X800 Universal Microplate Reader (Bio-Tek Instruments, Winooski, VT).

RNA Isolation

DNase I-treated total RNA (Ambion, Austin TX) was extracted from 20–30 mg of day 1 IUGR and sham rat pup livers using the NucleoSpin RNA and virus purification kit (BD Biosciences Clontech, Palo Alto, CA). Total RNA was quantitated using the Pharmasep-1700 UV absorbance spectrophotometer (Shimadzu Scientific Instruments, Torrance, CA). RNA integrity was confirmed by gel electrophoresis.

Real-Time RT-PCR

Liver mRNA levels of GR, 11β-HSD1, P53, and Bax were measured at day 1 with the real-time RT-PCR method as previously described (30). cDNA was synthesized using random hexamers and Superscript III RT (Life Technologies, Gaithersburg, MD) from 2.0 μg of DNase-treated total RNA as described above. Primers and probes for GAPDH, GR, and Bax were designed using Primer Express 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 (15). Real-time PCR quantification is then performed using the Taqman glyceraldehyde-3-phosphate dehydrogenase controls. Rat GAPDH was used as an internal control to correct for differences in cDNA loading. Relative quantification of PCR products are then based on value differences between the target and GAPDH control using the comparative Ct method (31). 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 triplicate.

Immunoblotting and Antibodies

Protein isolation. Whole livers were obtained from day 1 IUGR and sham rat pups. Total protein was isolated by homogenizing 30–50

| Table 1. Sequence of PCR primers for real-time PCR |
|-------|-------|-------|-------|
| Gene | Forward Primers | Reverse Primers | Probe Sequence |
| GR | 5’-GGACAGCTACTCCTTGG-3’ | 5’-TCACGGGCTCTTTCGATACCACCC-3’ | 5’-TGAACTCTCCACGGCTGACCTG-3’ |
| Bax | 5’-TGCTGCTCCCTCTTCTACTTCGC-3’ | 5’-TGATCACTGGCCTGATTTA-3’ | 5’-AACTTGGCTCAAGGCCTGTG-3’ |
| GAPDH | 5’-CAAGAGTTGGAAGGTCGGT-3’ | 5’-CACAGAGGGACAGCTGGT-3’ | 5’-GGTGCCCATACGGCCAAATCCG-3’ |

GR, glucocorticoid receptor.
mg 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). Enriched nuclear protein was isolated by pulverizing 50–100 mg of tissue under liquid nitrogen with a loose pestle of a Dounce homogenizer. Cytosolic supernatant was separated by centrifugation. Nuclei were suspended in elution buffer and incubated at 4°C. Samples were centrifuged postincubation at 10,000 g for 10 min and the supernatants were collected. The BCA protein assay kit method (Pierce, Rockford, IL) was used for protein quantitation.

Western blot analysis. Total protein or enriched nuclear protein (20 µg) and molecular weight markers were loaded and separated by 10% and 4–12% XT Criterion gels (Bio-Rad Laboratories, Hercules, CA). After gel electrophoresis, the proteins were transferred to nitrocellulose membranes (Millipore, Billerica, MA) in standard transfer buffer and incubated at 4°C. After blocking the membranes with either 5% milk or 5% BSA for 1 h, bound proteins were probed with primary antibodies: GR (Abcam), phospho-GR (serine 211) (Cell Signaling Technology), cyclin-A (Upstate Cell Signaling), CDK2 (Abcam), p53 (Cell Signaling Technology), and GAPDH (Abcam) overnight at 4°C. After multiple wash steps in Tris-buffered saline Tween (TBS-T), membranes were probed with anti-rabbit horseradish peroxidase antibody (Cell Signaling Technology) 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 Lightning enhanced chemiluminescence (PerkinElmer Life Sciences, Boston, MA) and quantified using Kodak Image Station 2000R (Eastman Kodak/SIS, Rochester, NY). GAPDH was used as a loading control in detection analysis.

Statistics

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

RESULTS

IUGR Increases Corticosterone Levels

Plasma corticosterone levels were measured by ELISA. Following UPI induced by bilateral uterine ligation, plasma corticosterone levels were significantly increased in IUGR pups (206 ± 24 vs. 161 ± 9.7 ng/ml in sham, P < 0.05) on day 1 of life.

IUGR Increases GR mRNA and Protein Levels in Liver, as Well as GR Phosphorylation

mRNA levels of GR were measured using real-time RT-PCR. Protein levels of total GR, and phospho-GR (serine 211) were quantified using Western blot analysis. GAPDH was used as an internal control. UPI significantly increased day 1 IUGR liver mRNA levels of GR to 196 ± 3% of sham values (P < 0.01). Concomitantly, IUGR liver protein analysis performed on day 1 showed increased levels of total liver GR in IUGR pups (216 ± 14% of sham, P < 0.05). Furthermore, UPI also increased hepatic phospho-GR (serine 211), and cyclinA/CDK2 levels (288 ± 28, 157 ± 10, and 147 ± 12% of sham, respectively, P < 0.01) (fig. 1), demonstrating increased levels of activated GR.

IUGR Decreases p53 Protein Levels and Bax mRNA Expression in Liver

Protein levels of p53 were quantified using Western blot analysis, and mRNA levels of Bax were measured using real-time RT-PCR. GAPDH was used as an internal control. The p53 protein levels and its downstream target, Bax mRNA levels, were decreased in association with UPI (75 ± 7% of sham, P < 0.05 and 70 ± 4% of sham, P < 0.001, respectively) (Fig. 2A).

IUGR Decreases 11β-HSD1 mRNA Levels in Liver

mRNA levels of 11β-HSD1 were measured using real-time RT-PCR. GAPDH was used as an internal control. In contrast to the above findings, real-time RT-PCR showed that IUGR pups had decreased liver mRNA levels of 11β-HSD1 (58 ± 12% of sham, P < 0.05) (Fig. 2B).

DISCUSSION

UPI is an important cause of IUGR in humans and is known to increase fetal serum cortisol levels (12). This study demonstrates that UPI in the rat similarly increases serum corticosterone levels. Interestingly, prenatal administration of dexamethasone in rats causes low birth weight, hypercorticosteronemia, and adult onset insulin resistance (29, 34, 44). Furthermore, rat fetal cortisol levels can be increased by inhibiting placental 11β-HSD2, resulting in reduced birth weight and fasting hyperglycemia and hyperinsulinemia (29).

In our rat model of UPI, hypercorticosteronemia increased liver GR mRNA and protein levels. These results correlates with previous reports of increased hepatic GR mRNA levels at 5 days, 3 wk, and 8 mo of life in rats whose dams received prenatal dexamethasone (7, 34). These findings contrast to the response observed in most adult cells and tissues, in which GR mRNA levels are negatively regulated by increased circulating corticosteroids during adulthood (19, 42).

A common characteristic of the different models of prenatal overexposure to glucocorticoids is “programming” of permanent physiological changes that lead to adult onset of impaired insulin resistance, hyperglycemia, and hyperinsulinemia through altered glucocorticoid receptor (GR) signaling in liver. A primary physiological effect of prenatal glucocorticoid exposure is increased liver GR protein and mRNA levels. This study demonstrates a significant increase in liver GR protein levels and mRNA levels in rats whose dams received prenatal dexamethasone (7, 34). These findings contrast to the response observed in most adult cells and tissues, in which GR mRNA levels are negatively regulated by increased circulating corticosteroids during adulthood (19, 42).

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IUGR affects glucocorticoid receptor biology in liver

glucose tolerance and hyperinsulinemia (29, 34, 48, 53). Our model of IUGR differs from the other animal models in that the primary insult also involves hypoxia, acidosis, hypoglycemia, and hypoinsulinemia. These characteristics are shared by both the rat and human fetus suffering from UPI (9–11). In contrast, the administration of glucocorticoids to the mother is likely to induce complications in the offspring that are not typically associated with IUGR in the human, such as hyperglycemia (49).

Our findings are of further interest because GR, a fundamental signaling molecule, may also play a significant role in the persistent changes in hepatic gene expression observed in IUGR rats (13, 30). One of the mechanisms through which GR can affect transcription is through the modification of chromatin structure (8). Changes in chromatin structure, such as histone hyperacetylation, are heritable from cell to cell and characterize the UPI-induced IUGR rat liver.

Although hormone binding is essential for GR activation, mounting evidence exists that posttranslational modification of GR by phosphorylation activates or enhances receptor function (6). In support of this, mutations in the CDK2 catalytic subunit p34 decrease GR-dependent transcriptional activation, demonstrating that phosphorylation is needed for full GR-mediated transcriptional enhancement (24). In the present study, we demonstrate that UPI activates GR by phosphorylation at serine 211, and this correlates with increased protein levels of cyclinA/CDK2.

Because previous studies (33, 47) have found that hypoxia and stress activate the GR, which subsequently sequesters the p53 protein in the cytoplasm and therefore facilitates p53 degradation, we measured hepatic p53 protein levels in control and IUGR rat livers. We found that UPI decreases hepatic IUGR p53 protein levels as well as mRNA levels of the p53 downstream target Bax. These findings suggest that UPI not only increases protein levels of GR and phospho-GR (serine 211) but also affects GR-mediated regulation of p53 within the IUGR liver.

UPI also decreased hepatic 11β-HSD1 mRNA in the day 1 IUGR livers. This enzyme is a second source of active glucocorticoids since it catalyzes the conversion of circulating inactive 11-dehydrocorticosterone to active corticosterone in several tissues, including liver, adipose tissue, and brain and in part regulates intracellular glucocorticoid access to GR. Expression of 11β-HSD1 is regulated by several factors including glucocorticoids, sex steroids, insulin, growth hormone, and IGF-1 (50). Interestingly, in our animal model, several of these regulatory factors are known to be affected in IUGR pups (10, 16).

Our finding of decreased hepatic 11β-HSD1 mRNA levels in the IUGR rat concurs with a previous report by Jamieson et al. (18) that demonstrated chronic stress and elevated levels of glucocorticoids reduce hepatic and hippocampal 11β-HSD1 mRNA expression in postnatal rats. Our findings suggest that circulating glucocorticoids in utero might also suppress 11β-HSD1 expression in certain tissues. Thus this homeostatic feedback mechanism could reduce excessive metabolic actions of corticosterone during stress in organs, such as the liver, while maintaining elevated circulating glucocorticoid levels to be used by other tissues, such as the immune system.

Caution must be applied of course when comparing data from an animal model to the human condition. The fetal rat is physiologically immature relative to the human, and the timing and impact of UPI experienced by humans range across a continuum. In contrast, the insult imposed on the fetal rat in this model of UPI is severe and specific.

In summary, we conclude that IUGR increases prenatal glucocorticoid levels and hepatic GR expression. This is associated with alteration in GR modulation as well as activity, with increased GR phosphorylation, increased levels of cyclin A/CDK2 complex, decreased liver 11β-HSD1 expression, and decreased hepatic p53 protein and Bax expression. We speculate that these alterations in liver GR biology and activity early in life contribute to long-term morbidities previously described in the IUGR population.

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Fig. 2. A: quantification of day 1 protein levels of hepatic p53 and quantification of Bax mRNA expression. Data are means ± SE of IUGR %control. Open bars are control values C, Control; I, IUGR. (*P < 0.05 and **P < 0.001). Western blots are of day 1 p53 protein. GAPDH is used as internal control. B: quantification of day 1 hepatic 11 β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) mRNA expression. Open bars are control values (*P < 0.05).
GRANTS

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