Uteroplacental insufficiency induces site-specific changes in histone H3 covalent modifications and affects DNA-histone H3 positioning in day 0 IUGR rat liver

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Fu, Qi, Robert A. McKnight, Xing Yu, Laiyi Wang, Christopher W. Callaway, and Robert H. Lane. Uteroplacental insufficiency induces site-specific changes in histone H3 covalent modifications and affects DNA-histone H3 positioning in day 0 IUGR rat liver. Physiol Genomics 20: 108–116, 2004. First published October 19, 2004; doi:10.1152/physiolgenomics.00175.2004.—Uteroplacental insufficiency and subsequent intrauterine growth retardation (IUGR) increase the risk of adult onset insulin resistance and dyslipidemia in humans and rats. IUGR rats are further characterized by postnatal alterations in hepatic PPAR-γ coactivator (PGC-1) and carnitine-palmitoyltransferase I (CPTI) expression, as well as overall hyperacetylation of histone H3. However, it is unknown whether the histone H3 hyperacetylation is site specific or relates to the changes in gene expression previously described in IUGR rats. We therefore hypothesized that uteroplacental insufficiency causes site-specific modifications in hepatic H3 acetylation and affects the association of acetylated histone H3 with PGC-1 and CPTI promoter sequences. Uteroplacental insufficiency was used to produce asymmetrical IUGR rats. IUGR significantly increased acetylation of H3 lysine-9 (H3/K9), lysine-14 (H3/K14), and lysine-18 (H3/K18) at day 0 of life, and these changes occurred in association with decreased nuclear protein levels of histone deacetylase 1 (HDAC1) and HDAC activity. Chromatin immunoprecipitation using acetyl-H3/K9 antibody and day 0 chromatin revealed that uteroplacental insufficiency affected the association between acetylated H3/K9 and the promoters of PGC-1 and CPTI, respectively, in IUGR liver. At day 21 of life, the neonatal pattern of H3 hyperacetylation persisted only in the IUGR males. We conclude that uteroplacental insufficiency increases H3 acetylation in a site-specific manner in IUGR liver and that these changes persist in male IUGR animals. The altered association of the PGC-1 and CPTI promoters with acetylated H3/K9 correlates with previous reports of IUGR altering the expression of these genes. We speculate that in utero alterations of chromatin structure contribute to fetal programming.

BARKER’S “fetal origins of adult disease hypothesis” proposes that fetal adaptation to a deprived intrauterine milieu leads to permanent changes in cellular biology and systemic physiology (3, 4). Intrauterine growth retardation (IUGR) predisposes affected newborns toward long-term morbidity from type 2 diabetes and dyslipidemia (3, 16). Uteroplacental insufficiency, a morbidity associated with many common complications of pregnancy such as preeclampsia, induces low ponderal index or asymmetrical IUGR. In the rat, uteroplacental insufficiency induced through bilateral uterine artery ligation of the pregnant dam also results in asymmetrical IUGR, and similar to the human, causes fetal hyposulinemia, hypoglycemia, acidosis, and hypoxia (6, 10, 11, 50, 51). Juvenile IUGR rats demonstrate insulin resistance, and adult animals suffer overt diabetes with fasting hypertriglyceridemia, hyperglycemia, and hyperinsulinemia (35, 59, 62).

These morbidities are associated with increased mRNA, as well as protein levels of hepatic PPAR-γ coactivator (PGC-1), and decreased mRNA levels carnitine-palmitoyltransferase I (CPTI) (35, 38). PGC-1 is a transcriptional coactivator that mediates hepatic glucose production by controlling mRNA levels of key gluconeogenic enzymes, such as glucose-6-phosphatase (G-6-Pase), phosphoenolpyruvate carboxykinase (PEPCK), and fructose-1,6-bisphosphatase (FBPase) (63). CPTI is a part of the carnitine shuttle and is considered to be a rate-limiting transporter in mitochondrial fatty acid β-oxidation (43). Altered mRNA levels of these genes characterize the IUGR liver at birth, and these changes persist postnatally (35, 38). Such persistent alterations in gene expression suggest that a mechanism exists through which the altered intrauterine environment induces a relatively static change in transcriptional regulation.

Chromatin structure plays a key role in transcriptional regulation, and important determinants of chromatin structure include histones and their covalent modifications (18, 46, 47). Posttranslational covalent modifications (acetylation, methylation, phosphorylation) of the NH2-terminal tail of histones allow regulatable contacts with the underlying DNA and make up the histone code that governs gene transcription (22). Previous studies have demonstrated that hyperacetylation of histone H3 (H3) characterizes IUGR liver; however, it is not known whether these changes are site specific (42).

Acetylation of H3 occurs on lysine-9 (H3/K9), lysine-14 (H3/K14), and lysine-18 (H3/K18) and is generally believed to be associated with actively transcribed genes (23, 58). Steady-state acetylation levels of the core histones result from a balance between the opposing activities of histone acetylases and histone deacetylases (HDAC). It is interesting that hypoxia, a component of the IUGR intrauterine milieu, affects HDAC1 gene expression: HDAC1 is a class I deacetylase that is necessary for embryonic survival and deacetylates H3/K14 (20, 30).

Based upon this background, we hypothesized that uteroplacental insufficiency causes site-specific modifications in hepatic H3 acetylation and alters gene expression of HDAC1, as well as HDAC activity. Because IUGR increases PGC-1 and decreases CPTI expression, we further hypothesized that site-specific modifications observed in IUGR animals would be

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differentially associated with the PGC-1 and the CPTI promoters. To prove this hypothesis, we performed bilateral uterine artery ligation (IUGR), a well-characterized model of uteroplacental insufficiency and IUGR, and sham surgery (control) on pregnant Sprague-Dawley rats on day 19 of gestation (term, 21.5 days), (6, 19, 31–41, 50–52, 54, 57, 59, 62). Levels of site-specific histone H3 acetylation relative to the total amount of histone H3 in control and IUGR pups at day 0 and day 21 of life were quantified by Western blot. HDAC activity was assessed from nuclear protein levels were measured by real-time RT-PCR and Western blot, respectively. HDAC1 mRNA and protein levels were measured by real-time RT-PCR and Western blot, respectively. HDAC activity was measured by a positive DNA amplification control in the typing experiments to demonstrate the quality and presence of DNA (forward primer, 5′-CTCCATGAACCTGGGTC; reverse primer, 5′-GAGCATCCCTCACTGGGTAATGAG) (Fig. 1A, top).

The day 21 animals were separated from their dams for 4 h, anesthetized, and killed (n = 6 litters con and IUGR, respectively). Pups were studied at this age because they have not yet developed overt insulin resistance or dyslipidemia that may confound our findings (35, 59, 62). At both ages, the liver was quickly removed, flash frozen in liquid nitrogen, and stored in −80°C.

Histone isolation and Western blotting. Histones were isolated from day 0 and day 21 liver tissue by acid extraction according to Galasinski et al. (15). Histone concentrations were determined using the Micro BCA protein assay kit (Pierce Biotechnology, IL). We separated 10–20 μg of histones on 15% SDS-PAGE gels and transferred by electroblotting to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Blocking was carried out with freshly prepared PBS plus 3% nonfat milk (PBS-MLK). After washing, the membrane was incubated overnight with primary antibodies diluted in PBS-MLK. Primary antibodies used included anti-acetyl-H3/K9 at 1:400 (Cell Signaling Technologies, Beverly, MA), anti-acetyl-H3/K14 at 1:5,000, anti-acetyl-H3/K18 at 1:500, and anti-histone H3 at 1:2,000 (Upstate Cell Signaling Solutions, Lake Placid, NY). Appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) were incubated for 1 h at room temperature. Signals were detected using ECL performed according to the manufacturer’s instructions (Amersham, Buckinghamshire, UK). The amount of acetylated H3 was quantified relative to the amount of total H3 in the sample. Although most studies focusing upon histone covalent modifications do not perform this step, we utilized this internal loading control because we are analyzing in vivo tissues that are potentially more heterogeneous in terms of histone acetylation than cell culture or clonal tumor cells.

METHODS

Animals. All procedures were approved by the University of Utah Animal Care Committee and are in accordance with the APS Guiding Principles (2). Surgical procedures have been described previously (31–41, 50–52, 54, 57, 59, 62). On day 19 of gestation, 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 that underwent identical anesthetic and surgical procedures except for the uterine artery ligation (n = 12 litters). The day 0 pups were delivered by cesarean section (n = 6 litters control and IUGR, respectively) at term, 2.5 days after the bilateral uterine artery ligation. The day 0 pups were genotyped using PCR for the spermatogenic gene (Sby) from the Y chromosome to ensure an equal distribution of each sex for each methodology (forward primer, 5′-ACTGTTCAAGCAGTCAGCCG; reverse primer, 3′-CGGCAGCTAGGCTGCA). The latter was performed to demonstrate the presence and quality of DNA. Lane 1 contains genomic DNA samples; lane 2, male rat (positive control); lane 3, no IP antibody; lane 4, 2 μg of histones from control litters; lane 5, IP with 8.8 μg of anti-acetyl-H3/K9; lane 6, IP with 110 μg of anti-acetyl-H3/K9; lane 7, no IP antibody; lane 8, 1:20 dilution of lane 6; lane 9, 1:20 dilution of lane 7; lane 10, 1:20 dilution of lane 8; lane 11, 1:20 dilution of lane 9; lane 12, 1:20 dilution of lane 10; lane 13, 1:20 dilution of lane 11; lane 14, 1:20 dilution of lane 12; lane 15, 1:20 dilution of lane 13; lane 16, 1:20 dilution of lane 14.

Fig. 1. A: representative agarose gels demonstrating the PCR products for the Sby gene (Y chromosome) (top) and carnitine-palmitoyl-transferase I (CPTI) (bottom). The latter was performed to demonstrate the presence and quality of DNA. Lane 1, known day 21 male rat (positive control); lane 2, known day 21 female rat (negative control); lane 3, blank; lanes 4, 6, 7, 10, 11, and 12 are male day 0 rat samples; lanes 5, 8, 9, 13, and 14 are female day 0 rat samples. B: representative Western blot of immunoprecipitated chromatin (IP) using anti-histone H3. Lane 1, 24 μg of acid extracted histone protein (positive control); lane 2, no IP antibody; lane 3, IP with 5 μg of anti-acetyl-H3/K14; lane 4, 1:20 dilution of lane 3; lane 5, IP with 10 μg of anti-acetyl-H3/K14; lane 6, 1:20 dilution of lane 5; lane 7, IP with 20 μg of anti-acetyl-H3/K14; lane 8, 1:20 dilution of lane 7; C: representative Western blot of IP using anti-histone H3. Lane 9, no IP antibody; lane 10, IP with 2.2 μl of anti-acetyl-H3/K9; lane 11, IP with 4.4 μl of anti-acetyl-H3/K9; lane 12, IP with 8.8 μl of anti-acetyl-H3/K9; lane 13, 1:20 dilution of lane 12; lane 14, IP with 22 μl of anti-acetyl-H3/K9; lane 15, 1:20 dilution of lane 14; lane 16, IP with 110 μl of anti-acetyl-H3/K9. Arrows indicate the position of histone H3.
RNA isolation. Total RNA was extracted from day 0 and day 21 liver using the RNaseasy Mini Kit (Qiagen, Valencia, CA), treated with DNase I (Qiagen), and then quantified. Gel electrophoresis confirmed the integrity of the samples.

Real-time PCR. Liver mRNA levels of HDAC1 were measured using real-time RT-PCR as previously described (37, 54). cDNA was synthesized from 2 μg of DNase-treated total RNA as previously described. Target primers and probes were designed using Primer Express software (Applied Biosystems, Foster City, CA) (Table 1); target probes were labeled with carboxyfluorescein (FAM) fluorescent reporter dye. cDNA, probe, and primers were added to TaqMan universal PCR master mix (Applied Biosystems), and samples were run on an ABI Prism 7900. Real-time RT-PCR quantification was then performed using the TaqMan glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. Relative quantification of PCR products was based on value differences between the target and GAPDH control using the comparative C_{t} method (TaqMan Gold RT-PCR manual; PE Biosystems, Foster City, CA). Cycle parameters were 50°C, 2 min, 95°C, 10 min, and then 40 cycles at 95°C, 15 s, 60°C, 60 s. For each set of reactions, samples were run in triplicate.

Nuclear protein extracts. A quantity of 100 mg of liver tissue was ground under liquid nitrogen. Tissue that was used to prepare nuclear extracts for the HDAC activity assay was first washed with ice-cold PBS to lyse red blood cells. The ground tissue was resuspended in 5 ml ice-cold PBS, then centrifuged at 2,000 g for 5 min. The washing step was repeated until all color was removed. The ground tissue was resuspended in 5 volumes of buffer C (1 mM DTT, 0.5 mM PMSF, 10 mM KCl, 10 mM HEPES, pH 7.9, 1.5 mM MgCl₂) and incubated for 15 min on ice, then centrifuged at 2,000 g for 5 min. The supernatant was removed by aspiration, and the tissue pellet was resuspended in 2 ml of buffer C. The tissue was disrupted with a Dounce homogenizer. Lysis was checked for every 10 strokes using trypan blue. The lysate was centrifuged at 1,000 g for 5 min. The nuclear pellet was resuspended in 0.5 ml of buffer D (1.5 mM MgCl₂, 1 mM DTT, 420 mM NaCl, 0.5 mM PMSF, 25% (vol/vol) glycerol, 0.2 mM EDTA, 20 mM HEPES, pH 7.9), incubated at 4°C for 30 min, then centrifuged at 17,000 g for 15 min. The supernatant was collected and stored at −80°C.

HDAC1 Western blotting. Cell lysates and nuclear extracts were used to do HDAC1 Western blots. Cells were lysed using lysis buffer (50 mM Tris-Cl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 μg/ml each aprotinin, leupeptin, and pepstatin, 1 mM NaVO₃, 1 mM NaF). Lysates were centrifuged, and the protein concentration of the supernatant was determined using the Micro BCA protein assay kit (Pierce Biotechnology). Thirty micrograms of protein from day 0 liver cell lysates or nuclear extracts was separated by 10% SDS-PAGE gels and transferred to PVDF membrane (Millipore). After transfer, membranes were blocked with 3% non-fat milk in PBS, then incubated in 1:50 primary antibody of HDAC1 (H-51: sc-7872, Santa Cruz Biotechnology) at 4°C overnight with agitation, washed three times, incubated with HRP-conjugated secondary antibodies (rabbit), and detected with ECL.

HDAC enzyme activity assay. The HDAC Activity Assay Kit (Abcam, Cambridge, MA) was used to measure HDAC activity in the day 0 liver nuclear extracts as described by the manufacturer. Fifty micrograms nuclear protein was added to each well of a 96-well plate. A 2-μl volume of 1 mg/ml of the HDAC inhibitor trichostatin A was added to each control well.

ChIP assay and real-time PCR. The protocol described by Oberley and Farnham (49) was used with the following modifications. A quantity of 100 mg day 0 liver tissue was ground in liquid nitrogen and fixed with formaldehyde, final concentration of 1%, for 10 min. The chromatin was sonicated (Sonic Dismembrator, model 100; Fisher Scientific, Pittsburgh, PA) 12 times for 10 s on ice at the highest level to generate chromatin fragments of 500–2,000 bp. The sonicated chromatin was quantitated on the basis of DNA content at A₂₆₀. The chromatin equivalent of 40 μg DNA based on the absorption at A₂₆₀ was used in each immunoprecipitation (IP). To determine the amount of antibody to use to IP formaldehyde cross-linked chromatin, each antibody was titrated against 24 μg of acid extracted histone protein and 48 μg sonicated chromatin in 110 μl of IP dilution buffer. For anti-acetyl-H3/K14 (Upstate Cell Signaling Solutions) antibody concentrations of 5, 10, and 20 μg were used (Fig. 1B). For anti-acetyl-H3/K9 (Cell Signaling Technologies) volumes of 2.2, 4.4, 8.8, 20, and 110 μl were used (Fig. 1C). Different units for the antibodies are presented because the two companies used different quantitation methods. Each immunoprecipitate was electrophoresed on a 15% polyacrylamide SDS-PAGE gel. The gel was stained with Ponceau S, blotted onto PVDF membrane, probed with anti-H3 antibody, and detected with ECL. From these results, 20 μg of anti-acetyl-H3/K14 and 110 μl of anti-acetyl-H3/K9 were used for each IP. The DNeasy Tissue Kit (Qiagen, Valencia, CA) was used to purify the DNA from the total amount of DNA extracted from either 40 μg of chromatin or immunoprecipitated from 40 μg of chromatin using anti-acetyl-H3/K9, anti-acetyl-H3/K14, anti-rabbit IgG-HRP, and no antibody, respectively. The latter two groups were run to verify the specificity of the ChIP antibodies. DNA was quantitated by measuring A₂₆₀/A₂₈₀. Real-time PCR was used to quantitate the amount of PGC-1, CPTI, and GAPDH promoter sequence immunoprecipitated (Table 1). The amount of PGC-1 and CPTI DNA in each sample was quantitated relative to the amount of GAPDH, which served as an internal control for the amount of DNA precipitated and amplified. The GAPDH internal control was used because promoters of weakly expressed housekeeping genes such as GAPDH have been shown to be associated with acetylated H3 (45). The use of the control was therefore necessary to determine whether the association of PGC-1 and CPTI DNA with the acetylated H3 sites was significantly different vs. this housekeeping gene.

Statistics. All data presented are expressed as mean percent of control ± SE. For real-time RT-PCR, real-time PCR, and Western blotting, statistical analyses were performed using ANOVA (Fisher’s protected least-significant difference) and Student’s unpaired t-test.

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<th>Gene</th>
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Table 1. PCR primers for real-time PCR
RESULTS

Histone H3 acetylation in day 0 and day 21 liver. Western blotting determined the amount of histone H3 acetylated at lysine-9 (H3/K9), lysine-14 (H3/K14), and lysine-18 (H3/K18); total histone H3 was used as an internal control. Uteroplacental insufficiency significantly increased acetylation of H3/K9, H3/K14, and H3/K18 in day 0 IUGR livers to 217 ± 60%, 132 ± 9%, and 140 ± 11%, respectively, vs. control values (all \( P < 0.05 \)) (Fig. 2A).

In day 21 males, IUGR similarly increased acetylation at H3/K9, H3/K14, and H3/K18 to 175 ± 60%, 154 ± 12% \((P < 0.0001)\), 154 ± 12% \((P < 0.01)\), and 121 ± 5% \((P < 0.01)\) of control values (Fig. 2B). Interestingly, in day 21 female IUGR livers, acetyl-H3/K9 and acetyl-H3/K18 were significantly decreased to 43 ± 4% and 69 ± 2% of control values, respectively (both \( P < 0.0001 \)), whereas no significant differences were noted for acetyl K14/H3 (Fig. 2B).

Significant differences were also noted at day 21 of life based solely on sex. In the control animals, hepatic H3/K9 acetylation was significantly decreased in the male rats relative to female rats, although in the IUGR male rats, H3/K9 acetylation was increased relative to the female IUGR rats (Fig. 3). Furthermore, H3/K14 and H3/K18 hepatic acetylation was significantly increased and decreased, respectively, in both groups of male animals relative to the female animals (Fig. 3).

HDAC1 gene expression and HDAC activity in day 0 liver. Real-time RT-PCR revealed that uteroplacental insufficiency significantly decreased day 0 mRNA levels of HDAC1 to 52 ± 3% \((P < 0.01)\) and HDAC nuclear activity to 61 ± 5% \((P < 0.05)\) of control values (Fig. 4). Total protein levels of HDAC1 were not affected by uteroplacental insufficiency and subsequent IUGR.
**HDAC1 gene expression and HDAC activity in day 21 liver.** At day 21 of age IUGR did not significantly affect HDAC1 mRNA levels in either male or female livers (Fig. 5A). Similarly, no significant differences were noted in HDAC1 total protein and HDAC1 nuclear protein levels between IUGR and control male livers; in contrast, increased HDAC1 total protein (173 ± 25; \(P < 0.05\)) and nuclear protein levels (178 ± 13; \(P < 0.01\)) characterized the IUGR female livers relative to controls (Fig. 5A). No significant differences were noted between the sexes in terms of HDAC protein levels.

Nuclear HDAC activity was unchanged in the female IUGR livers and was significantly decreased in the IUGR male livers (26 ± 9\%) vs. the sex-matched control animals (\(P < 0.01\)). Interestingly, relative to the IUGR males, nuclear HDAC activity increased 300 ± 48\% in the female IUGR livers (\(P < 0.01\)) (Fig. 5B). In contrast, nuclear HDAC activity increased only by 19 ± 4\% in the female control livers vs. the male controls (\(P < 0.05\)) (Fig. 5B).

**Chromatin immunoprecipitation assay.** Antibodies to H3/K9 and H3/K14 were used to precipitate control and IUGR day 0 chromatin. PCR was then used to measure whether uteroplacental insufficiency and IUGR increased the association between the PGC-1 or CPTI promoters and acetylated histones. Because housekeeping genes such as GAPDH associate with acetylated histone H3, the amount of GAPDH in the precipitated chromatin DNA was used to normalize the findings (45).

Precipitation with the acetyl-H3/K9 antibody demonstrated that uteroplacental insufficiency significantly increased association between the PGC-1 promoter and acetylated H3/K9 to 130 ± 8\% of control values (\(P < 0.05\)), whereas IUGR significantly decreased the association between the CPTI promoter and acetyl-H3/K9 to 69 ± 12\% of control values (\(P < 0.05\)) (Fig. 6). Performance of the ChIP assay using anti-acetyl-H3/K14 demonstrated no significant differences of the PGC-1 and CPTI promoter association with acetyl-H3/K14 between day 0 control and IUGR liver (Fig. 6).

**DISCUSSION**

Rats rendered IUGR via uteroplacental insufficiency have persistent alterations in hepatic gene expression and a predisposition toward diabetes; furthermore, male IUGR pups appear to be more severely affected relative to female IUGR pups (19, 31, 32, 35, 37, 38, 59, 62). Investigations into the mechanism behind these phenotypic changes suggest that epigenetic phenomena such as histone acetylation, as well as genome-wide and promoter-specific DNA methylation, alters gene expression and contributes to long-term consequences of uteroplacental insufficiency (42, 54). This manuscript focuses upon the effects of uteroplacental insufficiency upon site-specific hepatic histone H3 acetylation and the finding that site-specific histone hyperacetylation persists postnatally in IUGR male animals, but not in female IUGR rats. These findings are novel because they potentially initiate the unraveling of whether a “histone code” plays a role in the morbidities that afflict the bilateral uterine artery ligation model in vivo model of IUGR and adult onset diabetes.

The “histone code” hypothesis proposes that covalent modifications of histones contribute to transcriptional regulation and that these modifications further provide a mechanism for encoding information through successive generations of cell division (22). Because histone NH2-terminal tails can be modified by phosphorylation, methylation, ubiquitination, and acetylation, a large number of combinations exist that allow regulatable contacts with DNA. Moreover, multiple signaling pathways converge upon histones (7). A recent study by Kurdistani et al. (29) identified groups of genes in yeast that are expressed in response to stressful conditions such as oxidative stress, amino acid starvation, and temperature shock and are associated with specific combinatorial histone acetylation patterns. Moreover, similar functional classes and upstream DNA sequence motifs were associated with these clustered genes (29). Kurdistani et al. (29) also found that acetylation of H3/K9...

Additional evidence from cell culture studies shows that multiple pathways regulate acetylation of H3/K14 and potentially contribute to differential transcriptional regulation (8, 58). Similarly, acetylation of H3/K9 contributes to the promoter regulation of several genes (14, 27, 48). These latter studies utilized ChIP to determine the relationship between chromatin modifications and specific promoters. The present investigation differs from these studies through the use of the GAPDH promoter as a baseline control and the focus upon an in vivo model of prenatal nutrient deprivation and postnatal phenotypic changes (19, 32, 33, 35, 38, 59, 62).

These phenotypic changes include insulin resistance and hypertriglyceridemia, which occur in association with persistent postnatal reduction of CPTI mRNA and elevation of PGC-1 mRNA (35, 38). CPTI is a part of the carnitine shuttle and a rate-limiting step in mitochondrial fatty acid β-oxidation (43). Within the 6,139-bp rat liver CPTI promoter, there are 123 CpG sites, 20 of which are located in the 190-bp basal promoter (53). As is observed in nearly half of all promoters examined, CpG content significantly increases in this proximal promoter region vs. the rest of the promoter sequence. Sp1 binding sites occur three times in the liver CPTI promoter and

Fig. 5. A: quantification of day 21 hepatic HDAC1 gene expression and representative Western blots. Data are expressed as IUGR percent of control ± SE. White bars represent control values to demonstrate ± SE. Western blots for total hepatic HDAC1 protein utilize GAPDH as an internal control. C and I identify control and IUGR samples, respectively. B: female nuclear HDAC activity relative to the male values. Gray bars represent the male values to demonstrate the ± SE. *p < 0.05. †p < 0.01.

Fig. 6. Quantification of real-time PCR products from chromatin immunoprecipitation assay (ChIP) using the H3/K9 and H3/K14 antibodies, respectively. PPAR-γ coactivator (PGC-1) and CPTI primers encompass regions of their respective promoters known to contribute to transcriptional regulation. Data are expressed as IUGR percent of control ± SE. White bars represent control values to demonstrate ± SE. *p < 0.05.
contain four CpG sites. Just as methylation of transcription factor binding sites can block protein binding sites, transcription factor binding can affect DNA methylation (5, 66). PGC-1 potentiates hepatic insulin resistance through multiple mechanisms, including the control of gluconeogenesis and the induction of the mammalian tribbles homolog TRB-3 (28, 63). There are 14 CpG sites within the 1,784-bp promoter, although no Sp1 binding sites are evident. Because DNA methylation of CpG sites affects local histone acetylation and based upon our findings with the ChIP assay and presence of potential methylation sites, we speculate that DNA methylation of these promoters plays a role in their transcriptional regulation (21). The altered CPTI and PGC-1 promoter associations with acetylated H3/K9 demonstrate that at day 0 of life, uteroplacental insufficiency alters promoter DNA-histone H3 contacts of key hepatic genes. Our findings with PGC-1 are particularly intriguing in light of our sex-specific findings, because this coactivator potentiates the transcriptional response to several steroids in a receptor-specific manner (26).

Each sex responds in a specific manner to perinatal nutritional deprivation. Studies utilizing rats rendered IUGR through uteroplacental insufficiency demonstrate sex-specific differences in growth, serum triglycerides, and gene expression, with males appearing to be more severely affected than females (19, 35, 37). Studies focusing upon human outcomes also show sex differences. Flanagan et al. (13) found that decreased insulin sensitivity was common among 20-yr-old men who suffered IUGR, but not women. A study by Szathmari et al. (60) revealed that low-birth-weight young women experienced increased insulin resistance and hyperandrogenism in response to modest hypercortisolism, whereas low-birth-weight young men did not.

Our findings of altered HDAC1 and HDAC activity correlate with the sex-specific differences of altered histone H3 acetylation and emphasize the sex-specific response. HDACs, which play a major role in determining the acetylation state of histones, exist in three classes. Class I HDACs resemble the yeast RPD3 protein and are generally believed to be a component of large protein complexes in vivo that direct gene-specific regulation of transcription, hormone signaling, cell cycling, differentiation, and DNA repair (61). Class II HDACs resemble the yeast Hda1 protein and are expressed in highest levels in human heart, brain, and skeletal muscle (9, 65). Class III HDACs resemble the Sir2 protein and have been implicated in chromatin silencing and aging (61).

We initiated our studies on a class I HDACs because these proteins have been found complexed with DNA methyltransferase I, whose expression is decreased in IUGR liver in association with DNA hypomethylation (42, 55, 56). Class I HDACs are also intriguing because they contribute to the regulation of multiple processes, such as hormone signaling, which is altered IUGR rats. HDAC1 drew our specific focus because it is necessary for embryonic stem cell survival, since targeted disruption of HDAC1 cannot be compensated for by HDAC2 or HDAC3 (30). Furthermore, in vitro studies demonstrate that hypoxia decreases HDAC1 protein levels, whereas growth factors activate HDAC1 (17, 20). Although little is known about the in vivo regulation of hepatic HDAC1 and HDAC activity under the conditions of decreased levels of growth factors and hypoxia that characterize the IUGR intrauterine milieu, our in vivo findings of decreased HDAC1 mRNA, nuclear protein, and HDAC activity at day 0 of life correlate nicely with published in vitro studies.

At day 21 of life, hepatic HDAC total and nuclear protein levels were higher in IUGR female rats vs. controls; furthermore, relative to male IUGR rats, female IUGR rats demonstrate significantly increased HDAC activity. It is likely that a downregulation of HDAC activity results in the persistent site-specific histone H3 hyperacetylation observed in IUGR male rats, whereas the restitution of HDAC activity in IUGR female rats decreases histone H3 acetylation to the point of potential overcompensation. The hypoacetylation at H3/K9 and H3/K18 in IUGR female livers significantly differs from the controls. As a result, the response to the deprived IUGR intrauterine milieu results in a sex-specific “histone code” that evolves over the lifetime of the animal and results in sex-specific responses to the effects of aging, as well as environmental stimuli such as diet.

Examples in the literature demonstrate sex-specific differences in adult gene expression or phenotype in response to an early nutritional insult. In response to uteroplacental insufficiency, IUGR rats of both sexes decreased liver expression of CPTI at day 21 of postnatal life, but only the male IUGR rats decreased expression of CPTI at day 120 of postnatal life relative to the controls (35). Furthermore, this phenomenon is not limited to the liver. Skeletal muscle PGC-1 mRNA and protein levels were reduced in control and IUGR animals diverged at postnatal day 21 based in part upon sex (37). Other animal models have found similar patterns of response. In response to maternal malnutrition, the growth-retarded adult male guinea pig suffered dyslipidemia and insulin resistance, whereas female growth-retarded guinea pigs were relatively unaffected (24, 25). The differential sex response to environmental stresses is not surprising considering our findings of relative histone hyperacetylation at H3/K14 and histone hypoacetylation at H3/K18 and H3/9 in the control male livers vs. the female livers. These results suggest that the “histone code” is sex specific.

Debate does exist on whether a “histone code” actually exists. Kurdistani et al. (29) acknowledged this debate surrounding the “histone code” and speculated that different patterns of histone modifications may play a role in more general chromatin functions. Indeed, Zhang et al. (64) demonstrated that acetylation of particular histone H3 and H4 lysines are required for cell growth and that cell viability requires a critical overall level of histone acetylation. Furthermore, recruitment of the general transcription factor TFIIID requires acetylation of H3/K9 and H3/K14, and HDAC1 deficiency in embryonic stem cells reduced proliferation rates and decreased cyclin-associated kinase activities, although this may involve gene-specific effects on genes such as p21WAF1 (1, 12). We speculate that histone covalent modifications such as histone acetylation serve multiple purposes that include the transmission of specific genetic information (the “histone code?”) and regulation of more general chromatin functions such as cell proliferation and viability.

Caution is necessary when attempting to apply data from a rat model to human pathophysiology. The fetal and juvenile rat is physiologically immature relative to the human, and the insult imposed on the fetal rat in this model of uteroplacental...
insufficiency is severe and specific. In contrast, the timing and impact of uteroplacental insufficiency experienced by humans ranges across a continuum.

In summary, uteroplacental insufficiency and subsequent IUGR in the rat causes site-specific changes in histone H3 acetylation and alters the histone association with the promoter regions of PGC-1 and CPTI, two genes that we have previously demonstrated to be persistently altered in the IUGR rat. We speculate that the deprived prenatal environment associated with uteroplacental insufficiency triggers a cascade of events that contributes to the post natal morbidities associated with IUGR.

REFERENCES

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IUGR AFFECTS SPECIFIC HISTONE H3 SITES


