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11 beta-hydroxysteroid dehydrogenase 2 promoter methylation is associated with placental protein expression in small for gestational age newborns

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ABSTRACT

Small for gestational age infants have greater risk of developing metabolic diseases in adult life. It has been suggested that low birth weight may result from glucocorticoid excess *in utero*, a key mechanism in fetal programming. The placental enzyme 11-beta hydroxysteroid dehydrogenase type 2 (11β-HSD2, *HSD11B2* gene) acts as a barrier protecting the fetus from maternal corticosteroid deleterious effects. Low placental 11β-HSD2 transcription and activity have been associated with low birth weight, yet the mechanism regulating its protein expression is not fully understood. In the present study we aimed to analyze 11β-HSD2 protein expression in placentas of adequate and small for gestational age (AGA and SGA, respectively) newborns from healthy mothers, and to explore whether 11β-HSD2 protein expression could be modulated by DNA methylation. 11β-HSD2 protein levels were measured by western blot in placental biopsies from term AGA and SGA infants (n = 10 per group). DNA methylation was profiled both globally and in the *HSD11B2* promoter by liquid chromatography with UV detection and methylation-specific melting curve analysis, respectively. We found lower placental 11β-HSD2 protein expression and higher *HSD11B2* promoter methylation in SGA compared to AGA. Promoter methylation was inversely correlated with both protein expression and, importantly, birth weight. No changes in global placental methylation were found. In conclusion, lower 11β-HSD2 protein expression is associated with higher *HSD11B2* promoter methylation, correlating with birth weight in healthy pregnancy. Our data support the role of 11β-HSD2 in determining birth weight, providing evidence of its regulation by epigenetic mechanisms, which may affect postnatal metabolic disease risk.

1. Introduction

Birth weight constitutes an important health factor, not only for postnatal outcome [1,2], but also for programming metabolic health in adult life [3–5]. Being born small for gestational age (SGA) increases the risk of fetal, neonatal or infant death, and leads to impaired postnatal growth, immune and neuronal development alterations [2]. Low birth weight has also been related to several diseases in postnatal life and adulthood, such as glucose intolerance [6], metabolic syndrome [7], obesity [8], type 2 diabetes [7,9], cardiovascular disease, and chronic renal disease [10]. Hyperandrogenism and polycystic ovary syndrome in females [11], excessive adrenarch, precocious puberty, and infertility in males [5] have been associated with SGA as well.

Glucocorticoids are part of the mechanisms involved in growth, and thus, regulation of glucocorticoid exposure is crucial for normal fetal growth and development [12]. Fetal over-exposure to glucocorticoids leads to an impairment in fetal growth [12]. Glucocorticoid excess has been proposed as a linking mechanism between maternal factors such

Abbreviations: 11β-HSD2, 11-beta hydroxysteroid dehydrogenase type 2 enzyme; *HSD11B2*, 11-beta hydroxysteroid dehydrogenase type 2 gene; AGA, adequate for gestational age; SGA, small for gestational age; IUGR, Intrauterine growth retardation; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; BSA, bovine serum albumin; 5mdC, 5-methyl-2′-deoxycytidine; dC, 2′-deoxycytidine

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as nutrition [13], environmental factors [14] and stress [4], with low birth weight, and adverse metabolic and neurobehavioral outcome in adulthood [15,16], likely through reprogramming of the Hypothalamic-Pituitary-Adrenal (HPA) axis [17,18].

In mammals, circulating cortisol during pregnancy is higher in the mother than in the fetus, being the placenta the only barrier impeding maternal cortisol to reach the fetal circulation [15,19]. The placental enzymes 11 beta-hydroxysteroid dehydrogenases type 1 (11β-HSD1) and 2 (11β-HSD2) participate in this fine tuning of fetal exposure to glucocorticoids, inactivating cortisol and corticosterone by their oxidation into cortisone and 11-dehydrocorticosterone respectively (11β-HSD2) and converting inactive cortisone back to cortisol (11β-HSD1) [15,19–21]. Human placenta has a predominantly 11β-HSD2 activity [9,22,23], and variations in it are capable of affecting fetal glucocorticoid levels [24] and thus fetal growth [25,26]. Furthermore, mutations in the *HSD11B2* gene causing apparent mineralocorticoid excess have been associated to reduced birth weight [27], emphasizing the important physiological role of 11β-HSD2 protecting the fetus from maternal glucocorticoids and their deleterious effects [15,19].

11β-HSD2 deficiency and its regulation may be a key mechanism in developmental programming and fetal growth [28]. Several studies have shown that placental 11β-HSD2 activity or *HSD11B2* mRNA are reduced in intra-uterine growth retardation (IUGR) and SGA newborns [25,26,29–33]. Nevertheless, placental *HSD11B2* mRNA is not always in accordance with 11β-HSD2 protein expression [29] or activity [9]. The mechanisms regulating tissue-specific *HSD11B2* transcription have not yet been clarified. It has been shown that 11β-HSD2 cell-type specific transcription and activity are subject to regulation through epigenetic modification of its promoter region, i.e. DNA methylation [34]. Recent studies in placentas of IUGR and SGA newborns have found associations between mRNA levels and methylation in four particular CpG dinucleotides within the *HSD11B2* promoter [32,35,36]. However, whether methylation of *HSD11B2* in other sites of its promoter is related to placental 11β-HSD2 protein expression in SGA has not been elucidated.

In the present study, we aimed to evaluate the protein expression of 11β-HSD2 in placenta and its association with birth weight in SGA born from healthy uncomplicated pregnancies, exploring if differential methylation levels in novel methylation sites on the *HSD11B2* promoter may be related to this expression.

2. Materials and methods

2.1. Research design and study population

Twenty healthy women with singleton pregnancy were recruited from UMAE 48 IMSS Hospital and General Regional Hospital in the city of Leon, Guanajuato, Mexico. The study was approved by the ethics committee of the Department of Medical Sciences of the University of Guanajuato, and the research committees of the correspondent hospitals from which the samples were taken. All procedures were performed according to the Mexican General Health Laws and the Declaration of Helsinki. Participants signed informed consent prior to recruitment.

Mothers between 18 and 35 years of age and their term (37–40 weeks) newborns, born from vaginal delivery or non-elective caesarean section, without perinatal asphyxia or acute fetal suffering signs, and Apgar 7 at first minute were included in the study. Women diagnosed with preeclampsia, gestational or type 2 diabetes, antiphospholipid syndrome, connective tissue diseases, chronic infection, alcohol consumption or smoking habit during the current pregnancy were not included. Newborns were classified as born small for gestational age (SGA, lowest 10th percentile) or appropriate for gestational age (AGA), based on their birth weight and gestational age using the tables currently validated for Mexican population [37]. Within 1 h after delivery, a 5 × 5 cm piece of placental tissue, including fetal and maternal sides, was cut halfway between the cord insertion and the placental border. Separate transversal cuts from this biopsy were made to perform protein expression and DNA methylation analysis. Placental tissue was immediately snap frozen in dry ice and refrigerated at −70 °C until use.

2.2. Western blot of placental 11β-HSD2

Approximately 150 mg of placental tissue were washed in cold PBS and homogenized in cold lysis buffer (10 mM HEPES, 320 mM sucrose, 100 μM EDTA, 1.5 mM DTT) with protease inhibitors (Complete, Roche). Protein quantification was performed by Lowry assay [38]. Fifty ug of protein for each sample, or 75 ug of AGA or SGA protein pool were loaded per lane. Four ug of Hydroxysteroid Dehydrogenase, 11-beta Type II, Human control synthetic peptide (USBiologicals) were loaded to confirm antibody specificity (Supplementary Fig. A.1). Proteins were resolved by 10% SDS-PAGE gel electrophoresis and electrotransferred to nitrocellulose membranes. Membranes were blocked overnight in 5% skimmed milk to reduce non-specific binding. Blots were then incubated 2 h with Anti 11β-HSD2 antibody (1:1000, USBiologicals), washed, and incubated with *anti*-rabbit IgG HRP (1:2000, Santa Cruz) for 1 h. Bands were detected by chemiluminescence using Luminol ECL Plus Western Blotting Detection System (Amersham, GE Healthcare). Band intensities were quantified by densitometry in a Chemidoc XRS + Molecular Imager®, using Lab Image software 3.0 (Bio-rad). Normalization of band intensity was done with *anti*-alpha tubulin antibody (1:8000, Sigma) using *anti*-mouse IgG HRP as secondary antibody (1:4000, Sigma). 11β-HSD2 placental concentration was estimated for each sample as well, using densitometric measure of control peptide, adjusting by total protein load (Supplementary Fig. A.2).

2.3. DNA extraction and DNA conversion with bisulfite

Genomic DNA from placental tissue was extracted using the DNA purification Wizard Genomic kit (Promega), according to the manufacturer instructions. DNA quality was evaluated by 1% agarose gel electrophoresis, and DNA quantification was performed using a Gene QuantII spectrophotometer (Pharmacia Biotech). Genomic DNA was treated with sodium bisulfite, which converts unmethylated cytosine to uracil, whereas methylated cytosine residues remain unmodified [39]. Briefly, DNA (1.5 μg) was digested with 1.5 μg of *Eco*RI (Invitrogen), 1.45 μL of 10X enzyme buffer and 200 ng BSA/μg DNA, for 6 h at 37 °C. DNA in 20 μL final volume was then denatured with 2 μL of NaOH 3 M, incubating for 5 min at 37 °C. Next, 12 μL of hydroquinone (10 mM) and 208 μL of sodium bisulfite (2 M) was added and the mix was incubated for 16 h at 55 °C. Wizard DNA clean-up System (Promega) was used to recover the modified DNA, which was treated with 5.5 μL NaOH 3 M and precipitated with 20 μg of glycogen, 33.3 μL of ammonium acetate (3 M, pH 7) and 300 μL 100% ethanol, at 70 °C for 4 h. The DNA pellet was dissolved in 30 μL bi-distilled water and stored at −20 °C until used [40].

2.4. HSD11B2 promoter methylation

To determine *HSD11B2* promoter methylation, primers were designed to amplify both methylated and unmethylated bisulfite-converted DNA using the open access MethPrimer software (http://www.urogene.org/methprimer/index1.html). The primers used were 5′-TTTTTGTTTTAGGTAGGTTTTGTGG-3′ (forward) and 5′-CTCAAATAAACACATACCACTCAC-3′ (reverse). The primers amplify a 289 bp region (chr16:67,430,457–67,430,745; human GRCh38 genome) mapping to the distal promoter (position −660 to −372 relative to the transcription start site) of *HSD11B2*, containing 31 CpGs. Those CpGs have been previously shown to be subject to methylation in normal placental tissue [34]. CpGs profiled in previous studies [32,35,36] are excluded from the region evaluated in the present study,