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Reduced Placental 11β-Hydroxysteroid Dehydrogenase Type 2 mRNA Levels in Human Pregnancies Complicated by Intrauterine Growth Restriction: An Analysis of Possible Mechanisms

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11β-Hydroxysteroid dehydrogenase type 2 (11β-HSD2) inactivates cortisol to cortisone. In the placenta 11β-HSD2 activity is thought to protect the fetus from the deleterious effects of maternal glucocorticoids. Patients with apparent mineralocorticoid excess owing to mutations in the 11β-HSD2 gene invariably have reduced birth weight, and we have recently shown reduced placental 11β-HSD2 activity in pregnancies complicated by intrauterine growth restriction. This is reflected in the literature by evidence of hypercortisolemia in the fetal circulation of small babies. In this study we have determined the levels of placental 11β-HSD2 mRNA expression across normal gestation (n = 86 placentae) and in pregnancies complicated by intrauterine growth restriction (n = 19) and evaluated the underlying mechanism for any aberrant 11β-HSD2 mRNA expression in intrauterine growth restriction. 11β-HSD2 mRNA expression increased more than 50-fold across gestation, peaking at term. Placental 11β-HSD2 mRNA levels were significantly decreased in intrauterine growth restriction pregnancies when compared with gestationally matched, appropriately grown placentae [*e.g.* at term ΔCt (11β-hydroxysteroid dehydrogenase type 2/18S) 12.8 ± 0.8 (mean ± SE) *vs.* 10.2 ± 0.2, respectively, $P < 0.001$]. These differences were not attributable to changes in trophoblast mass in intrauterine growth restriction placentae, as assessed by parallel analyses of cytokeratin-8 mRNA expression. No mutations were found in the 11β-HSD2 gene in the intrauterine growth restriction cohort, and imprinting analysis revealed that the 11β-HSD2 gene was not imprinted. Although the underlying cause is unknown, 11β-HSD2 gene expression is reduced in intrauterine growth restriction pregnancies. These data highlight the important role of 11β-HSD2 in regulating fetal growth, a known factor in determining fetal morbidity but also the subsequent development of cardiovascular disease in adulthood. (*J Clin Endocrinol Metab* 86: 4979–4983, 2001)

FETAL GROWTH RESTRICTION is an important cause of perinatal morbidity and mortality (1) but, through as yet ill-defined "programming" mechanisms, has also been linked to the development of prevalent adult diseases including hypertension, diabetes, and coronary artery disease (2). The endocrine control of fetal growth, therefore, seems likely to have a major impact on both neonatal and adult well-being. Glucocorticoid excess *in utero* has been shown to impair fetal growth in rodents (3) and man (4). It is also of note that in both animal models (5) and human pregnancies (6, 7), small-for-gestational-age babies appear to have elevated cortisol levels. A key factor in determining glucocorticoid hormone action is the "prereceptor" role played by two distinct isozymes of 11β-hydroxysteroid dehydrogenase (11β-HSD) that interconvert hormonally active cortisol to inactive cortisone (8, 9). 11β-HSD1 is a low-affinity, nicotinamide adenine dinucleotide phosphate-dependent dehydrogenase/11-oxo-reductase (10, 11), which is expressed in the maternal decidua but is low or absent in human fetal tissues including trophoblast (12). 11β-HSD2 is a high-affinity nicotinamide adenine dinucleotide-dependent dehydrogenase (13) that is principally expressed in the kidney and colon (12, 14) where it serves to protect the MR from cortisol excess, enabling aldosterone to interact with the MR (8, 9). In addition, the enzyme is widely distributed in human fetal tissues (12), including the placental trophoblast where it is expressed in particularly high abundance (15–20). Unlike the kidney, however, the precise role of placental 11β-HSD2 is unknown; the placenta is not an obvious mineralocorticoid target tissue and it seems likely that the enzyme may modulate cortisol exposure to the GR rather than the MR (21). Specifically in the placenta, the concept has emerged that 11β-HSD2 inactivates the much higher maternal cortisol concentrations across gestation, thereby facilitating fetal growth (22). In support of this, significant correlations between birth weight and placental 11β-HSD2 activity have been reported in some human studies (18) but not others (19).

Furthermore, patients with mutations in the HSD11B2 gene, located on human chromosome 16, present with a severe form of mineralocorticoid hypertension [the syndrome of apparent mineralocorticoid excess (AME)] (8) but, in addition, have impaired fetal growth with low birth weight (23). Genetic studies have documented the associa-

Abbreviations: AME, Apparent mineralocorticoid excess; 11β-HSD, 11β-hydroxysteroid dehydrogenase; IUGR, intrauterine growth restriction; SNP, single nucleotide polymorphism.

tion between intrauterine growth restriction (IUGR) and confined placental mosaicism, and chromosome 16 has been shown to be involved. Furthermore, trisomy 16 is the most common trisomy associated with both miscarriage (10% of trisomies found) and IUGR (24). In recent studies we have reported reduced placental 11β-HSD2 activity in pregnancies complicated by severe IUGR (25). The aim of this study was to further examine the role of placental 11β-HSD2 across normal gestation and in pregnancies complicated by IUGR. Having documented reduced placental 11β-HSD2 mRNA levels in IUGR, we also explored the underlying basis for this by screening the HSD11B2 gene for any disease-causing mutations similar to those seen in AME and determined the imprinting status of the gene in IUGR placentae.

Materials and Methods

Samples

The study had the approval of the local hospital ethics committee. Placental samples were collected from either termination tissues (in accordance with the Polkinghorne report) or elective cesarean sections. A total of 86 placental samples were collected and grouped as follows: early first trimester (6–8 wk gestation, n = 19), late first trimester (9–12 wk gestation, n = 16), second trimester (13–17 wk gestation, n = 6), early third trimester (27–34 wk gestation, n = 4), and term (n = 22). Nineteen cases of IUGR were diagnosed prospectively, having at least three of the four following characteristics determined from ultrasound examination: 1) ultrasound measurement of fetal abdominal circumference less than or equal to the third percentile for gestational age, 2) abnormal fetal growth velocity (Δabdominal circumference <1.5 SD over 14 d), 3) severe oligohydramnios (amniotic fluid index less than or equal to the third percentile for gestational age), 4) absent or reversed velocities in umbilical artery Doppler waveforms. The IUGR group was divided into early and late gestation groups, 15 patients being 25–32 wk gestation and 4 patients 37–38 wk gestation.

In each case, placental samples were obtained by dissecting the placental tissue off the chorionic plate. No membrane was left adherent to the placental samples that were snap frozen in liquid nitrogen and stored at −80 C until analysis.

RNA extraction

RNA was extracted from placentae using the StrataPrep total RNA miniprep kit (Stratagene, Amsterdam, The Netherlands), which included a *DNase* digestion step to remove any contaminating genomic DNA. One microgram of RNA from each sample was reverse transcribed using AMV reverse transcriptase (Promega Corp., Southampton, UK) and random hexamers in 20-μl reaction volumes according to the manufacturer's instructions.

Quantitative PCR

11β-HSD2 mRNA levels were analyzed using an ABI 7700 sequence detection system, which employs TaqMan chemistry for highly accurate quantification of mRNA levels. Briefly, a fluorogenic TaqMan probe consists of an oligonucleotide with a 5′ reporter and a 3′ quencher dye. Fluorescent quenching of the reporter dye depends on the spatial proximity of its quencher. PCR amplification releases the reporter into solution, away from its quencher, yielding a signal that is read by a laser and CCD camera. One such event occurs for each PCR product generated, enabling real-time detection of cDNA amplification.

RT-PCR was performed in 25-μl volumes on 96-well plates, in reaction buffer containing TaqMan universal PCR master mix, 3 mM $Mn(Oac)_2$, 200 μM dNTPs, 1.25 U AmpliTaq Gold polymerase, 1.25 U AmpErase UNG, 100–200 nmol TaqMan probe, 900-nmol primers and 25–50 ng cDNA. All reactions were multiplexed with the housekeeping gene 18S, provided as a preoptimized control probe (PE Biosystems, Warrington, UK) enabling data to be expressed in relation to an internal reference to allow for differences in reverse transcription efficiency. Data were obtained as Ct values according to the manufacturer's guidelines (the cycle number at which logarithmic PCR plots cross a calculated threshold line) and used to determine ΔCt values (ΔCt = Ct of the target gene minus Ct of the housekeeping gene). Measurements were carried out on at least three occasions for each sample. All target gene probes were labeled with the fluorescent label FAM, and the housekeeping gene with the fluorescent label VIC. Reactions were as follows: 50 C for 2 min, 95 C for 10 min, and then 44 cycles of 95 C for 15 sec and 60 C for 1 min.

As a further control, RT-PCR was performed on all the placental samples to determine the levels of cytokeratin-8 mRNA expression, a trophoblast epithelial marker (26).

Quantitative primer and probe sequences for 11β-HSD2 were forward primer GGGCCTATGGAACCTCCAA, reverse primer GACCCACGTTTCTCACTGACTCT, and TaqMan probe CCGTGGCGCTACTCATGGACACA and for cytokeratin-8 were forward primer ATGTGGATGAAGCATACATGAACA, reverse primer TCCCGGATCTCCTCTTCATACA, and TaqMan probe CCGACGAGATCAACTTCCTCAGGCA.

To exclude potential bias owing to averaging data that had been transformed through the equation $2^{-\Delta\Delta Ct}$, all statistics were performed at the ΔCt stage. Statistical analysis of comparisons among groups was undertaken using an unpaired *t* test.

DNA extraction and 11β-HSD2 gene studies

DNA was extracted from IUGR and normal term placentae using the Nucleon Bacc II DNA extraction kit (Tepnel Life Sciences, Manchester, UK) according to the manufacturer's instructions. PCR primers were designed for each of the five exons and the promoter of 11β-HSD2 as previously reported by our group (27). PCR was performed on DNA from 10 IUGR placentae using 200 ng DNA, 25-pmol primers, and 2.5 U *Taq* polymerase (Promega Corp.) according to standard procedures. The cycling conditions were 94 C for 5 min for one cycle, 94 C for 20 sec, 55–60 C for 20 sec, and 72 C for 20–30 sec for 30–32 cycles depending on the region being amplified. The amplicons were electrophoresed on agarose gels and then purified using the Qiaex II gel purification kit (QIAGEN, Crawley, UK). Amplicons were sequenced using BigDye terminator sequencing chemistry (PE Biosystems) and sequences analyzed and compared with both GenBank data (accession numbers U27317 and U27318) and our previous sequencing results.

Imprinting analyses used two single nucleotide polymorphisms (SNPs) present in exon 3 of the 11β-HSD2 gene at codon positions 178 (GAG to GAA) (28) and 196 (GCG to GCA) (29). Exon 3 was amplified from DNA of 22 term placentae and the amplicons electrophoresed, gel purified, and sequenced as detailed previously. RNA was extracted from three placentae that were heterozygous for the SNPs; 1 μg of RNA from each sample was reverse transcribed, and exon 3 was amplified using a primer spanning the exon 3–4 boundary (5′ATATGGCATGTCCCCCGCTG3′) and an exon 2 primer. This in conjunction with the *DNase* treatment of the RNA excludes the possibility of genomic DNA contamination in the PCR.

Results

mRNA expression studies

Normal gestation. Relative fold increases in 11β-HSD2 mRNA expression across gestation were calculated using the early first trimester group as a reference (arbitrary value of 1). The 11β-HSD2 mRNA expression was similar in the late first and second trimester groups (relative fold increase: late first trimester 0.65; second trimester 0.48) as it was in the early first trimester group. There was, however, a 12-fold (ΔCt 12.46, $2^{-\Delta\Delta}$Ct 11.98) increase in 11β-HSD2 mRNA expression at gestational wk 27–34, and a 56-fold (ΔCt 10.24, $2^{-\Delta\Delta}$Ct 55.7) increase at term when compared with early first trimester (Fig. 1).

IUGR. The 11β-HSD2 mRNA levels were significantly reduced in placentae from IUGR pregnancies. When analyzed as a whole group, the IUGR placentae demonstrated only a 4-fold increase in 11β-HSD2 mRNA expression, compared

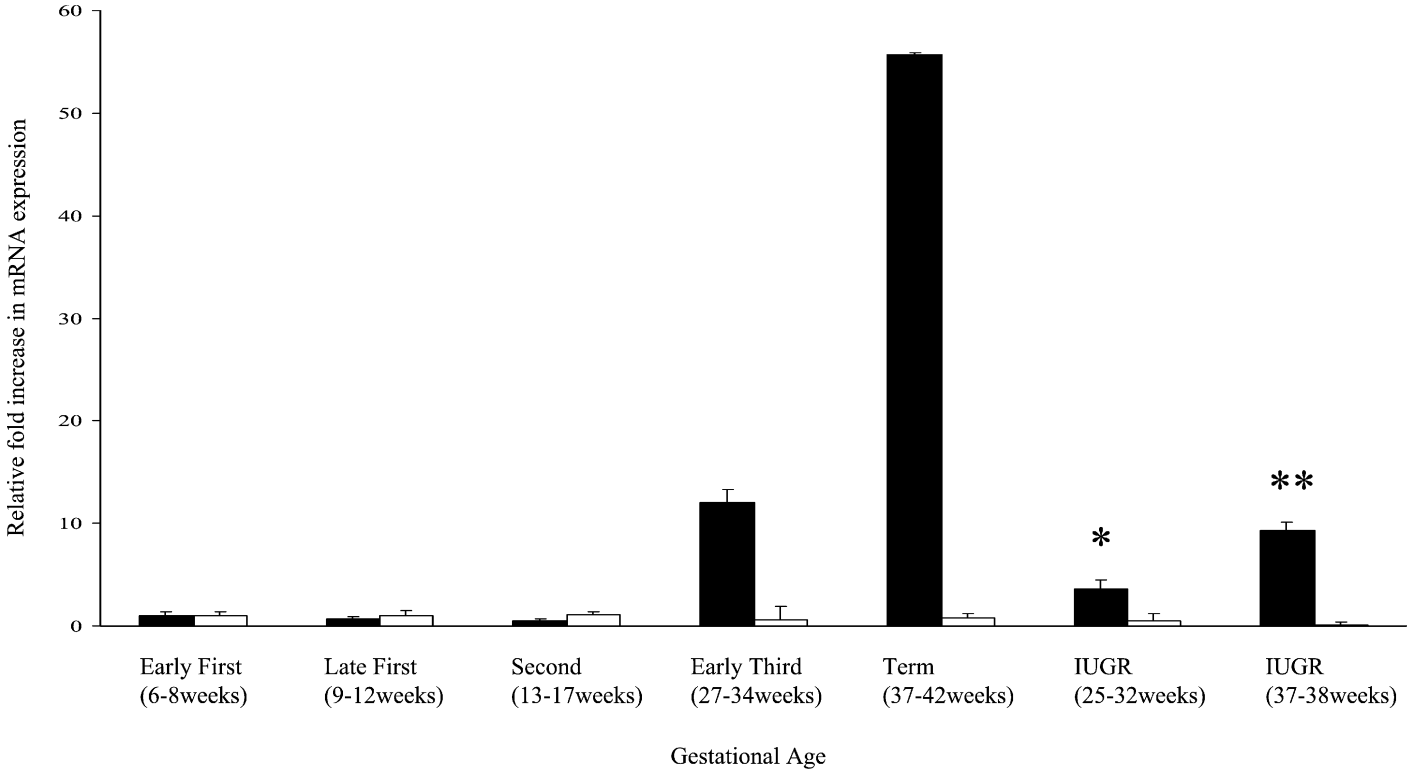

FIG. 1. The relative fold increase in 11β-HSD2:18S *(black)* and cytokeratin 8:18S *(white)* mRNA expression, across gestation and in IUGR-complicated placentae. Statistically significant differences were observed between the early third trimester and early IUGR placentae ($P = 0.04$) and between the term and late IUGR placentae ($P < 0.001$).

with early first trimester (a 12- to 56-fold increase in normal placentae was observed). When the IUGR cohort was analyzed on the basis of gestational age, the early subgroup (25–32 wk) had reduced 11β-HSD2 mRNA levels, compared with gestational-matched normally grown placentae (early third trimester group) [ΔCt 14.2 ± 0.9 *vs.* 11.3 ± 1.0 (mean ± SE), respectively, $P = 0.04$]. The later IUGR subgroup (37–38 wk) also had reduced 11β-HSD2 mRNA levels, compared with term placentae (ΔCt, 12.8 ± 0.8 *vs.* 10.2 ± 0.2, $P < 0.001$) (Fig. 1).

There were no significant differences in the levels of cytokeratin-8 mRNA across gestation (early first trimester ΔCt 12.66 ± 0.44; second trimester ΔCt 12.47 ± 0.31; term ΔCt 13.04 ± 0.42) or, importantly, in the IUGR subgroup (25–32 wk, ΔCt 13.54 ± 0.69; 37–38 wk, ΔCt 14.55 ± 0.21), indicating that the reduction in 11β-HSD2 mRNA levels could not be explained by changes in the amount of trophoblast tissue (Fig. 1).

11β-HSD2 gene studies

All five exons and the promoter region (−860 bp) of the 11β-HSD2 gene were amplified and sequenced in 10 placentae from pregnancies complicated with IUGR. No mutations were identified.

The two SNPs in exon 3 of 11β-HSD2, positions 178 (GAG to GAA) and 196 (GCG to GCA), were examined in 22 term placentae. Three of the placentae were informative at one of these loci, and cDNA was prepared from them and sequenced to determine which allele was being expressed. Both alleles of the SNP were present in all three placentae (Fig. 2), indicating that the parental allele was not silenced suggesting that 11β-HSD2 is not an imprinted gene.

Discussion

Across normal human pregnancy, levels of 11β-HSD2 mRNA expression were similar in the first and second trimester groups but thereafter significantly increased in the early third trimester (12-fold) to term (56-fold increase). These data support earlier placental enzyme activity analysis carried out in a separate pregnancy cohort (25) and indicate that the exponential increase in placental 11β-HSD2 expression as term progresses is mediated at the transcriptional level. In the baboon placenta, E increases 11β-HSD2 enzyme expression (30) (as it does in the rodent kidney), and the rising E levels that occur across pregnancy may account for the changes in 11β-HSD2 gene expression. Conversely, at least in late gestation, progesterone levels fall and *in vitro* studies have shown that progesterone inhibits 11β-HSD2 enzyme activity (31). Other factors within the placenta may mediate these striking changes in 11β-HSD2 expression. Thus, although the 11β-HSD2 protein is identical in adult and fetal tissues (32), the use of alternate transcriptional start sites results in placental 11β-HSD2 (as determined in the human choriocarcinoma cell, JEG-3 cells) but not adult kidney 11β-HSD2 being regulated by cAMP and its analogs (33–35). Additionally, nitric oxide inhibits 11β-HSD2 activity and mRNA levels in cultured human term trophoblast cells (34).

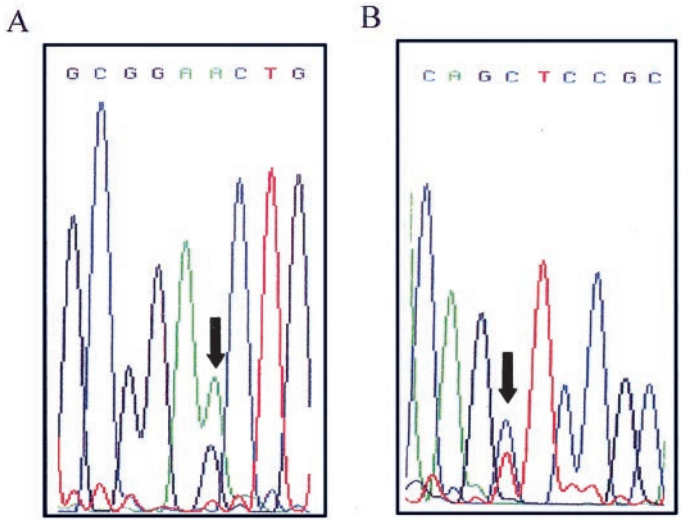

FIG. 2. Imprinting analysis of term placentae. A, Sequence of DNA from exon 3 11β-HSD2 shown in the forward orientation. SNP at position 178 is indicated by an arrow, with both A and G present at the same position. B, Sequence of cDNA from exon 3 11β-HSD2 shown in the reverse orientation. SNP at position 178 is indicated by an *arrow*, with both C and T present at the same position.

Placental 11β-HSD2 activity studies together with selective sampling of the placental vascular beds indicate the immense capacity of the placenta to inactivate cortisol to cortisone (18, 19, 25, 36). Despite this impressive activity, the definitive role of placental 11β-HSD2 remains unknown. Studies in the baboon, supported by data in humans, suggest that the placental inactivation of cortisol facilitates the development and "awakening" of the fetal hypothalamopituitary-adrenal axis. Thus, the removal of the suppressive influence of cortisol (be it maternal or fetal in origin) is the stimulus to activate fetal hypothalamic corticotrophin-releasing factor and pituitary POMC synthesis and secretion (30, 37). Alternatively, it has been suggested that placental 11β-HSD2 may serve as a barrier, protecting the developing fetus from the possible deleterious effects of maternally derived glucocorticoids (3, 36). Evidence from rodent, primate, and human studies support the notion that glucocorticoid excess *in utero* impairs fetal growth (3, 4), thereby providing a potential mechanism to explain the Barker hypothesis—the inverse correlation between birth weight and prevalence of adult hypertension, diabetes, and coronary artery disease (2). Placental 11β-HSD2 expression may, in turn, be crucial to this process. Thus, patients with AME owing to mutations in the 11β-HSD2 gene invariably have reduced birth weight (23), and correlations between placental 11β-HSD2 activity and birth weight have been reported across normal pregnancies in some (18) but not all (19) studies.

Several rodent studies have attempted to address this issue, and the adverse programming of insulin sensitivity and blood pressure has been documented in adult rats following maternal treatment with carbenoxolone, an 11β-HSD2 inhibitor (38). However, recombinant mice lacking the 11β-HSD2 gene are of normal birth weight (39); similarly, we were unable to alter birth weight in the mouse by inhibiting fetal 11β-HSD2 expression across gestation (40). However, the ontogeny of 11β-HSD2 in the rodent is notably different from that reported here across human pregnancy; in both rats and mice, 11β-HSD2 gene transcription is effectively silenced in mid- and late gestation in fetal tissues including the placenta in contrast to the exponential increase observed in man (41, 42). The applicability of rodent models in this setting, therefore, is highly questionable.

In placentae from IUGR-complicated pregnancies, 11β-HSD2 mRNA levels were significantly reduced when compared with term and gestationally matched, appropriately grown pregnancies. Importantly, this could not be attributed to obvious changes in trophoblast mass between IUGR and normal placentae in view of the similar amounts of the trophoblast marker, cytokeratin-8, present in each group of samples. Previous data from our group have noted that 11β-HSD2 is the predominant isoform expressed in trophoblast tissue (12). However, other groups have noted the expression of 11β-HSD1 on "intermediate villi and endothelial cells on the fetal vasculature" (20). Our own data from cultured primary cytotrophoblast cells, although confirming the type 2 isoform as the predominantly expressed 11β-HSD2 in trophoblast, have noted low levels of mRNA encoding 11β-HSD1 in these cultured cells (43). It is possible, therefore, that maintenance of some 11β-HSD1 activity and attenuated 11β-HSD2 would account for elevated fetal cortisol concentrations seen in IUGR pregnancies. These data support our earlier posttranscriptional (activity) studies performed on a separate cohort of patients with IUGR (25) and provide impressive, circumstantial evidence for a role of 11β-HSD2 in regulating fetal growth. Whether this is mediated by glucocorticoid excess *in utero* remains to be proven; it should be noted that the expression of 11β-HSD2 is widespread in many fetal tissues in addition to trophoblast, suggesting that developing fetal tissues can protect themselves in an autocrine fashion (12, 21). An alternative hypothesis, therefore, is that 11β-HSD2 is directly involved in the placentation process itself.

The explanation for the reduction in placental 11β-HSD2 gene expression in IUGR remains unknown. We have not identified any mutations in the 11β-HSD2 gene from DNA extracted from IUGR placentae. Several cases of IUGR have been reported in association with confined placental mosaicism. In particular, a high incidence of maternal uniparental disomy for chromosome 16 (the chromosomal localization of the 11β-HSD2 gene) has been found in confined placental mosaics of IUGR placentae (24). Imprinting of a gene on chromosome 16 therefore could result in distorted expression of that gene product. For example, if 11β-HSD2 was an imprinted gene and expressed only on the paternal chromosome, maternal uniparental disomy for chromosome 16 would lead to zero expression of 11β-HSD2; if this occurred in a confined placental mosaic, it would lead to areas of the placenta without 11β-HSD2 activity. Additionally, it is established that a mechanism used in imprinting is methylation. Methylation of CpG sites within GC-rich promoters can effectively, and heritably, lock genes in the off condition (44). Both the promoter region and exon 1 of the 11β-HSD2 gene are extremely GC rich and therefore a candidate for gene silencing by imprinting. However, by evaluating informative encoding polymorphisms within the 11β-HSD2 gene, we can conclude that the gene is not imprinted and

therefore any uniparental expression of 11β-HSD2 would not result in varying levels of enzyme expression. It is still possible that methylation may result in epigenetic silencing of 11β-HSD2 in IUGR placentae, and this needs further evaluation.

In summary, we have demonstrated that placental 11β-HSD2 gene expression increases across normal human gestation but is significantly decreased in pregnancies complicated by IUGR. The cause of the reduction in mRNA expression cannot be explained on the basis of mutations in the 11β-HSD2 gene or this gene being imprinted. This study supports the concept that placental 11β-HSD2 plays a crucial role in regulating fetal growth across human pregnancy. Defects in expression of the enzyme in fetal life may have considerable ramifications for both neonatal and adult well-being.

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