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OUTSTANDING CONTRIBUTION

11β-hydroxysteroid dehydrogenase type 2 in human pregnancy and reduced expression in intrauterine growth restriction

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The type 2 isoform of 11β-hydroxysteroid dehydrogenase (11β-HSD2), which inactivates cortisol (F) to cortisone (E), has been suggested to play a role in the ontogeny of the fetal pituitary–adrenal axis and also protect the developing fetus from the deleterious effects of circulating maternal glucocorticoids. The abundance of 11β-HSD2 in the placenta and other fetal tissues was inferred from the F/E ratio in 17 term deliveries in both umbilical arterial (1.73 ± 0.24, mean ± SE) and umbilical venous blood (1.16 ± 0.14) compared with adult peripheral venous blood (7.76 ± 0.57, *n* = 70). Using sensitive assays for 11β-HSD2 and an in-house human 11β-HSD2 antibody, the expression and activity of this enzyme in fresh frozen human placenta increased progressively from first (8–12 weeks, *n* = 16) and second (13–20 weeks, *n* = 9) to third trimester (term) pregnancies (39–40 weeks, *n* = 50). Placental 11β-HSD2 activity was significantly reduced in deliveries complicated by intrauterine growth restriction (IUGR) [25–36 weeks, *n* = 12, activity 380 pmol/mg/h median (225–671; 95% confidence interval)], compared with the term deliveries [888 (725–1362)] and with appropriately grown pre-term deliveries [27–36 weeks, *n* = 14, activity 810 (585–1269)], *P* < 0.05. In human pregnancy placental 11β-HSD2 activity increases markedly in the third trimester of pregnancy at a time when maternal circulating levels of glucocorticoid are rising. The finding of attenuated placental 11β-HSD2 activity in IUGR suggests that glucocorticoids may, in part, contribute to impaired fetal growth and that this is closely controlled in normal gestation through placental 11β-HSD2 expression.

Key words: cortisol/cortisone/11β-hydroxysteroid dehydrogenase/fetal growth/placenta

Introduction

Fetal growth restriction is an important cause of perinatal morbidity and mortality (West Midlands Perinatal Audit, 1996). Although fetuses with intrauterine growth restriction (IUGR) form a heterogeneous group, a major aetiological factor is abnormal placentation (Khong *et al.*, 1986). The trophoblast facilitates the vascular connection between mother and fetus, a process which is essential for fetal growth, by secondary migration of extravillous trophoblast to surround, invade and transform the decidua and intramyometrial portions of the spiral arteries, converting them into low resistance blood vessels (Pijnenborg, 1996). A wide spectrum of clinical disorders, including late spontaneous abortion, pre-eclampsia and IUGR, are associated with a reduced or failed transformation of the uteroplacental arteries by this extravillous trophoblast. Severe compromise of umbilical blood flow in IUGR with absent end-diastolic flow velocities (AEDFV) in umbilical arteries results in increased downstream impedance in the fetoplacental circulation secondary to abnormal development of the villous vasculature (Jackson *et al.*, 1995). In addition to the abnormal vasculature, there has been considerable interest in the role of adrenocorticosteroids and the control of fetal growth. Part of this impetus has come through an attempt to explain the underlying inverse correlation between fetal growth and the development of adult diseases such as hypertension and diabetes (Barker *et al.*, 1990; Hales *et al.*, 1991), implying prenatal programming. It has been argued that the placental inactivation of cortisol by an enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD) may protect the developing fetus from the deleterious effects of maternally-derived cortisol (Benediktsson *et al.*, 1993). Two distinct isozymes of 11β-HSD have been cloned and characterized (Stewart, 1996; White *et al.*, 1997). 11β-HSD1 is a low affinity, NADP(H)-dependent dehydrogenase/11oxo-reductase (Tannin *et al.*, 1991; Stewart *et al.*, 1994a), whereas 11β-HSD2 is a unidirectional high affinity NAD-dependent dehydrogenase (Albiston *et al.*, 1994). 11β-HSD2 is principally expressed in kidney and colon in human adult tissues (Albiston *et al.*, 1994; Whorwood *et al.*, 1995) where it serves to protect the mineralocorticoid receptor (MR) from cortisol excess, enabling aldosterone to interact with the MR. The enzyme is more widely distributed in human fetal tissues (Stewart *et al.*, 1994b), including the placenta where it is expressed in particularly high abundance (Murphy *et al.*, 1974; Lopez-Bernal *et al.*, 1980; Blasco *et al.*, 1986; Brown *et al.*, 1993; Stewart *et al.*, 1995; Rogerson *et al.*, 1997; Sun *et al.*, 1997). Although 11β-HSD1 is expressed in human decidua, very little, if any 11β-HSD1 is expressed in human placenta (Stewart *et al.*, 1995; Sun *et al.*, 1997).

Table I. Demographic details of pregnancies studied. Where appropriate results are expressed as median [95% CI]

	First trimester	Second trimester	Pre-term (not IUGR)	IUGR	Term
No. studied	16	9	14	12	50
GA (weeks)	10 [9–10]	15 [14–18]	34 [29–35]	33 [28–36]	40 [39–40]
Placental weight (g)			450 [327–761]	138 [101–232]	625 [571–703]
Fetal weight (g)			2070 [1291–2500]	1847 [713–2050]	3390 [3285–3508]
AEDFV (*n*)			0/14	11/12	0/50
Oligohydramnios (*n*)			0/14	10/12	0/50

IUGR = intrauterine growth restriction; GA = gestational age; AEDFV = absent end-diastolic flow velocities.

In support of a 'protective' role for placental 11β-HSD2, the administration of high doses of glucocorticoids to pregnant rats has resulted in hypertensive offspring with reduced birthweight (Lindsay *et al.*, 1995). Furthermore, patients with a form of inherited hypertension (apparent mineralocorticoid excess) due to mutation in the 11β-HSD2 gene were shown to have had reduced birth weights (Kitanaka *et al.*, 1996). Finally, in some studies correlations have been reported between term placental 11β-HSD2 activity and birth weight (Benediktsson *et al.*, 1993; Stewart *et al.*, 1995). Given the proposed importance of 11β-HSD2 in regulating fetal growth and development, we have analysed 11β-HSD2 expression and activity across normal human pregnancy, and in pregnancies complicated by IUGR with AEDFV in the fetoplacental circulation.

Materials and methods

Subjects

We studied 101 women with singleton pregnancies of known gestational age (confirmed by ultrasonography). All subjects were on no regular medication and were normotensive at booking and throughout pregnancy. The study had the approval of the South Birmingham Ethics Committee. Placentae were collected from three groups of pregnancies: (i) first trimester following surgical termination of pregnancy (8–12 weeks, $n = 16$); (ii) second trimester following surgical termination of pregnancy (13–20 weeks, $n = 9$); and (iii) preterm deliveries of appropriately grown babies (gestational age 27–36 weeks, $n = 14$). Of the fetuses, 10 were delivered vaginally and a further four by Caesarean section; (iv) term deliveries (39–40 weeks of gestation, $n = 50$). 90% were delivered vaginally ($n = 45$) and the remainder by Caesarean section; (v) fetal growth restriction (gestational age 25–37 weeks, $n = 12$). Our definition of IUGR was prospective and rigorous, defined by at least three of the four ultrasound-derived criteria: (i) an abdominal circumference $<$3rd centile for gestational age; (ii) abnormal umbilical artery Doppler waveforms (AEDFV); (iii) oligohydramnios; and (iv) a growth velocity < 1.5 SD/week. No fetuses had congenital anomalies or aneuploidy (Chang *et al.*, 1993).

In each case, fresh trophoblast tissue was dissected from fetal membranes and either frozen in liquid nitrogen and stored at –70°C or fixed in 10% formaldehyde prior to embedding in paraffin wax.

11β-HSD2 activity studies

Placental tissue was thawed and homogenized in 0.154 M phosphate buffer using an Ultraturrax homogenizer. Homogenates were centrifuged for 10 min at 1000 *g* to sediment large tissue fragments and a protein assay (Biorad, Richmond, CA, USA) was performed on the supernatant. Activity studies were undertaken under conditions of first order kinetics as previously reported (Stewart *et al.*, 1995). Briefly, homogenates (50 μg protein/ml) were incubated in 0.5 ml of phosphate-buffered saline (PBS; 0.1 M, pH 7.6) containing 50 000 c.p.m. [^{3}H]F, 100 nmol/l F and 100 nmol/l of nicotinamide-adenide dinucleotide (NAD) for 15 min at 37°C in a shaking water bath. The reaction was terminated by the addition of 10 volumes of dichloromethane. Steroids were extracted in dicholoromethane, concentrated, and separated by thin layer chromatography (TLC) using ethanol:chloroform (8:92) as mobile phase. The TLC plates were analysed on a Bioscan imaging detector (Lablogic, Sheffield, UK) and the fractional conversion of F to E calculated. All incubates were carried out in triplicate and the experiment was repeated three times. Activity was expressed in picomoles of E formed/mg homogenate protein/h.

Immunocytochemistry

Sections (4 μm) of formalin-fixed, paraffin-embedded placentae were mounted onto superFrost Plus-coated microscope slides (Surgipath; London, UK) and heat fixed. Sections were deparaffinized in xylene, rehydrated and washed in PBS. Endogenous peroxidase activity was quenched by incubating tissue sections in 3% hydrogen peroxide (in PBS) for 30 min. After washing in PBS, sections were pre-incubated for 30 min in 10% normal goat serum and subsequently for 60 min at room temperature with a 1:100 dilution of a polyclonal sheep antibody (Ab) specific for human 11β-HSD2 (Shimojo *et al.*, 1997). After washing in PBS, a 1:25 dilution of a donkey anti-sheep peroxidase conjugate (Binding Site, Birmingham, UK) was applied to the sections for 30 min. Immunoreactivity was visualized by the addition of 0.5 mg/ml of diaminobenzidine with 0.01% hydrogen peroxide in PBS. Sections were washed with distilled water, counterstained with Carazzi's haematoxylin (R.A.Lamb; London, UK) and mounted. In each case positive controls (human kidney) and negative controls (human liver, use of Ab pre-incubated with immunising peptide) were performed simultaneously.

Steroid assays

Cortisol and cortisone were measured by radioimmunoassay in umbilical arterial and venous blood from 17 of the 50 term deliveries and from peripheral venous blood in 70 normal adults. Serum cortisone was extracted into chloroform and dried extracts were analysed by radioimmunoassay using antiserum N-137 and a 21-acetyl-cortisone-3CMO-histamine [^{125}I] tracer (Wood *et al.*, 1996). Cortisol was measured by a direct assay using Guildhay antiserum

HPS 631-IG and a cortisol-3CMO-histamine [^{125}I] tracer (Moore *et al.*, 1985).

Statistical analysis

F/E ratios were expressed as mean ± SEM and compared using Student's paired and unpaired *t*-test. Placental 11β-HSD2 activity did not exhibit Gaussian distribution and thus results were expressed as median [95% CI] and non-parametric statistical analysis carried out using the Mann–Whitney *U*-test. All correlations were made using Spearman's rank correlation test.

Results

Demographic data of all the pregnancies studied are shown in Table I. Using an anti-human 11β-HSD2 antibody, an immunoreactive species of 41 kDa, in keeping with the predicted size of human 11β-HSD2, was expressed in human placental homogenates throughout gestation (data not shown). Using this same Ab, in first trimester placentae, 11β-HSD2 immunoreactivity was variably localized to either the inner cytotrophoblast layer (Figure 1A) or the outer syncytiotrophoblast layer (Figure 1B) within any given villus. At this gestational age, staining in both layers within a villus was not observed. In term placentae, intense 11β-HSD2 immunoreactivity was localized to the fused syncytiocytotrophoblast layer (Figure 1C). 11β-HSD2 was not expressed in any other cell type of the human placenta.

Placental homogenates from first, second and third trimester pregnancies showed the presence of high levels of 11β-dehydrogenase activity (i.e conversion of F to E). There was marked individual variability (Figure 2), but 11β-HSD2 activity was significantly higher in term placenta [888 (725–1362) pmol E/mg/h (median [95% confidence interval]) $n = 50$) than first trimester (476 [376–904] pmol E/mg/h, $n = 16$, $P < 0.01$) and second trimester (286 [221–617] pmol E/mg/h, $n = 9$, $P < 0.001$) pregnancies (Figure 3). In the 50 term deliveries there was no correlation between placental 11β-HSD2 activity and either birth ($r = 0.21$) or placental ($r = 0.35$) weight. As previously demonstrated (Lopez-Bernal *et al.*, 1982), the mode of delivery (vaginal or Caesarean section) did not affect 11β-HSD2 activity (data not shown).

Compared with term pregnancies, 11β-HSD2 activity was reduced in placentas obtained from IUGR deliveries (360 [225–671] pmol E/mg/h, $P < 0.05$). Although the gestational age of these IUGR deliveries was 25–37 weeks, significant reduction in 11β-HSD2 activity was also seen when a comparison was made with gestationally-matched pre-term, but appropriately grown pregnancies (810 [585–1269] pmol E/mg/h, $n = 14$, $P < 0.05$; Figure 3). Furthermore as demonstrated from regression plots, placental activity tended to fall in IUGR pregnancies as term approached in contrast to normal pregnancy (Figure 2).

The serum cortisol (F)/cortisone ratio (E) was 7.76 ± 0.57 (mean ± SE) in adult peripheral venous blood ($n = 70$). In 17 of the 50 term deliveries, this contrasted with a ratio of 1.72 ± 0.23 ($P < 0.001$) in umbilical arterial and 1.16 ± 0.14 ($P < 0.001$) in umbilical venous blood. The ratio was significantly lower in umbilical venous compared with umbilical arterial blood ($P < 0.05$, Table II). Furthermore in

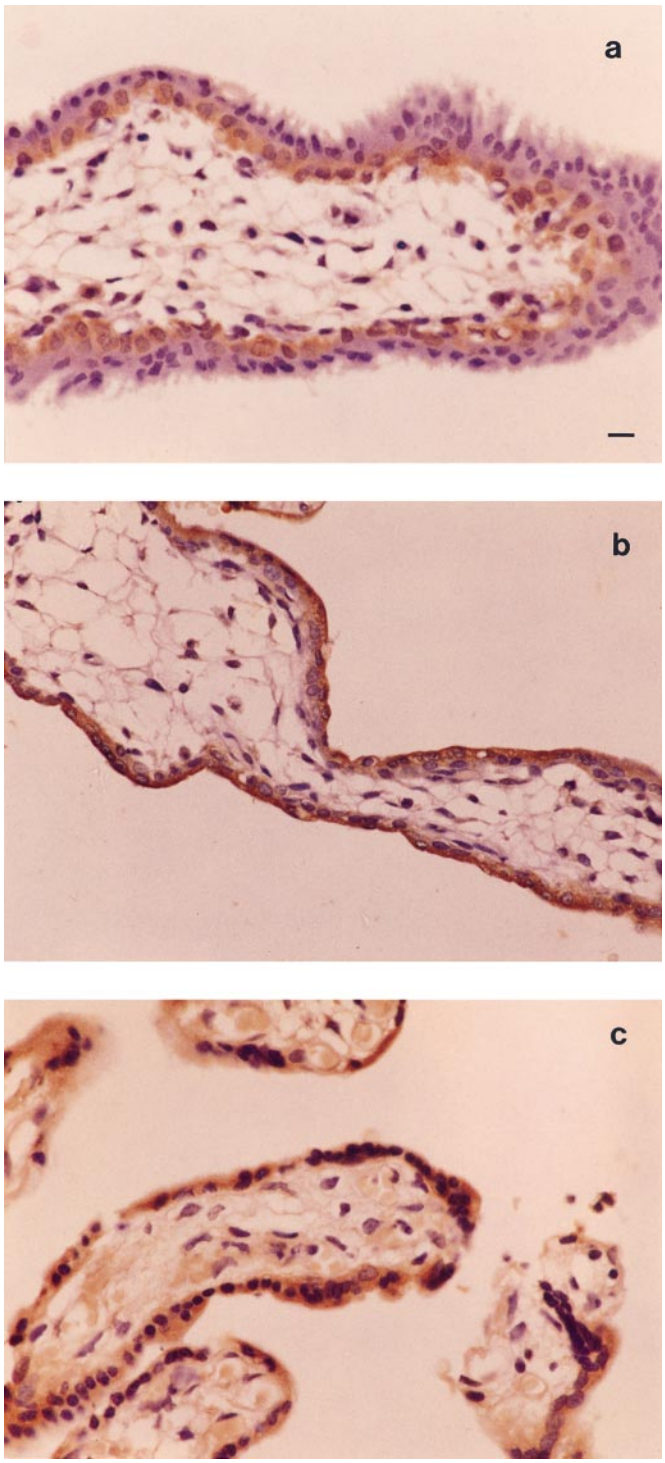

Figure 1. Expression of 11β-hydroxysteroid dehydrogenase (11β-HSD2) in human placenta obtained from a first trimester pregnancy (**A** and **B**) and term pregnancy (**C**). Immunocytochemistry results are shown using an antibody against human 11β-HSD2. In early gestation (6 weeks), immunoreactivity (brown stain) of either the inner cytotrophoblast layer (**A**), or the outer syncytiotrophoblast layer (**B**) is seen within a given placenta. At term (**C**) intense immunoreactivity is observed in the fused syncytiocytotrophoblast layer. Other surrounding structures are negative (scale bar = 100 μm; original magnification ×400).

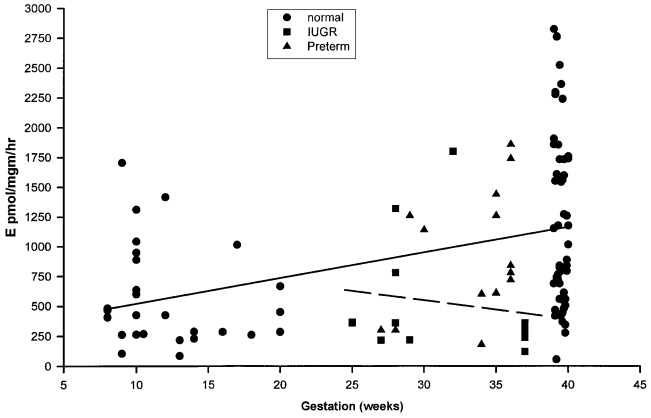

Figure 2. Scatter plot diagram showing 11β-hydroxysteroid dehydrogenase (11β-HSD2) activity [expressed as pmoles cortisone (E) formed/mg protein/h] in normal human placenta across gestation ($n = 75$), in placenta from pre-term pregnancies from appropriately grown fetuses ($n = 14$) and in pregnancies complicated by intrauterine growth restriction (IUGR) ($n = 12$). Each point represents the mean of at least three separate analyses on any given placenta. The continuous line represents the line of regression for normal placenta, whereas the dashed line represents the line of regression for the IUGR placenta.

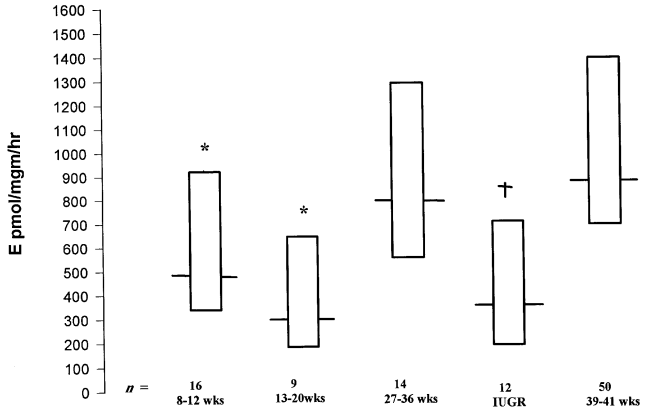

Figure 3. 11β-hydroxysteroid dehydrogenase (11β-HSD2) activity in human placentae across gestation and in intrauterine growth restriction (IUGR) placentae. Gestational age is given together with the number of placentae studied. Pre-term deliveries (27–36 weeks) were all appropriately grown for gestational age. The values are expressed as picomoles of cortisone (E) formed/mg protein/h (median with 95% confidence interval). $^{*}P < 0.01$ versus term placenta (39–41 weeks). $^{\dagger}P < 0.05$ versus term and gestationally-matched pre-term placentas.

these 17 individuals there was a positive correlation between placental 11β-HSD2 activity and the umbilical venous F/E ratio ($r = 0.52$, $P = 0.05$) but not umbilical arterial F/E ratio ($r = 0.13$).

Discussion

With the exception of earlier studies prior to the characterization of the two isozymes of 11β-HSD (Murphy *et al.*, 1974; Lopez-Bernal *et al.*, 1980; Blasco *et al.*, 1986) experimental data relate to placental 11β-HSD activity at term. This is the first study to localize placental 11β-HSD2 protein and its activity throughout gestation and to demonstrate altered expression in

Table II. Serum cortisol (F), cortisone (E) and F/E ratio in 70 adult peripheral venous samples and 17 term umbilical arterial and venous samples. Results are expressed as mean ± SE

	Peripheral vein ($n = 70$)	Umbilical artery ($n = 17$)	Umbilical vein ($n = 17$)
F (nmol/l)	409 ± 13.3	419 ± 60.2	307 ± 37.4*
E (nmol/l)	48.3 ± 2.7	255 ± 24.2**	287 ± 33**
F/E ratio	7.76 ± 0.57	1.73 ± 0.24 **	1.16 ± 0.14**

$^{*}P < 0.05$, $^{**}P < 0.001$ compared with peripheral vein.
Umbilical artery F/E compared with umbilical vein F/E, $P < 0.05$.

pathology. Activity was present from the first trimester and increased significantly as term approached. These data are in keeping with published results across baboon pregnancy (Pepe *et al.*, 1996), but are in direct contrast to rodent studies (Brown *et al.*, 1996; Condon *et al.*, 1997) where activity and expression of 11β-HSD2 is switched off in late gestation. At term we have demonstrated that the expression of 11β-HSD2 is confined to the fused syncytiocytotrophoblast layer in keeping with earlier observations (Krozowski *et al.*, 1995).

In the first trimester within any given placenta, expression was seen in the inner cytotrophoblast layer in some villi, or the outer syncytiotrophoblast in others. In no cases, however, could immunoreactivity to both layers be demonstrated within a given villus. The implications of this observation are unclear but suggest that the expression of 11β-HSD2 may be closely linked to the differentiation of trophoblast tissue.

The capacity of the placenta to inactivate glucocorticoids through the expression of 11β-HSD2 is immense (Stewart *et al.*, 1995; Benediktsson *et al.*, 1997; Rogerson *et al.*, 1997); indeed per mg of wet weight of tissue, the placenta is the most abundant source for 11β-HSD2. This was endorsed in our study, both in terms of the high specific enzyme activity observed across gestation, and the serum cortisol/cortisone ratios in differing vascular beds. Thus, compared with adult venous blood where the circulating cortisol/cortisone ratio was 7.7, circulating cortisone levels were much higher in the fetal circulation giving an F/E ratio of < 2. This F/E ratio in umbilical venous blood was directly correlated with placental homogenate 11β-HSD2 activity. Despite this impressive activity, the definitive role of placental 11β-HSD2 across gestation remains unknown. It has been suggested that it may serve as a 'barrier' (Benediktsson *et al.*, 1993, 1997), protecting the developing fetus from the possible deleterious effects of maternally derived glucocorticoids. The localization of 11β-HSD2 to the 'maternally-bathed' syncytiotrophoblast layer in gestation is in keeping with such a role, though one could argue that the expression of the enzyme in the inner cytotrophoblast layer in some placental villi in early gestation, at a time when fetal development is critical, is incongruous for such a protective role. However, it should be noted that the expression of 11β-HSD2 is widespread in many fetal tissues, other than trophoblast, in early gestation (Stewart *et al.*, 1994b); if required they could 'protect' themselves from cortisol in an autocrine fashion. This was also demonstrated in our circulating F/E ratios. Whilst there was a significant difference in the F/E ratio between umbilical vein and umbilical artery, no doubt

directly reflecting placental 11β-HSD2 activity, this ratio was still highly significantly reduced in the umbilical artery compared with adult levels. One must assume, therefore, that numerous fetal tissues, in addition to trophoblast, contribute to the higher circulating cortisone concentration in human fetal life (Bro-Rasmussen *et al.*, 1962).

It has also been suggested that the high metabolic clearance rate of cortisol may facilitate the ontogeny of the fetal hypothalamo–pituitary–adrenal axis. This conclusion is largely based on the elegant studies of Pepe and Albrecht (1995) in primate models. In human pregnancy, we have also proposed that the placental metabolism of fetally-derived cortisol by 11β-HSD2 does serve to maintain high fetal ACTH levels, fetal adrenal hyperplasia and the continued secretion of DHEA, the precursor for maternal oestrogen production across gestation (Stewart *et al.*, 1995). In this study, we observed marked individual variability in placental 11β-HSD2 activity as previously reported (Stewart *et al.*, 1995; Rogerson *et al.*, 1997), but the ontogeny of placental 11β-HSD2, with an increase in activity as term approached, is in keeping with such a role, facilitating the development of an autonomous fetal hypothalamo–pituitary–adrenal axis.

The mechanisms behind this increase in activity as term approaches are unclear. It is possible that the rise in activity may be secondary due to the rise in oestrogen levels that occur in late pregnancy or, in contrast, because of a reduction in progesterone activity in late gestation. In-vivo and in-vitro studies have shown that oestrogen stimulates 11β-HSD2 activity in the primate placenta and fetus (Baggia *et al.*, 1990), whilst progesterone inhibits enzyme activity (Bujalska *et al.*, 1997).

One of the aims of the study was to analyse 11β-HSD2 in pregnancies complicated by IUGR. Growth-restricted fetuses may develop profound problems in the antenatal and neonatal period (West Midlands Perinatal Audit, 1996). Furthermore, there is considerable interest in the control of fetal growth and the programming of adult disease processes such as hypertension and glucose intolerance (Law and Barker, 1994). Glucocorticoids may be an aetiological factor in explaining this link given that, *in utero*, glucocorticoids have been shown to inhibit fetal growth in human (Reinisch *et al*., 1978), primate (Novy and Walsh, 1983) and rodent (Benediktsson *et al.*, 1993) pregnancies. Furthermore, patients with apparent mineralocorticoid excess who are known to have mutations in the human 11β-HSD2 gene and low placental 11β-HSD2 activity (Stewart *et al.*, 1996), have been documented to have low birth weights (Kitanaka *et al.*, 1996). Correlation studies in rodents (Benediktsson *et al.*, 1993) and in some human studies (Stewart *et al.*, 1995) demonstrated positive correlations between birth weight and placental 11β-HSD2 activity, suggesting that reduced placental 11β-HSD2 activity may impair fetal growth, presumably through failure to inactivate maternally-derived glucocorticoid. In this study we were unable to demonstrate such a relationship in keeping with data from Rogerson *et al*. (1997). We did, however, demonstrate a significant reduction in 11β-HSD2 activity in placentas from growth-restricted fetuses compared with both term and gestationally-matched, but appropriately grown deliveries. Unfortunately we were not able to obtain umbilical arterial or venous blood for cortisol/cortisone estimation, but this may not have been conclusive. Any reduction in placental 11β-HSD2 activity as seen in IUGR may result in an increase in maternally-derived cortisol in the fetal circulation, but this may not change circulating steroid concentrations. Analogous to the normal circulating levels seen in patients with apparent mineralocorticiod excess (AME) who have a totally inactive 11β-HSD2 enzyme (Stewart *et al.*, 1988), one could postulate that any defect in placental 11β-HSD activity *per se* may suppress the fetal hypothalamo–pituitary–adrenal axis with no net change in cortisol in the fetal circulation. If the defective placental 11β-HSD2 activity in IUGR reflects a more generalized defect in other fetal tissues, then a role in modulating growth can occur at an autocrine level through altered tissue concentrations of active glucocorticoid, but circulating levels may be misleading. In fact where this has been studied, circulating ACTH and cortisol levels were found to be elevated in growth-restricted fetuses and this was thought to be due to enhanced placental corticotrophin releasing hormone (CRH) secretion (Goland *et al*., 1993). In contrast to adult hypothalamic CRH secretion, glucocorticoids stimulate placental CRH secretion (Riley *et al.*, 1991) and impaired 11β-HSD2 activity within the placenta would increase this.

In summary we have demonstrated that 11β-HSD2 expression increases across normal human gestation. Activity is reduced in pregnancies complicated by IUGR, suggesting that 11β-HSD may play an important role in regulating fetal growth.

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