Steroidogenic enzyme expression in the human fetal liver and potential role in the endocrinology of pregnancy

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ABSTRACT: The human feto-maternal unit produces large amounts of steroid hormones, particularly estrogens, during the second and third trimesters. The fetal adrenal gland and the placenta are considered the principal tissues driving steroid production but the fetal liver is likely to play an essential role in this process. This study was designed to measure transcript expression of proteins involved in steroid synthesis, metabolism, conjugation and signalling in the human fetal liver and to examine sex differences and effects of maternal smoking. Liver samples were taken from 55 normal fetuses from women undergoing second trimester elective termination. Levels of 23 mRNA transcripts encoding steroid synthesis/metabolic/conjugation enzymes and steroid receptors were measured by real-time PCR. The expression of representative proteins was confirmed by western blotting and immunohistochemistry. The human fetal livers expressed high levels of CYP19A1, SULT2A1, SULT1E1, HSD17B2, SRD5A3 and CYP3A7. Lower levels of SULT1A1, STS, UGT2B17, GPER, AKR1C3, UGT2B15, AR, CYP11A1, CYP21A2, HSD17B3, HSD17B1 and SRD5A1 were also detectable. The expression of ESR, ESR2, CYP17A1 and HSD3B transcripts was undetectable in most fetal livers, although HSD3B was shown to be present by western blotting. Sex differences were limited to SRD5A3 (lower in females) and UGT2B17 (higher in females). Maternal smoking increased the expression of CYP19A1, SULT2A1, UGT2B17, HSD17B2 and AKR1C3 and reduced the expression of SRD5A3 in the male fetal liver. This study shows that the human fetal liver is likely to have an extensive effect on circulating steroid levels in the human fetus and mother. The most important of these effects will be alterations to the species, conjugation and availability of estrogens in the fetus. Maternal smoking is likely to reduce circulating androgen bioactivity in male fetuses.

Key words: steroidogenesis / conjugation / estrogen / androgen / placenta

Introduction

In the second and third trimesters of human pregnancy steroid hormone production and metabolism is dominated by the activity of the placenta and the fetal adrenal gland (Diczfalusy, 1969). The fetal adrenal secretes high levels of pregnenolone and dehydroepiandrosterone (DHEA) (and their sulphated derivatives) (Kaludjerovic and Ward, 2012) which are converted to progesterone and estrogens by the placenta. The functions of the high CYP19A1 (aromatase) (gene names and genbank accession numbers are shown in Table I) activity in the placenta are unclear but one role is protection of female fetuses (and mothers) from exposure to androgens derived from the fetal adrenal (Belgorosky et al., 2009). Previous studies have indicated that most other human fetal tissues, apart from the gonads (O’Shaughnessy et al., 2007; Fowler et al., 2011), express low levels of the major steroid biosynthetic enzymes such as HSD3B and CYP17A1 (Pezzi et al., 2003). Activity in these tissues may act to alter local levels of specific steroids but are unlikely to affect overall circulating levels in the fetus. One likely exception, however, is the fetal liver, which has been shown to contain high levels of CYP19A1 transcript and to express appreciable aromatase activity (Pezzi et al., 2003; Yamamoto et al., 1984; Doody and Carr, 1989). In addition, the human fetal liver expresses very high levels of CYP3A7 (Lacroix et al., 1997; Stevens et al., 2003; O’Shaughnessy et al., 2011), which acts as a steroid 16a-hydroxylase and is a component enzyme in the production of estriol, the major estrogenic species in the maternal and fetal circulation during pregnancy (Smith et al., 1979; Pasqualini, 2005). It appears probable, therefore, that the human fetal liver normally contributes to steroid hormone synthesis in pregnancy and, given that it also expresses steroid-conjugating enzymes, is likely to have an extensive effect on circulating levels of specific steroids in the fetus.
<table>
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<th>Gene</th>
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<th>Forward primer/reverse primer</th>
<th>Protein function</th>
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<td>Cytochrome P450 11B1</td>
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<td>Steroid 11β hydroxylase, generates cortisol from deoxycorticisol</td>
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<td>17α-hydroxylase/C17-20 lyase enzyme, converts C17 steroids to C19 androgens</td>
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*aThis is not an exhaustive list of possible activities of the translated protein but states the likely activity with respect to fetal liver function and steroid synthesis, metabolism or function.*
enzymes (Leakey et al., 1987; Stanley et al., 2005; Duanmu et al., 2006), it is also likely to regulate the bioactivity of steroid hormones produced by the placenta or the testes. For these reasons it is, perhaps, surprising that a detailed analysis of steroidogenic enzymes present in the human fetal liver has not previously been reported. This means that we lack an overall picture of the activity and likely importance of the fetal liver in the steroid endocrinology of pregnancy. To address this issue, we have used a large cohort of human fetal livers to carry out a comprehensive analysis of the transcript and protein levels of enzymes and receptors primarily involved in C19 and C18 steroid synthesis, metabolism, conjugation and activity. The fetal liver cohort studied here has previously been used to characterize the expression of phase 1 and 2 metabolic enzymes during liver development (O’Shaughnessy et al., 2011).

During gestation, maternally derived xenotoxins from the environment or from maternal lifestyle (e.g. cigarette smoking) pose a significant threat to normal fetal development (Rogers, 2008). Around 70% of the blood supply to the human fetal liver is from the umbilical vein and, so, directly from the fetomaternal interface. This means that the fetal liver is exposed to the highest concentrations of maternally derived toxicants and, along with the placenta, is the primary fetal defence against such exposure (O’Shaughnessy et al., 2011). We have shown previously that maternal smoking alters the transcript expression of several hepatic proteins involved in liver function and toxicant inactivation [e.g. cytochrome P450 1A1, epoxide hydrolase 1, NAD(P)H dehydrogenase quinone 1, glutaredoxin 2; O’Shaughnessy et al., 2011]. This study has also, therefore, examined the effects of maternal smoking on steroidogenic enzyme activity in the fetal liver to determine whether smoking may alter the steroid endocrinology of pregnancy.

Materials and Methods

Ethical approval

The collection of fetal liver material was approved by the National Health Service Grampian Research Ethics Committees (ECO04/00802/21) (O’Shaughnessy et al., 2007; Fowler et al., 2011).

Tissue samples

In total, livers from 55 fetuses were collected as described previously (O’Shaughnessy et al., 2011). Liver samples were snap frozen in liquid nitrogen and stored at −80 °C or were fixed overnight in normal-buffered formalin.

Cotinine assay

Cotinine, a metabolite of nicotine and a marker of smoking, was determined with a commercial kit using previously frozen liver samples (Cochart Plc, Abingdon, Kent, UK) (O’Shaughnessy et al., 2011).

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from frozen fetal liver samples (10–20 mg) using TRIzol (Life Technologies, Paisley, UK). Reverse transcription, primer design and real-time PCR were carried out as previously described (Baker and O’Shaughnessy, 2001; O’Shaughnessy et al., 2002; O’Shaughnessy et al., 2007). The primers used are shown in Table I. Note that the primers designed to amplify SULT1A1 will also amplify SULT1A2 and so have been designated SULT1A1/2. For most transcripts data were collected from 48 samples without prior DNase treatment of the RNA and primers were designed which did not amplify genomic DNA. It was necessary, however, to DNase-treat RNA samples prior to reverse transcription in order to measure GPER, SRD5A1 and SRD5A3 because of the presence of processed pseudogenes lacking introns in the human genome. DNase treatment was carried out using DNA-free (Life Technologies). Data were normalized against a combination of housekeeping genes as described previously (O’Shaughnessy et al., 2011).

Western blot

Proteins were extracted from fetal liver slices (~30 mg) and used for western blotting as described previously (O’Shaughnessy et al., 2011). The antibodies used were (i) HSD3B2 (1:2000: rabbit polyclonal; Papacleovoulou et al., 2009) which will detect both HSD3B1 and HSD3B2, (ii) CYP11A1 (1 µg/ml: rabbit polyclonal, LS-B3997, LifeSpan Biosciences, Inc, Seattle, WA, USA), (iii) SRD17B2 (1:1,100, rabbit polyclonal, gift from Ian Mason), (iv) CYP21A2 (1:250: rabbit polyclonal, AP7880c, Abgent Europe, Oxfordshire, UK), (v) SULT1A1 (1:1000: mouse monoclonal, H00006817, Abnova GmbH, Heidelberg, Germany), (vi) SULT2A1 (1:500: rabbit polyclonal, Ab38417, Abcam Ltd., Cambridge, UK), (vii) STS (1:500: rabbit polyclonal, HPA002904, Sigma-Aldrich Company Ltd, Poole, UK) and (viii) AKR1C3 (1:1000: rabbit polyclonal, Ab84327, Abcam).

Immunohistochemistry

Immunohistochemistry was used to examine protein localization in the fetal liver. Liver sections (5 µm) were mounted on to superfrost slides and stained as described previously (Fowler et al., 2011). The antibodies used were (i) CYP19A1 (1:3000: mouse monoclonal, Novartis clone no. 667 (Nicol et al., 2009), (ii) CYP3A7 (3 µg/ml: mouse monoclonal, Ab55840, Abcam Ltd.), (iii) AR (1:200: rabbit polyclonal, Ab74272, Abcam Ltd.), (iv) CYP21A2 (1:50: AP7880c, rabbit polyclonal, Abgent Europe), (v) HSD17B3 (1:30: rabbit polyclonal, HPA015307, Sigma-Aldrich Company Ltd.), (vi) GPER (1:70: rabbit polyclonal, HPA027052, Sigma-Aldrich Company Ltd) and (vii) SULT2A1 (0.01 µg/ml: rabbit polyclonal, Ab38416, Abcam Ltd.). IgG-negative sections, exposed to non-immune rabbit and mouse serum in the absence of primary antibody, were included in all immunohistochemistry runs and showed no positive immunostaining.

Statistical analysis

Pearson’s correlation or Spearman’s rank correlation coefficient was used to determine whether there was a correlation between transcript expression and gestational age. Normally distributed data (with or without log transformation as necessary) were analyzed by three-factor ANOVA with multiple regression (fetal sex, weeks of gestation and maternal smoking) and if group or interaction differences were significant (P < 0.05), individual groups were compared by t-tests using the pooled error variance. Transcripts that deviated significantly from a normal distribution were analysed using the Kruskal–Wallis test. Data were analysed using JMP 9.03 software (Thomas Learning, London, UK).

Results

Cytochrome P450 enzymes

Transcripts encoding CYP11A1, CYP21A2 and CYP19A1 were detected in most fetal liver samples with the expression of CYP19A1 particularly marked (Fig. 1). Only levels of CYP21A2 in non-smoking females showed a significant change with fetal age (Supplementary data, Fig. S1). Transcripts encoding CYP17A1 and CYP11B1 were
undetectable in most liver samples. Maternal smoking increased CYP19A1 levels in male fetuses (to reach levels seen in the female fetuses), although transcript levels of CYP11A1 and CYP21A2 were unaffected by smoking (Fig. 1). There were no sex differences in the expression of any of the measured cytochrome P450 enzyme transcripts.

**Oxidation/reduction enzymes**

Transcripts encoding HSD17B2, SRD5A3 and AKR1C3 were detectable at a high level in all samples (Fig. 2). The expression of SRD5A1 and HSD17B3 transcripts was detectable in most samples though at a lower level. The expression of HSD17B1 was detectable in 13 samples (24%) at a low level, while HSD3B was only detectable in four samples (7%). The expression of SRD5A2 was not detectable in any samples (not shown). There was a significant age-dependent increase in smoking-exposed female HSD17B3 levels during the second trimester (Supplementary data, Fig. S1) but no age-dependent changes in other transcripts of this group. Maternal smoking increased HSD17B2 and AKR1C3 liver transcript levels in males but had no significant effect in female fetuses (Fig. 2). Smoking also acted to reduce levels of SRD5A3 in male fetal livers without significantly affecting female livers. Levels of SRD5A3 were higher in males than in females but there were no other sex differences in the expression of transcripts encoding oxidation/reduction enzymes.
**Figure 2** Expression of selected transcripts encoding oxidation and reduction enzymes in the human fetal liver and effects of gender and maternal smoking. Real-time PCR was used to measure specific transcript levels as described in the text. Data from individual fetuses (11–16 fetuses per group) are shown and the horizontal bar represents the mean of the group. Significant differences between groups are indicated by letters in the boxes above each graph. Within each transcript, groups that do not share a letter are significantly ($P < 0.05$) different. 'Control' represents fetuses from non-smoking mothers; 'Sm' represents fetuses from mothers who smoked during pregnancy.
Conjugation/deconjugation enzymes

Transcripts encoding the steroid sulphation enzymes SULT1E1, SULT2A1 and, to a lesser extent, SULT1A1/2 were expressed at high levels in most liver samples (Fig. 3). Transcript expression of the sulphatase enzyme STS was also detectable in almost all samples though at a lower level. Transcripts encoding the androgen and estrogen glucuronidation enzymes UGT2B15 and UGT2B17 were present in all female fetal livers and most male livers. Levels of SULT2A1 transcript declined with gestation age in both control (non-smoke-exposed) and smoke-exposed female fetuses, SULT1A1/2 declined in smoking-exposed male fetuses and expression of UGT2B15 increased in non-exposed male fetuses (Supplementary data, Fig. S1). There were no other age-dependent changes in this group. Maternal smoking increased SULT2A1 and UGT2B17 levels in males but had no effects in females (Fig. 3). Overall expression of UGT2B17 transcript was higher in control (non-smoke-exposed) females than in control males with no other sex differences apparent.

Steroid receptors

Expression of AR transcript was detectable in most samples, although there was no effect of maternal smoking and no sex differences in expression (Fig. 4). Expression of AR increased with gestation age in

Figure 3  Expression of selected transcripts encoding conjugating enzymes in the human fetal liver and effects of gender and maternal smoking. Real-time PCR was used to measure specific transcript levels as described in the text. Data from individual fetuses (11–16 fetuses per group) are shown, and the horizontal bar represents the mean of the group. Significant differences between groups are indicated by letters in the boxes above each graph. Within each transcript, groups that do not share a letter are significantly (P < 0.05) different. ‘Control’ represents fetuses from non-smoking mothers; ‘Sm’ represents fetuses from mothers who smoked during pregnancy.
smoking-exposed female fetuses (Supplementary data, Fig. S1). Transcripts encoding the estrogen receptors ESR1 and ESR2 were undetectable in most fetal liver samples (Fig. 4), although GPER was detectable in all samples. Smoking reduced GPER in females with no effect in males.

Protein expression

Protein expression of representative transcripts (HSD3B, HSD17B2, CYP11A1, CYP21A2, SULT1A1, SULT2A1, STS and AKR1C3) was examined qualitatively by western blotting (Supplementary data, Fig. S2). Proteins were selected for study because of their likely importance to overall steroid synthesis/metabolism and availability of suitable antibodies. Clear bands of the correct size for AKR1C3, HSD17B2, SULT1A1 and SULT2A1 were seen in both male and female samples, while fainter bands for HSD3B, CYP11A1, CYP21A2 and STS were present. Using immunohistochemistry, CYP19A1, HSD17B3, CYP3A7, UGT2B15 and SULT2A1 were clearly localized in the hepatocytes (Fig. 5). GPER was also detectable in most hepatocytes with staining predominant around the cell membrane and some other cells showing a high level of protein expression. Expression of AR protein was divided between nuclear and/or cytoplasmic localization in some hepatocytes and nuclear/cytoplasmic localization in many developing haematopoietic cells (Fig. 5C and D).

Discussion

The steroid endocrinology of fetal development and pregnancy in the human is unique with sustained very high levels of circulating progesterone and estrogens present from the end of the first trimester (Fowler et al., 1998). As in other species, progesterone in pregnancy acts to reduce myometrial contractility, although the role that estrogens might play in human fetal development is less clear. In a non-human primate model estrogens are required in pregnancy for normal gonadal and placental development and for maturation of the hypothalamic–pituitary–adrenal axis (Pepe et al., 1990; Zachos et al., 2002; Albrecht et al., 2009). Critically, there is also evidence of abnormal gonadal development in aromatase-deficient humans (Belgorosky et al., 2009). The primary source of fetal estrogens is the placenta which uses DHEA and DHEAS derived from the fetal adrenal as the initial substrate (Pasqualini, 2005). From the extensive data reported here, and from earlier results (Lacroix et al., 1997; Stanley et al., 2005; Duanmu et al., 2006), it is now clear that the fetal liver is likely to have a significant effect on both the relative...
amounts of each estrogenic species in the circulation and the overall level of estrogen conjugation. In addition, in the male fetus, where circulating levels of androgen are high (~10 nmol/l of total testosterone; O’Shaughnessy et al., 2007), the liver probably contributes significantly to overall estrogen levels and to androgen bioactivity through reduction/conjugation reactions and production of plasma-binding proteins.

Figure 6 shows the steroidogenic pathways that are likely to be active or inactive in the human fetal liver based on enzyme, transcript and protein data reported here and by others (Boite et al., 1966; Huhtaniemi 1974; Leakey et al., 1987; Lacroix et al., 1997; Takeyama et al., 2000; Pezzi et al., 2003; Stanley et al., 2005; Duanmu et al., 2006; Holinka et al., 2008). The figure shows that the major substrate inputs to the liver will be DHEA from the fetal adrenal and androgens from the fetal testis. The relatively low levels of CYP11A1 mean that the synthesis of pregnenolone will be limited. We were also unable to measure consistent expression of HSD3B and CYP17A1 transcripts in the human fetal liver, although western blotting did show that small amounts of HSD3B protein (which the same antibody appropriately localized to fetal Leydig cells as a positive control) were present, consistent with an earlier study (Pezzi et al., 2003). The human fetal liver

Figure 5 Immunolocalization of CYP19A1, HSD17B3, AR, CYP3A7, UGT2B15, GPER and SULT2A1 in the human fetal liver. Top left hand corner insets to photomicrographs (A), (B), (D–H) show the absence of immunostaining in the presence of non-immune serum instead of primary antibody. The lower inset in (A) shows the detection of CYP19A1 in pre-granulosa cells of the human fetal ovary. The inset in (C) shows the expected detection of AR primarily in the peritubular myoid cells of a fetal human testis (Shapiro et al., 2005). In all images the stain is DAB (brown) and the counterstain is haematoxylin (blue). Labelled black bars show the scale in each image. In all images except non-immune serum insets, yellow arrows show immune-positive hepatocytes, while white arrows highlight hematopoietic cells, whether stained or not. The grey arrows show immune-negative hepatocytes and in (D), the blue arrow shows a hepatocyte that expresses AR mostly within its cytoplasm.
probably, therefore, has a very limited capacity for conversion of pregnenolone or adrenal DHEA to active androgen but this is unlikely to be physiologically relevant, especially not in the male. The most significant contribution of the fetal liver to overall fetal steroid endocrinology in both sexes is likely to be a combination of 16α-hydroxylation of DHEA or estrogen through CYP3A7 (Lacroix et al., 1997; O'Shaughnessy et al., 2011) and estrogen sulphation through the SULT enzymes (Duanmu et al., 2006). This will influence the estrogenic species present in the fetal circulation as well as the overall estrogen bioactivity. In addition, the fetal human liver secretes very high levels of alpha fetoprotein, which acts to bind estrogens in the circulation and limit levels of bioactive-free estrogen (Bader et al., 2004). This means that, along with the placenta, the liver will be the main determinant of estrogenic exposure in the fetus. The relatively high levels of CYP19A1 and aromatase reported here, and by others (Yamamoto et al., 1984; Doody and Carr, 1989), also means that the liver can contribute to the overall levels of estrogens in the fetal and maternal circulation. However, the lack of HSD3B means that de novo androgen synthesis by the fetal liver, and conversion of adrenal DHEA to the Δ4 pathway, will be very limited. In male fetuses, androgens produced by the testes will be subject to aromatization and to conjugation and so the overall effect of the fetal liver will be to reduce bioactive androgens in the fetal and maternal circulation. The principal pathways likely to be active in the fetal liver are conversions between estrogenic species resulting primarily in formation of E2 and E3 and conjugations of those estrogens, primarily by sulphation but also by glucuronidation. The fetal liver will also generate 16α-hydroxyDHEA and its sulphate as substrate for aromatization by the liver. Lack of HSD3B and CYP17A1 means that de novo androgen synthesis by the fetal liver, and conversion of adrenal DHEA to the Δ4 pathway, will be very limited. In male fetuses, androgens produced by the testes will be subject to aromatization and to conjugation and so the overall effect of the fetal liver will be to reduce bioactive androgens in the fetal and maternal circulation. Steroid abbreviations: A4, androstenedione; DHEA, 16α-hydroxyDHEA, 16α-hydroxydehydroepiandrosterone; 16OHDHEA, 16α-hydroxydehydroepiandrosterone sulphate; DHT, dihydrotestosterone; DHTG, dihydrotestosterone glucuronide; DOC, deoxycorticosterone; E1, estrone; E2, 17β-estradiol; E3, estriol; E4, estetrol; 16OHE, 16α-hydroxyestrone; P4, progesterone; P5, pregnenolone; PSS, pregnenolone sulphate; T, testosterone; TG, testosterone glucuronide.
(DHT). SRD5A3 has a relatively low affinity for testosterone (Uemura et al., 2008), the preferred substrate being polyprenols, but the high transcript levels in the fetal liver suggest that it is likely to contribute along with SRD5A1 to overall DHT formation. On the other side of the equation, both UGT2B15 and UGT2B17 catalyze the glucuronidation of the 17β-hydroxy position of DHT and testosterone. This is an irreversible step, which abolishes affinity for the androgen receptor and so the overall effect of the fetal liver is likely to be to limit androgen action through the removal of bioactivity by glucuronidation.

We have shown previously that maternal smoking alters the expression levels of many enzymes involved in xenobiotic metabolism by the fetal liver (O'Shaughnessy et al., 2011). The effects of maternal smoking on enzymes involved in fetal hepatic steroidogenesis and steroid metabolism are less marked, although smoking increased transcript levels of five enzymes in males (CYP19A1, SULT2A1, UGT2B17, HSD17B2 and AKR1C3) and reduced transcript levels of one enzyme (SRD5A3). With the exception of AKR1C3, the overall effect of these changes would be to reduce the levels of circulating androgens and/or androgen bioactivity. Maternal smoking is associated with impaired reproductive health in exposed men (Virtanen et al., 2011). It was speculated that this difference may be due to androgen expression of AR transcripts in the fetal hepatocytes reported here (O'Shaughnessy et al., 2011) and the consistent expression of AR transcripts in the fetal hepatocytes reported here would support this hypothesis.

Results reported here show that the human fetal liver expresses high levels of transcripts and proteins involved in steroid synthesis, metabolism, conjugation and detection. These include CYP3A7, CYP19A1, AKR1C3, SULT2A1, SULT1E1, SRD5A3, HSD17B2 and UGT2B17. The major, and possibly critical, role of the human fetal liver in steroid endocrinology is likely to be the determination of estrogen species in the circulation as well as regulating overall levels of estrogen/androgen conjugation and plasma protein binding.

**Supplementary data**

Supplementary data are available at http://molehr.oxfordjournals.org/.

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**Authors’ roles**

P.O.S. was involved in design of the study, analysis of the data and drafting the manuscript. A.M. carried out real-time PCR studies and analysed data. S.B. was involved in the design of the study and provision of study materials. M.J.F. carried out proteomics analysis studies and data analysis. P.A.F. was involved in conception and design of the study, in data analysis and preparation of the manuscript. All authors contributed to writing and approved the final version of the manuscript.

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**Conflict of interest**

None declared.

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