Effects of cortisol and oestradiol on hepatic 11 β -hydroxysteroid dehydrogenase type 1 and glucocorticoid receptor proteins in late-gestation sheep fetus

S Gupta¹, N Alfaidy¹, A C Holloway¹, W L Whittle¹, S J Lye¹,
W Gibb² and J R G Challis¹

¹CIHR Institute of Human Development, Child and Youth Health, Departments of Physiology, and Obstetrics and Gynecology, University of Toronto, Canada M5S 1A8

²CIHR Institute of Human Development, Child and Youth Health, Departments of Cellular and Molecular Medicine, and Obstetrics and Gynecology, University of Ottawa, Ottawa, Canada K1H 8L6.

(Requests for offprints should be addressed to S Gupta, Department of Physiology, University of Toronto, 1 King's College Circle, Medical Sciences Building Room 3344, Toronto, Ontario M5S 1A8, Canada; Email: shalini.gupta@utoronto.ca)

Abstract

In the late-gestation sheep, increased fetal plasma cortisol concentration and placental oestradiol (E₂) output contribute to fetal organ maturation, in addition to the onset of parturition. Both cortisol and E₂ are believed to regulate the enzyme 11 β -hydroxysteroid dehydrogenase type 1 (11 β -HSD1), which interconverts bioactive 11-hydroxy glucocorticoids and their inactive 11-keto metabolites. 11 β -HSD1, abundantly expressed in fetal liver, operates primarily as a reductase enzyme to produce bioactive cortisol and thus regulates local hepatic glucocorticoid concentrations. Cortisol acts through the glucocorticoid receptor (GR) present in the liver. In this study, we examined the effects of cortisol and E₂ on hepatic 11 β -HSD1 and GR in the liver of chronically catheterized sheep fetuses treated with saline ($n=5$), cortisol (1.35 mg/h; $n=5$), saline + 4-hydroxyandrostendione, a P450 aromatase inhibitor (4-OHA; 1.44 mg/h; $n=5$), or cortisol + 4-OHA ($n=5$). Cortisol infusion resulted in increased plasma concentrations of fetal cortisol and E₂; concurrent administration of 4-OHA attenuated the

increase in plasma E₂ concentrations. Using immuno-histochemistry, we showed that fetal hepatocytes expressed both 11 β -HSD1 and GR proteins. Cortisol treatment increased GR in both cytosol and nuclei of hepatocytes; concurrent administration of 4-OHA was associated with distinct nuclear GR staining. Western blot revealed that cortisol, in the absence of increased E₂ concentrations, significantly increased concentrations of 11 β -HSD1 (34 kDa) and GR (95 kDa) proteins. 11 β -HSD1 enzyme activity was measured in the liver microsomal fraction in the presence of [³H]cortisone (10⁻⁶ M) or [³H]cortisol (10⁻⁶ M) and NADPH (reductase activity) or NADP⁺ (dehydrogenase activity) respectively. 11 β -HSD1 reductase activity was significantly greater in the presence of cortisol. In summary, we found that, in sheep during late gestation, cortisol increased both 11 β -HSD1 and GR in the fetal liver, and these effects were accentuated in the absence of increased E₂.

Journal of Endocrinology (2003) **176**, 175–184

Introduction

It is well established that parturition in the sheep is dependent on activation of the fetal hypothalamic–pituitary–adrenal axis during late gestation and a concurrent increase in fetal plasma cortisol concentrations (Bassett & Thorburn 1969, Liggins 1976, Thorburn & Challis 1979). There is also an increase in production of oestradiol (E₂) from the placenta that follows the increase in circulating glucocorticoid concentrations (Challis & Brooks 1989). During late pregnancy, fetal cortisol plays a major part in maturation of several fetal organ systems, including the lung, liver, kidneys and gut (Fowden *et al.* 1998). In

the fetal liver, glucocorticoids regulate hepatic functions, including lipid and carbohydrate metabolism (Silver 1990, Jamieson *et al.* 1999). Cortisol reaches the liver from the systemic circulation, or may be produced locally under the influence of the enzyme 11 β -hydroxysteroid dehydrogenase (11 β -HSD). The regulation of hepatic 11 β -HSD in fetal life, and its contribution to bioactive glucocorticoid production in the fetal liver, are currently unknown.

11 β -HSD is a microsomal enzyme responsible for the interconversion of physiologically active glucocorticoids (cortisol in many species, including sheep and human; corticosterone in rodents) with their inactive 11-keto metabolites (cortisone in many species, including sheep

and human; 11-dehydrocorticosterone in rodents), thus regulating the access of active glucocorticoids to their receptors (Monder & White 1993). Two distinct types of 11 β -HSD have been cloned and characterized (Rusvai & Naray-Fejes-Toth 1993, Yang & Yu 1994, Brown *et al.* 1996). 11 β -HSD type 1 is a reversible NADP/NADPH-dependent isoenzyme that catalyses the interconversion of physiologically active cortisol and its inactive metabolite cortisone (Stewart & Krozowski 1999). In comparison, 11 β -HSD type 2 is a unidirectional NAD⁺-dependent dehydrogenase that converts cortisol to cortisone (Rusvai & Naray-Fejes-Toth 1993). Thus the expression of 11 β -HSD isoenzymes and the direction of activity may be important in the local regulation of corticosteroid concentrations within glucocorticoid target tissues (Whorwood *et al.* 1993, Rajan *et al.* 1996) such as liver (Tannin *et al.* 1991, Jamieson *et al.* 1995, Tomlinson & Stewart 2001), lung (Hundertmark *et al.* 1995, Wood & Srun 1995), placenta (Yang *et al.* 1997), adipose tissue (Bujalska *et al.* 1997, Rask *et al.* 2001), gonad (Ricketts *et al.* 1998b) and the central nervous system (Kim *et al.* 1995). In the fetal sheep liver 11 β -HSD1 was highly expressed, whereas 11 β -HSD2 mRNA and activity were undetectable (Yang *et al.* 1992). 11 β -HSD1 has a greater affinity for cortisone than cortisol, and generally acts in the reductase direction to produce cortisol from cortisone. Although some dehydrogenase activity has been detected in the fetal liver (Jamieson *et al.* 2000), the net production of bioactive cortisol predominates (Yang *et al.* 1995, Ricketts *et al.* 1998a).

Local cortisol exerts its actions via the glucocorticoid receptor (GR) expressed in glucocorticoid target tissues. The liver expresses relatively high numbers of receptors (Raddatz *et al.* 1996). In the ligand-unbound state, GR is found in the cytosol bound to a complex of heat shock proteins and other stabilizing molecules (Bamberger *et al.* 1996, Galigniana *et al.* 2001). Binding of cortisol causes dissociation of GR from the stabilizing molecules and allows translocation of the newly formed ligand–receptor complex to the nucleus. This newly formed ligand–receptor complex recognizes and binds to the glucocorticoid response element (GRE) to regulate gene transcription (Bamberger *et al.* 1996) of gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase (PEPCK) (Nyirenda *et al.* 1998).

Previous studies have revealed that 11 β -HSD1 and GR mRNA are present in the fetal sheep liver as early as day 60 of gestation, and increase throughout the rest of pregnancy and postpartum (Yang 1992, Yang *et al.* 1992, McMillen *et al.* 2000). There is also evidence that increased cortisol and oestradiol can affect local hepatic glucocorticoid actions by regulating 11 β -HSD1 and GR expression (Yang *et al.* 1994, Wang *et al.* 1997). Increased cortisol has a stimulatory effect on 11 β -HSD1, but the data concerning the effects of oestradiol on 11 β -HSD1 are equivocal. Fetal infusion of glucocorticoid and oestradiol

have been found to stimulate 11 β -HSD1 mRNA expression and enzyme activity (Wang *et al.* 1997, Sloboda *et al.* 2001). In contrast, other researchers reported that infusion of dexamethasone (a synthetic glucocorticoid) and oestradiol decreased hepatic 11 β -HSD1 mRNA and reductase activity in the male rat (Jamieson *et al.* 1999, Nwe *et al.* 2000). Thus the separate effects of cortisol and oestradiol on hepatic 11 β -HSD1 and GR in the late-gestation sheep fetus remain unclear. In the present study, we determined the effects of cortisol in the presence or absence of increased oestradiol on hepatic 11 β -HSD1 and GR expression in fetal sheep during late gestation.

Materials and Methods

Animal preparation

All animal use and surgical procedures were approved by the University of Toronto Animal Care Committee, in accordance with the guidelines of the Canadian Council for Animal Care. Singleton ewes ($n=20$) of mixed breed and known gestational ages were used. Gestational age was calculated from the date of insemination (day of mating = day 0; term = 147–149 days of gestation), and the number of fetuses was confirmed by ultrasonography. Surgery was performed with the animal under general anaesthesia as described previously (Manchester *et al.* 1979). Briefly, polyvinyl catheters were inserted into the maternal femoral artery and vein, fetal carotid artery and fetal jugular vein on days 120–123 of gestation as described elsewhere (Whittle *et al.* 2000). Stainless steel electrodes were implanted into the myometrium to monitor uterine muscle activity. After 5 days of postoperative recovery, animals entered the experimental procedure.

Experimental procedure

Beginning on day 125–128 of gestation, fetuses received a continuous infusion of saline (3 ml/h; $n=10$) or cortisol (1.35 mg/h; $n=10$; Steraloids Inc., Wilton, NH, USA) in the same volume of infusate. After 24 h of infusion, five animals in each group received an additional intra-fetal infusion of 1.44 mg/h 4-hydroxyandrostendione (4-OHA; Lentaron, Ciba-Geigy; Basel, Switzerland), a competitive, suicide inhibitor of the P450 aromatase enzyme (Brodie 1991). Fetal and maternal arterial blood samples were collected at 12-h intervals beginning 24 h before the start of the infusion procedure and continuing through the infusion period. Blood samples were collected into syringes previously rinsed with heparinized saline, centrifuged at 4 °C, and the plasma stored at –80 °C until required for analysis.

Monitoring of uterine activity began 24 h before the start of the infusion procedure and continued for the infusion period. From preliminary studies (data not shown)

Table 1 Terminal fetal plasma cortisol and oestradiol concentrations, as published by Holloway *et al.* (2001) and Whittle *et al.* (2000) respectively. Values are means $\pm$ S.E.M.

	Cortisol (ng/ml)	Oestradiol (pg/ml)
Saline	7.2 $\pm$ 2.64	37 $\pm$ 14
Cortisol	70.6 $\pm$ 20.34	259 $\pm$ 33
Saline + 4-OHA	6.1 $\pm$ 1.83	38 $\pm$ 11
Cortisol + 4-OHA	56.7 $\pm$ 11.06	94 $\pm$ 11

* $P < 0.05$ (two-way ANOVA followed by Tukey's pairwise comparison test).

it was determined that an intrafetal infusion of cortisol for a period of 80 h was sufficient to induce a pattern of uterine contraction consistent with labour. After completion of an 80-h infusion period, a terminal plasma sample was taken. Infusion of cortisol resulted in increased fetal plasma cortisol and oestradiol concentrations, whereas concurrent infusion of 4-OHA attenuated the increase in fetal plasma oestradiol (Table 1; Holloway *et al.* 2001, Whittle *et al.* 2000). Animals were killed with an overdose of Euthanyl (sodium pentobarbital; MTC Pharmaceuticals, Cambridge, Canada), and fetal liver tissue was collected. Tissues were either fixed in 4% paraformaldehyde + 0.02% glutaraldehyde for immunohistochemical analysis, or snap-frozen in liquid nitrogen for subsequent Western blot and enzyme assay analyses.

Immunohistochemical analysis

Pieces of fetal liver were embedded in paraffin, sectioned at 5 μ m on a microtome (Histocut, Reichert-Jung, Cambridge Instruments, W. Germany), placed on Superfrost Plus slides (Fisher Scientific, Fair Lawn, NJ, USA) and processed for immunohistochemistry as described elsewhere (Hsu *et al.* 1981). Briefly, slides were incubated in xylene (VWR Conlab, Mississauga, ON, Canada) to remove the paraffin, and then re-hydrated in a graded series of ethanol dilutions and given a final wash in 0.01 M PBS (pH 7.4; 150 mM NaCl, 19 mM Na₂HPO₄; 1.5 mM NaH₂PO₄). Slides were then incubated with rabbit anti-sheep 11 β -HSD1 (1:500 dilution in PBS; antibody from Dr Kaiping Yang, University of Western Ontario), or rabbit anti-human GR (1:100 dilution in PBS; M-20, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) polyclonal primary antibody. The avidin-biotin-peroxidase technique (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA, USA) for immunostaining was utilized with diaminobenzidine (Sigma Chemical Co., St Louis, MO, USA) as the chromagen (Riley *et al.* 1992). Slides were counterstained with Carazzi's haematoxylin, dehydrated in a graded series of ethanols, cleared in xylene substitute, and mounted with permount (Fisher

Scientific). As negative control, sections of fetal liver were incubated without the primary antibody.

Assay of 11 β -HSD1 activity

Reagents and supplies Cortisol (80 Ci/mmol) was purchased from Du Pont Canada Inc. (Mississauga, ON, Canada). [1,2,6,7-³H(N)]Cortisone was prepared from [1,2,6,7-³H(N)]cortisol in our laboratory as described previously (Alfaidy *et al.* 2001). Non-radioactive steroids were obtained from Steraloids Inc. Cofactors NADPH and NADP were purchased from Sigma Chemical Co. Polyester-backed thin-layer chromatography plates were obtained from Fisher Scientific (Unionville, ON, Canada). All solvents used were OmniSolv grade from BDH Inc. (Toronto, ON, Canada).

Hepatocyte microsomal preparation Fetal liver tissues were homogenized in buffer (10% glycerol, 300 mM NaCl, 1 mM EDTA, and 0.02 M Tris-HCl; pH 7.4). For microsomal preparation, tissue homogenate from each liver sample was centrifuged at 4 °C at 790 *g* for 10 min and the supernatant then centrifuged at 4 °C at 25 000 *g* for 40 min. This supernatant was centrifuged at 4 °C at 110 000 *g* for 60 min and the pellet containing the microsomal fraction was resuspended in 200–300 μ l 11 β -HSD1 homogenization buffer and stored until required for protein concentration determination using the Bio-Rad protein assay kit with BSA (Bio-Rad Laboratories, Inc., Richmond, CA, USA) as standard and protein absorbance measured at 595 nm (Bradford 1976).

Assay of 11 β -HSD1 reductase and dehydrogenase activities 11 β -HSD1 reductase activity was assessed by measuring the rate of conversion of [³H]cortisone to [³H]cortisol. In preliminary experiments, incubations were conducted for various times between 0 and 120 min to ensure measurement of levels of enzyme activity within the linear range; the optimal incubation period was determined to be 60 min. To assess 11 β -reductase activity, microsomal fractions (100 μ g microsomal protein) were incubated in 250 μ l buffer containing cortisone (10^{-6} M) and [³H]cortisone as tracer, in the presence of NADPH (10^{-3} M) at 37 °C for 60 min. The dehydrogenase activity was determined by incubating 200 μ g/250 μ l liver microsomal fraction with cortisol (10^{-6} M), and [³H]cortisol as tracer, in the presence of NADP (10^{-3} M) at 37 °C for 60 min. All reactions were stopped by immediate transfer of the tubes to ice and the addition of ethyl acetate (750 μ l in a final volume of 1 ml; Sigma). The steroids were extracted using ethyl acetate, and separated on silicon-coated thin-layer chromatography plates (Fisher Scientific) using chloroform:ethanol (95:5 vol:vol). Radioactivity was counted using a β -counter. 11 β -HSD1 activity was expressed as pmol cortisol or cortisone formed/ μ g microsomal protein per min.

Figure 1 Immunohistochemical localization of ir11 β -HSD1 (A–B) and GR proteins (D–F) in the liver of ovine fetuses infused with (A,D) saline (S), and (b,e) cortisol (F). (C) Negative control. (A–E) Slides are counterstained with Carazzi's haematoxylin; (F) slide not counterstained. Arrowheads indicate haematopoietic cell clusters.

Western blot analysis

Frozen liver samples were homogenized on ice for 1 min in RIPA lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% (wt/vol) sodium deoxycholate, 0.1% SDS, 100 mM sodium orthovanadate (Sigma), 1% (vol/vol) Triton X-100 (Fisher Scientific) and Complete MiniEDTA-free protease inhibitors (Roche Molecular Biochemicals; Dorval, Canada)]. Homogenates were centrifuged at 4 °C at 15 000 *g* for 15 min, and supernatants were collected. Protein concentrations were determined by the Bradford assay (Bradford 1976).

Polyacrylamide gels were prepared (stacking gel 4%; separating gel 12% for 11 β -HSD1, 8% for GR). Proteins

(70 μ g/well) were separated by PAGE, and then transferred electrophoretically to a 0.45- μ m pore nitrocellulose membrane (Bio-Rad Laboratories, Inc.). Transfer was confirmed by protein visualization with Ponceau S (Sigma). Blots were washed with PBS-T [150 mM NaCl, 10 mM Na₂HPO₄, 1.5 mM NaH₂PO₄, and 0.1% Tween-20 (Sigma); pH 7.5] and incubated overnight with blocking solution (5% skim milk powder in PBS-T). Subsequently, blots were incubated with primary antibody for rabbit anti-sheep 11 β -HSD1 (1:1000 dilution in blocking solution), or anti-human GR (1:250 dilution in blocking solution; Santa Cruz Biotechnology, Inc.) for 1 h. For negative controls, the membranes were incubated

without primary antibody. GR antibody was also pre-absorbed with a corresponding peptide sequence against which the antibody had been raised (10 μ g blocking peptide per 1 μ g antibody per ml 5% blocking solution; Affinity Bioreagents Inc., Neshanic Station, NJ, USA) overnight at 4 °C (shaking) before being used for Western blot analysis. All blots were then rinsed six times for 5 min each with PBS-T and incubated with secondary rabbit antiserum conjugated with horseradish peroxidase (1:1000 dilution in blocking solution; Amersham Pharmacia Biotech) for 1 h. Blots were washed six times, 5 min each, and the antibody-antigen complex was detected using the Amersham Pharmacia Biotech enhanced chemiluminescence detection system. Blots were exposed to X-ray film (Eastman Kodak Co.) for visualization. The intensity of the protein signal was quantified using computerized image analysis software (Image Research Inc., laser scanner from Molecular Dynamics, Inc.; ImageQuant software) and expressed as relative optical density (ROD). Protein bands were digitized and the mean pixel density for each band was analysed to obtain ROD units for each protein. In order to compare measurements between different blots, a control liver sample was included in each gel.

Statistical analysis

Data were analysed by two-way analysis of variance (ANOVA). When significance was reached, data were compared further using Tukey's pairwise comparison test and significance was set at $P < 0.05$. Results are presented as the mean ROD $\pm$ S.E.M. of averages for $n = 5$ animals per treatment group.

Results

Localization of 11 β -HSD1 protein

Immunohistochemical analysis revealed localization of the immunoreactive (ir) 11 β -HSD1 protein to the fetal hepatocytes of all treatment groups (Fig. 1A,B). In some tissue sections, hepatic ir11 β -HSD1 protein was observed in a radiating pattern around the central and portal veins in addition to some bile ducts, although this was not a consistent finding (data not shown). Liver sections of fetuses infused with saline or saline+4-OHA contained clusters of haematopoietic cells that were large in number and cluster size (Fig. 1A,D; arrowhead); the number and size of clusters decreased markedly in liver sections of animals infused with cortisol (arrowheads in Fig. 1B,E). The negative control, obtained by incubating the sections without primary antibody, revealed no immunoreactivity in the tissues (Fig. 1C).

Level of expression of 11 β -HSD1 protein

Western blot analysis revealed bands with molecular weights of 32, 34 and 68 kDa in homogenates of fetal liver

from all groups of animals (Fig. 2A). Expression of the 32 kDa molecular weight band did not change across the treatment groups (Fig. 2B). In comparison, the 34 kDa form increased with the infusion of cortisol in the presence or absence of 4-OHA, although this was only significant in the absence of increased oestradiol production (Fig. 2C). The 68 kDa band had a similar pattern of change as the 34 kDa band, although the mean values were not significantly different from each other (Fig. 2D).

11 β -HSD1 reductase and dehydrogenase activities

Enzyme activity assay indicated bidirectional 11 β -HSD1 isoenzyme activity in the fetal liver (Fig. 3). However, 11 β -reductase activities were significantly greater than dehydrogenase activities in all groups (Fig. 3). Infusion of cortisol, with or without concurrent administration of 4-OHA, increased the rate of conversion (expressed as pmol/ μ g microsomal protein per min), and this increase was significant in the absence of increased oestradiol concentrations.

Localization of GR protein

Immunoreactive irGR was localized to the cytosol and nuclei of fetal hepatocytes (Fig. 1D-F). Tissue sections were either counterstained with Carazzi's haematoxylin to visualize the nuclei (Fig. 1D,E) or left without counterstain (Fig. 1F) to visualize nuclear irGR localization. Infusion of cortisol (Fig. 1E) appeared to increase both cytosolic and nuclear expression compared with control (Fig. 1D).

Level of expression of GR protein

Western blot analysis revealed a major band of approximately 95 kDa, as well as additional bands of 45 and 57 kDa molecular weights (Fig. 4A). The intensity of the 95 kDa (GR protein) band increased significantly ($P < 0.05$) after infusion of cortisol in the presence of 4-OHA (Fig. 4B). The double 45 kDa and 57 kDa molecular weight bands were analysed together; the RODs obtained indicated a similar pattern of change as the 95 kDa band. Negative control, performed either by substituting the primary antibody with pre-immune rabbit serum (data not shown) or by preabsorbing the primary antibody with GR blocking peptide (Fig. 4A), eliminated or markedly reduced band intensity.

Discussion

We demonstrated that both 11 β -HSD1 and GR proteins are localized to fetal sheep hepatocytes in late gestation and that expression of both proteins was increased in animals infused with cortisol, especially in the absence of increased

Figure 2 Western blot analysis of 11 β -HSD1 protein expression in fetal liver after infusion of cortisol (F) in the presence or absence of 4-OHA, a P450 aromatase inhibitor. (A) Representative Western blot autoradiographic film; (B–D) mean RODs of hepatic 11 β -HSD1. Levels of expression of (B) 32 kDa, (C) 34 kDa and (D) 68 kDa proteins after treatment with saline (S), cortisol (F), saline+4-OHA (S+), or cortisol+4-OHA (F+). Loading control, blot incubated with S Ponceau. Values are mean RODs $\pm$ S.E.M. * $P < 0.05$, two-way ANOVA followed by Tukey's pairwise comparison test.

oestradiol concentrations. Therefore, we suggest that the prepartum increase in fetal cortisol not only increases responsiveness to glucocorticoids by increasing GR, but also increases the ability of the liver to generate cortisol locally from cortisone, through increased 11 β -HSD1 reductase activity. These effects were diminished in the presence of 4-OHA, suggesting that the prepartum increase in oestrogen may attenuate these effects of cortisol.

11 β -HSD1 was generally present in a radiating pattern around the central veins, portal veins and bile ducts. Ricketts *et al.* (1998b) have previously reported a similar radiating pattern of 11 β -HSD1 localization in the human liver, with maximal expression around the central veins. Brereton *et al.* (2001) recently reported the isoenzyme to be concentrated around the central vein, while being absent from the vicinity of bile ducts of rat liver. In addition, our findings of cellular and nuclear localization for ir11 β -HSD1 protein in hepatocytes are consistent with recent findings of its localization to the endoplasmic reticulum and the nuclear membrane (Brereton *et al.* 2001, Filling *et al.* 2001).

Western blot analysis revealed double bands of 32 and 34 kDa molecular weight, possibly corresponding to different glycosylated forms of 11 β -HSD1 proteins as previously described (Blum *et al.* 2000). Expression of the 34 kDa glycosylated form of the enzyme is believed to reflect a more mature, physiologically active form, whereas the 32 kDa non-glycosylated form is proposed to be immature and to require further processing before being rendered functionally active. However, Blum *et al.* (2000) have reported that human 11 β -HSD1 is enzymatically active regardless of its degree of glycosylation. Expression of the immature 32 kDa form remained relatively constant, with increased cortisol in the presence or absence of increased oestradiol concentrations. However, cortisol stimulated the mature 34 kDa form; this response was further enhanced in the absence of increased oestradiol concentrations. Similar stimulatory effects were observed for 11 β -HSD1 enzyme activity. We also detected an additional protein of 68 kDa molecular weight, which may correspond to a dimeric form of the enzyme. Changes in the 68 kDa molecular weight form followed a similar pattern of change as the 34 kDa band, further supporting

Figure 3 β -HSD1 reductase ($\square$) and dehydrogenase ($\blacksquare$) activities in the fetal liver after intrafetal infusion of saline (S), cortisol (F), saline + 4-OHA (S+), or cortisol + 4-OHA (F+). Values are mean $\pm$ S.E.M. * P < 0.05, two-way ANOVA followed by Tukey's pairwise comparison test.

a role for cortisol and oestradiol in the post-translational regulation of hepatic 11 β -HSD1. The stimulatory effects of cortisol on hepatic expression of 11 β -HSD1 protein and enzyme activity are consistent with previous findings of changes in mRNA and enzyme activity. Increased hepatic 11 β -HSD1 mRNA and enzyme activity during gestation that correlate with increased systemic cortisol concentrations in the late-gestation sheep fetus have been reported (Yang *et al.* 1992, Langlois *et al.* 1995, McMillen *et al.* 2000). Administration of cortisol or betamethasone has been shown to stimulate hepatic expression of 11 β -HSD1 mRNA and protein, and enzyme activity, in late-gestation fetal sheep liver (Yang *et al.* 1994, Sloboda *et al.* 2001). Dexamethasone has also been shown to stimulate 11 β -HSD1 activity in primary culture of rat hepatocytes (Liu *et al.* 1996). In addition, it has been shown that expression of 11 β -HSD1 is increased after occlusion of the ductus venosus and concomitant increase in plasma cortisol concentrations (Tchirikov *et al.* 2001).

Our finding of an apparent attenuation of stimulated 11 β -HSD1 by oestrogen is consistent with previous evidence for the inhibition of hepatic 11 β -HSD1 mRNA and activity in the fetus by oestradiol (Low *et al.* 1993, Seckl & Walker 2001). In the rat, males have significantly greater hepatic expression of 11 β -HSD1 mRNA than females, and the levels in males can be suppressed by the administration of oestradiol (Low *et al.* 1994, Albiston *et al.* 1995). These inhibitory effects of oestradiol on 11 β -HSD1 may be mediated indirectly by growth hormone (GH).

Figure 4 Western blot analysis of expression of GR protein in ovine fetal liver after fetal infusion of cortisol in the presence or absence of 4-OHA, a P450 aromatase inhibitor. (A) Representative Western blot autoradiographic film; (B,C) mean RODs of hepatic GR. Levels of expression of (B) 95 kDa and (C) double 45–57 kDa protein after treatment with saline (S), cortisol (F), saline + 4-OHA (S+), and cortisol + 4-OHA (F+). Values are mean RODs $\pm$ S.E.M. * P < 0.05, two-way ANOVA followed by Tukey's pairwise comparison test.

Low *et al.* (1994) further demonstrated that continuous administration of GH to male rats (mimicking the pattern of secretion of GH in the female) suppressed 11 β -HSD1 activity, whereas pulsatile administration of GH (mimicking the pattern of secretion of GH in the male) had no effect (Stewart *et al.* 2001). Similarly, GH has been shown to inhibit 11 β -HSD1 activity in primary cultures of rat hepatocytes (Liu *et al.* 1996). However, Wang *et al.* (1997) have reported increased hepatic 11 β -HSD1 mRNA after the administration of oestrogen to late-gestation fetal sheep, suggesting that there may be differences in response depending upon the duration and magnitude of the oestrogen administration. It is also possible that, in the present study, the P450 aromatase inhibitor affected 11 β -HSD1 directly, or that its mechanism of action results in accumulation of C19 steroids – oestrogen precursors that influence 11 β -HSD1. The simplest explanation for the apparent discrepancy between the present results and those of Wang *et al.* (1997), and for the paradox of potentially opposing effects of cortisol and oestrogen in the fetus at term, may lie in the different experimental models

used in these studies, or in effects of 4-OHA that are additional to decreasing stimulated oestrogen concentrations. In addition, examination of the regulation of 11 β -HSD1 by oestradiol and GH is plausible in fetal sheep after intrafetal administration of GH (Bauer *et al.* 2000).

Assessment of 11 β -HSD1 enzyme activity revealed markedly greater 11 β -reductase activity (cortisone to cortisol) compared with dehydrogenase activity (cortisol to cortisone). This is compatible with previous findings (Yang *et al.* 1994) and reinforces the concept that the liver is a potential extra-adrenal source of fetal cortisol during term gestation. Cortisol stimulated reductase activity, and thus acts in a positive feed-forward mechanism to enhance local hepatic production of cortisol. Although increased oestradiol appeared to inhibit local conversion of cortisone to cortisol, these effects were minimal and only apparent in the presence of increased cortisol.

The GR, expressed highly in the fetal liver, is another important mediator of the local action of hepatic cortisol. In the inactive state, ligand-free GR monomer is stabilized in the cytoplasm by binding to a complex of molecules, including heat shock proteins (hsp70, hsp90) and immunophilin FK binding proteins (i.e. FKBP52 and FKBP51 in rodents) (Bamberger *et al.* 1996). Upon binding of the ligand, GR monomers are believed to dissociate from the stabilizing complex. Only the newly formed receptor-ligand complexes are believed to dimerize and translocate to the nucleus, where they recognize and bind to the GRE and initiate specific gene transcription.

We found GR to be localized to the cytosol and nuclei of fetal hepatocytes. Increased GR immunoreactivity in the cytoplasm and nuclei after infusion of cortisol suggests increased synthesis, in addition to activation and nuclear translocation of the receptor. Using Western blot analysis, we found bands of 45, 57 and 95 kDa molecular weight in the fetal liver. The smaller 45 and 57 kDa bands may reflect receptor fragments or metabolites. Saoud & Wood (1996) have also previously reported a 45 kDa band in sheep pituitary and hypothalamus. The presence of these additional bands suggests possible tissue-specific pathways of post-translational GR metabolism, but their functional significance is unclear. The larger 95 kDa band reflects the full GR protein. Intrafetal infusion of cortisol had an overall stimulatory effect on GR, although this was significant only in the absence of increased oestradiol. These findings contrast with the classical negative feedback regulatory mechanism seen in adults and suggest altered regulation of the steroid receptor in the fetus compared with the adult (Bamberger *et al.* 1996). Infusions of cortisol and dexamethasone to the fetus have previously been shown to stimulate GR mRNA in the rat liver (Cleasby *et al.* 2001). Evidence of increased placental and pituitary expression of GR mRNA and protein after the infusion of cortisol (Holloway *et al.* 2001, Whittle *et al.* 2001) suggests a similar pattern of regulation in late gestation trophoblast, in addition to fetal tissue. Previous findings of a correlation

between the degree of receptor phosphorylation and functional significance provide evidence for a post-translational mechanism of receptor processing in mediating the action of cortisol in the fetal liver (Orti *et al.* 1992, Bamberger *et al.* 1996).

Prenatal exposure to glucocorticoids in rats has been shown to induce hyperglycaemia in adult offspring, and both altered lipid metabolism and increased insulin sensitivity in the fetus (Lindsay *et al.* 1996, Nyirenda *et al.* 2001). Glucocorticoids have been shown to oppose the actions of insulin in the liver by upregulating expression of the rate-limiting enzyme PEPCK (Low *et al.* 1994). Oestradiol, only in the presence of glucocorticoids, also downregulates expression of PEPCK (Jamieson *et al.* 1999). Morton *et al.* (2001) and others have shown that 11 β -HSD1-null mice have increased hepatic insulin sensitivity, and increased glycaemic control for similar insulin concentrations. Increased hepatic expression of 11 β -HSD1 and reductase activity, and a subsequent increase in local hepatic cortisol production, may result in induction of glucocorticoid-specific genes in the liver. Our findings also suggest that these effects would be propagated further by increased local hepatic expression of GR. In light of these results, we speculate that fetal sheep exposed to increased cortisol might show a predisposition to hepatic changes leading to hyperglycaemia, insulin resistance and later programming of inappropriate glycaemic control (Dodic *et al.* 1999).

In conclusion, we have demonstrated steroid-mediated regulation of the expression of 11 β -HSD1 and GR in fetal sheep liver. The expression of 11 β -HSD1 protein allows for local regulation of physiologically active glucocorticoids. Upregulation of GR in the presence of cortisol infusion is consistent with increased hepatic responsiveness to glucocorticoids in late gestation. The apparent attenuation by oestradiol of cortisol-induced expression of 11 β -HSD1 and GR protein may reflect a balance of these steroid hormone actions on local glucocorticoid regulation during normal pregnancy. We speculate that inappropriate upregulation of these proteins in response to precocious changes in fetal plasma cortisol may contribute to mechanisms by which fetal hepatic function is altered in a way that affects postnatal glucose homeostasis.

Acknowledgement

This research was funded by Canadian Institutes of Health Research (CIHR), Institute of Human Development, Child and Youth Health, Grant No. MOP-14097.

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Received in final form 28 October 2002

Accepted 4 November 2002