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Chronic Hypoxemia Selectively Down-Regulates 11β-Hydroxysteroid Dehydrogenase Type 2 Gene Expression in the Fetal Sheep Kidney[1]

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ABSTRACT

The present study was designed to examine the effects of chronic fetal placental embolization on the expression of 11β-hydroxysteroid dehydrogenase (11β-HSD) types 1 and 2, the intracellular enzymes responsible for the metabolism of glucocorticoids. Twelve instrumented fetal sheep were randomly allocated on Day 110 (term = 147 days) to either a control (n = 6) or embolized (n = 6) group. Embolized fetuses received daily injections of nonradioactive microspheres into the abdominal aorta for 21 days to decrease arterial oxygen content by 40–50% of the pre-embolization values. At the end of the experiment, fetal liver and kidney tissues as well as placental cotyledons were collected, and tissue levels of 11β-HSD mRNA and activity were determined by standard Northern blot analysis and radiometric conversion assay, respectively. There was a 44% reduction ($p < 0.01$) in the level of renal 11β-HSD2 mRNA in the embolized group as compared with the control group. Moreover, this reduction in mRNA was carried through to 11β-HSD2 protein, since there was a corresponding decrease in the level of 11β-HSD2 activity (4.5 ± 0.2 vs. 2.9 ± 0.1 pmol/min per milligram protein, $p < 0.01$). In contrast, levels of both 11β-HSD1 mRNA and activity in the fetal liver remained unchanged. Moreover, both 11β-HSD types 1 and 2 mRNA and activity in the placenta were not altered by the fetal placental embolization. In conclusion, chronic hypoxemia selectively inhibits renal 11β-HSD2 mRNA expression and enzyme activity in the ovine fetus, which may contribute, at least in part, to the mechanisms leading to fetal hypertension.

INTRODUCTION

In mammals, glucocorticoids play an important role in fetal organ growth and maturation [1, 2]. For example, it is well established that maternal administration of glucocorticoids is associated with enhancement in fetal lung maturation [3]. The bioavailability of glucocorticoids at the tissue level is regulated by the intracellular enzyme 11β-hydroxysteroid dehydrogenase (11β-HSD), which is responsible for the interconversion of bioactive glucocorticoids and their inactive metabolites [4, 5]. Two distinct isozymes of 11β-HSD have been characterized and cloned [6]. One isozyme, 11β-HSD1, possesses both dehydrogenase (conversion of cortisol to cortisone) and reductase (conversion of cortisone to cortisol) activities and is most abundant in the fetal liver [7]. In contrast, 11β-HSD2 has only dehydrogenase activity, and its expression is restricted to the placenta and to aldosterone-target organs such as the kidney [6]. In the kidney, 11β-HSD2 helps to confer aldosterone specificity for the nonselective mineralocorticoid receptors by inactivating glucocorticoids locally [8, 9]. Thus, deficiencies in this enzyme activity, either congenital or acquired through liquorice ingestion (glycyrrhetinic acid, the active ingredient of liquorice, is a potent inhibitor of 11β-HSD), lead to the syndrome of apparent mineralocorticoid excess in which cortisol acts as a mineralocorticoid causing hypertension and hypokalemia [6]. In the placenta, 11β-HSD enzymes may function to regulate the transplacental transfer of maternal glucocorticoids to the fetus [10–12].

We have recently demonstrated that chronic fetal placental embolization for 21 days causes progressive fetal hypoxemia and asymmetrical intrauterine growth restriction (IUGR). It is also associated with the development of chronic fetal hypertension and myocardial hypertrophy [13], in addition to a 5- to 6-fold increase in basal fetal plasma cortisol levels during the last 48 h of embolization [14]. Moreover, fetal cortisol levels are slightly elevated in human pregnancies complicated with IUGR [15]. It is possible that aberrations in fetal kidney 11β-HSD2 expression would favor an increase in kidney cortisol availability leading to the development of chronic fetal hypertension. Since the liver and kidney in fetal sheep are known to contain the greatest amount of 11β-HSD types 1 and 2, respectively [7, 16], the first objective of this study was to test the hypothesis that chronic fetal hypoxemia and IUGR in the ovine fetus are associated with changes in the fetal hepatic 11β-HSD1 and renal 11β-HSD2 gene expression and enzyme activity in a direction that would favor an increase in cortisol tissue availability. 11β-HSD enzymes in the placenta modulate the transplacental transfer of maternal glucocorticoids to the fetus and thus may potentially contribute to the control of glucocorticoid levels in the fetus. The second objective was therefore to examine the hypothesis that alterations in the expression of 11β-HSD types 1 and 2 in the placenta are associated with chronic fetal hypoxemia and may contribute to the increased fetal cortisol.

MATERIALS AND METHODS

Surgical Preparation

Twelve pregnant sheep of Western cross-breed with a singleton fetus, and of the same flock, were surgically prepared between 104 and 106 days of gestation. These sheep and the surgical procedures are the same as those described in previous publications [13, 14]. Briefly, under general anesthesia the uterus was exposed and a hind limb was exteriorized through a uterine incision. A polyvinyl catheter (V4; Bolab, Lake Havasu City, AZ) was implanted via the fetal femoral artery into the descending abdominal aorta. The catheter tip was placed below the renal arteries and approximately 1–2 cm above the common umbilical artery. A second catheter was introduced into the fetal inferior

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vena cava through the femoral vein. The correct position of the fetal catheters was confirmed at postmortem examination. A polyvinyl catheter (V11; Bolab) was sutured to the exterior of the fetal hind limb and left free-floating in the amniotic cavity to record amniotic pressure. After surgery, the sheep were housed in individual metabolic cages, with hay and water available ad libitum. Ewes were maintained on a 12L:12D cycle and were allowed at least 4 days to recover from surgery before the experiments began. This study was approved by the Animal Care Committees of St. Joseph's Health Centre and of the University of Western Ontario in accordance with the guidelines of the Canadian Council on Animal Care.

Experimental Protocol

The experimental protocol and associated changes in fetal morphometry, fetal arterial blood pressure, blood gases, and fetal hormones have been described in detail in previous publications [13, 14]. Briefly, on the 4th day postrecovery (range 108–110 days), animals were assigned randomly to either an embolized (n = 6) or a control (n = 6) group. After a 2-h recording period (0800–0100 h), nonradiolabeled carbonized latex microspheres suspended in dextran and diluted with sterile saline were injected every 15 min into the experimental fetuses over a 2-h period (0100–1200 h), through the descending abdominal aorta, for a total of 21 days. The number of microspheres injected was adjusted in order to decrease the fetal arterial oxygen content (CaO_2; mM) by 40–50% of the pre-embolization value. Control fetuses were injected over the same period for the same duration with the vehicle diluted in sterile saline. Paired maternal and fetal femoral artery blood samples were taken daily at 0900 h for measurement of CaO_2, PO_2, PCO_2, pH, and hemoglobin.

The ewes were killed on Day 21 at 1600 h, and the fetuses were delivered and their organs dissected rapidly. Kidney (upper third) and liver (segment of left lobe), as well as cotyledonary (large cotyledons near the umbilical cord insertion) tissues, were snap frozen in liquid nitrogen and stored at −70°C until analysis.

RNA Extraction and Northern Blot Analysis

Total cellular RNA was extracted using lithium chloride/urea [17]. The size as well as the relative abundance of 11β-HSD types 1 and 2 mRNAs was assessed by Northern blot analysis as described previously [7, 18]. Briefly, denatured RNA samples (20 μg) were subjected to agarose gel (1%) electrophoresis in the presence of formaldehyde and were transferred overnight by capillary blotting to a Zeta-Probe membrane (Bio-Rad Canada Ltd., Mississauga, ON, Canada). The RNA was fixed by UV cross-linking (Gene Cross-Linker; Bio-Rad) to the membrane, which was then baked under vacuum at 80°C for 60 min. The blot was hybridized at 42°C for 16 h in the presence of formamide (50%) and ^{32}P-sheep 11β-HSD types 1 [7] or 2 [19] cDNA prepared by random priming [20] with [^{32}P]dCTP (Du Pont, Markham, ON, Canada; 3000 Ci/mmol). We used a cDNA for mouse 18S rRNA as an internal control for gel loading and efficiency of RNA transfer, as described previously [7].

Assay of 11β-HSD Activity

Reagents and supplies. [1,2,6,7-^{3}H(*N*)]-Cortisol (80 Ci/mmol) was purchased from Du Pont Canada Inc. [1,2,6,7-^{3}H(*N*)]-Cortisone was prepared from [1,2,6,7-^{3}H(*N*)]-cortisol in our laboratory as described previously [21]. Nonradioactive steroids were obtained from Steraloids Inc. (Wilton, NH). Cofactors (NAD and NADPH) were purchased from Sigma Chemical Company (St. Louis, MO). 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).

Preparation of tissue homogenates. Fetal liver and kidney tissues (0.4–0.5 g) were homogenized in 20 vol of ice-cold 10 mM sodium phosphate buffer, pH 7.0, containing 0.25 M sucrose (buffer A). The homogenate was used immediately in assays as described below.

Protein estimation. Protein concentration was determined by the Bradford method using a Bio-Rad protein assay kit with BSA as standard.

Assay of 11β-HSD1 reductase and dehydrogenase activity. In fetal and adult sheep liver, it has been established that the reductase activity of 11β-HSD1 is always higher than the dehydrogenase activity [18, 22]. Therefore, in the present study, only the reductase activity of 11β-HSD1 in the fetal liver was determined by measuring the conversion rate of cortisone to cortisol as previously described [21, 22]. Briefly, the assay tubes contained approximately 100 000 cpm of the labeled cortisone, 1.0 μM of nonradioactive cortisone, and 250 μM of NADPH. Buffer C (0.1 M sodium phosphate buffer, pH 6.0) was added to bring the volume up to 0.4 ml. After 10-min incubation at 37°C, 100 μl of tissue homogenate containing 100–300 μg protein was added. After incubation for 15–30 min (preliminary studies indicated that the rate of reaction was linear with time from 5 to 120 min, and with the amount of tissue homogenates containing between 0.1–1.2 mg protein), the reaction was arrested, and the steroids were extracted with 4 ml ethyl acetate containing a 40-μg mixture of nonradioactive cortisol and cortisone as carrier steroids. The extracts were dried, and the residues were resuspended in 100 μl methanol. A fraction of the resuspension was spotted on a thin-layer chromatography plate, which was developed in chloroform/methanol (9:1, v:v). The bands containing the labeled cortisol and cortisone were identified by UV light of the cold carriers, cut out into scintillation vials, and counted in Scintisafe Econol 1 (Fisher Scientific, Toronto, ON, Canada). The rate of cortisone-to-cortisol conversion was calculated from the specific activity of the labeled cortisone and the radioactivity of cortisol, and results were expressed as the amount of cortisol (picomoles) formed per minute per milligram protein.

Since the ovine placenta expresses both 11β-HSD types 1 and 2, and the dehydrogenase activity of 11β-HSD1 is always higher than the reductase activity [23], the 11β-HSD1 dehydrogenase activity in the placenta was determined in a manner similar to that described above except that cortisol (1.0 μM) was used as substrate, NADP was used as cofactor, and buffer B (0.1 M phosphate buffer, pH 7.5) was used instead of buffer C.

Assay of 11β-HSD2 activity. The unidirectional 11β-HSD2 dehydrogenase activity in the placenta and fetal kidney was determined by measuring the conversion rate of cortisol to cortisone in a manner similar to that described above, except that 100 nM of cortisol was used as substrate, NAD was used as cofactor, and buffer B (0.1 M phosphate buffer, pH 7.5) replaced buffer C.

Data Analysis

To determine the relative abundance of 11β-HSD types 1 and 2 mRNA as well as 18S rRNA, the radioactive sig-

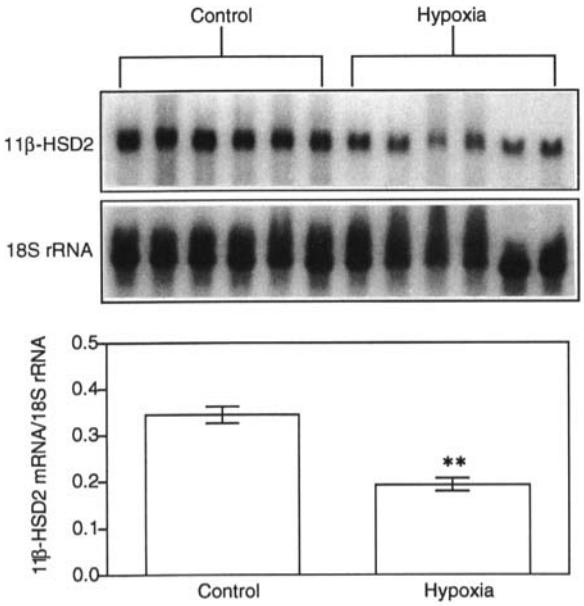

FIG. 1. Changes in 11β-HSD2 mRNA in the fetal kidney after 21-day fetal placental embolization. **Top**) Northern blot of 11β-HSD2 mRNA. **Bottom**) Mean ± SEM of the ratio of 11β-HSD2 mRNA to 18S rRNA in the control (n = 6) and embolized (n = 6) groups. **, $p < 0.01$.

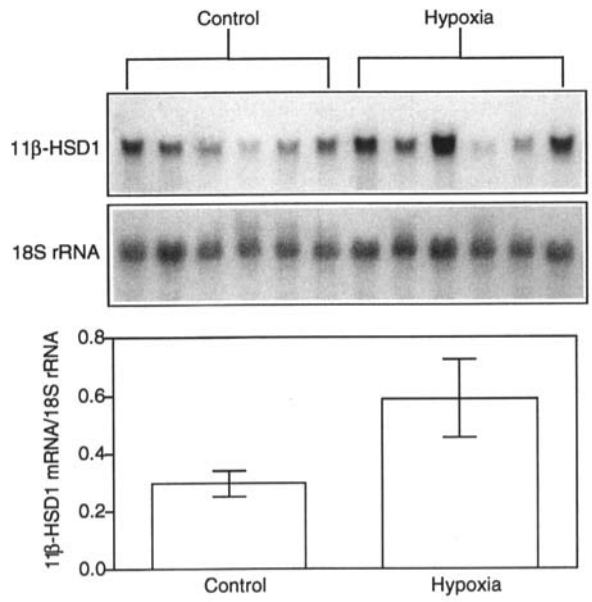

FIG. 3. Changes in 11β-HSD1 mRNA in the fetal liver after 21-day fetal placental embolization. **Top**) Northern blot of 11β-HSD1 mRNA. **Bottom**) Mean ± SEM of the ratio of 11β-HSD1 mRNA to 18S rRNA in the control (n = 6) and embolized (n = 6) groups ($p = 0.064$).

nals were measured directly from the blots by a phosphor imager system (Molecular Dynamics, Sunnyvale, CA). Thereafter, the blots were exposed to x-ray films for producing photographs. For each RNA sample, the ratio of 11β-HSD types 1 and/or 2 mRNA signal to 18S rRNA signal was calculated, and grouped mean was obtained. Statistical significance for the 11β-HSD types 1 and 2 mRNA as well as the corresponding activity data was determined by the use of a *t*-test for small samples with variances not assumed to be equal as described by Bailey [24], with confidence limits based on Student's *t*-distribution.

RESULTS

11β-HSD2 mRNA and Activity in the Fetal Kidney

When total RNA samples extracted from kidney tissues were subjected to Northern blot analysis using the ovine cDNA as probe, a single 2.0-kilobase (kb) transcript was detected in all the samples from both the control and embolized groups of fetuses (Fig. 1). The relative abundance of the 11β-HSD2 mRNA, expressed as the ratio of 11β-HSD2 mRNA to 18S rRNA, was decreased by 44% ($p < 0.01$) in the embolized group when compared with controls (Fig. 1).

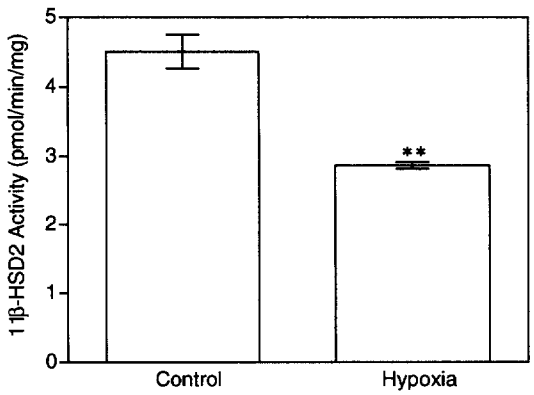

FIG. 2. Changes in 11β-HSD2 enzyme activity in the fetal kidney after 21-day fetal placental embolization. Each bar represents group mean ± SEM of the renal level of 11β-HSD2 activity in the control (n = 6) and embolized (n = 6) groups. **, $p < 0.01$.

To determine whether this decrease in the mRNA level was carried through to 11β-HSD2 protein, the level of 11β-HSD2 enzyme activity in kidney tissue homogenates was measured and compared between the two groups. There was a corresponding decrease ($p < 0.01$) in the level of 11β-HSD2 enzyme activity from 4.5 ± 0.2 pmol/min per milligram protein in the control group to 2.9 ± 0.1 pmol/min per milligram protein in the embolized group (Fig. 2).

11β-HSD1 mRNA and Activity in the Fetal Liver

When total RNA samples from liver tissues were analyzed, a single 1.8-kb 11β-HSD1 mRNA was detected in all the samples from both groups of fetuses. The intensity of the 11β-HSD1 mRNA signal was variable, and when expressed as the ratio of 11β-HSD1 mRNA to 18S rRNA, it displayed no significant change between the control and embolized groups, although there was a trend toward an increase ($p = 0.064$; Fig. 3). Since we have previously shown that elevated fetal glucocorticoid levels are associated with increases in the fetal hepatic 11β-HSD1 mRNA abundance [22], and fetal cortisol levels were elevated during the last 48 h of fetal placental embolization in the present study, we determined whether a positive correlation existed between fetal cortisol levels and fetal hepatic 11β-HSD1 mRNA abundance. Our results indicated that for all the 12 fetuses studied, their hepatic 11β-HSD1 mRNA correlated significantly ($p < 0.02$) with their cortisol levels in circulation (data not shown).

To determine whether changes occurred in the level of 11β-HSD1 enzyme activity, levels of the reductase activity in liver tissue homogenates were assessed by a standard radiometric conversion assay. In spite of a trend toward an increase in the 11β-HSD1 mRNA abundance, levels of 11β-HSD1 reductase activity in the control and embolized groups of fetuses were similar (Fig. 4).

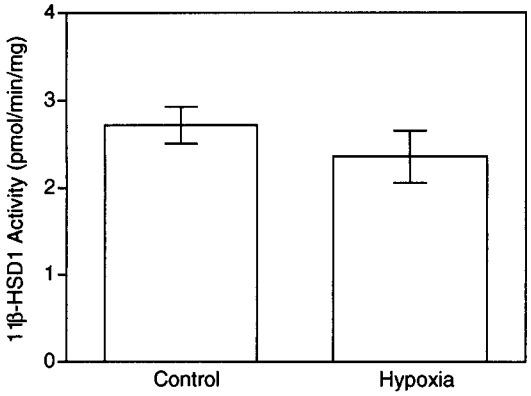

FIG. 4. Changes in 11β-HSD1 reductase activity in the fetal liver after 21-day fetal placental embolization. Each bar represents group mean ± SEM of the hepatic level of 11β-HSD1 activity in the control (n = 6) and embolized (n = 6) groups. $p > 0.05$.

11β-HSD types 1 and 2 mRNA and Activity in the Placenta

When total RNA samples from placental tissues were analyzed, a single 1.8-kb 11β-HSD1 mRNA was detectable in most but not all of the samples from both groups of fetuses. The intensity of the 11β-HSD1 mRNA signal was variable, and when expressed as the ratio of 11β-HSD1 mRNA to 18S rRNA, it displayed no significant change between the control and embolized groups (Fig. 5). When the same blot was stripped and reprobed with 11β-HSD2 cDNA probe, a single 2-kb mRNA with variable intensity was detectable in most but not all of the samples from both groups of fetuses, even after an extended period of exposure up to one month (Fig. 5).

To determined whether chronic fetal placental embolization altered 11β-HSD types 1 and/or 2 activity, levels of 11β-HSD1 dehydrogenase activity and of 11β-HSD2 activity in the placental tissue homogenates were measured and compared between the two groups. Our results revealed no significant changes in the tissue level of 11β-HSD types 1 and 2 enzyme activity (Fig. 6).

DISCUSSION

The present study has demonstrated that there is a reduction in the level of 11β-HSD2 mRNA and enzyme activity in kidneys of fetal sheep following progressive fetal hypoxemia induced by fetal placental embolization for 21 days. In contrast, levels of 11β-HSD1 mRNA and activity in the fetal liver, as well as both 11β-HSD types 1 and 2 mRNA and activity in the placenta, were unchanged, indicating that chronic fetal hypoxemia selectively down-regulates renal 11β-HSD2 expression in the fetal sheep. This is consistent with our previous studies in which a selective down-regulation of renal 11β-HSD2 mRNA but not enzyme activity was observed following a hypoxemia-induced acidosis, sustained for 8 h, in preterm fetal sheep [25].

Although we previously speculated that a resetting of the baroreceptor reflex and vasomotor tone could lead to chronic arterial hypertension during umbilical-placental insufficiency [13], a down-regulation in 11β-HSD2 mRNA and enzyme activity in the fetal kidney in response to chronic hypoxemia as shown in the current study could also play a major role in the development of fetal hypertension that could persist in adulthood. The diminished 11β-HSD2

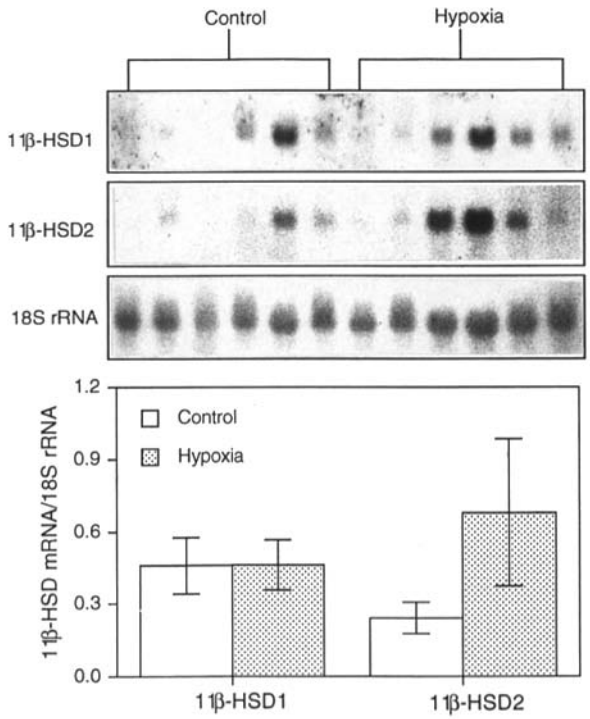

FIG. 5. Changes in 11β-HSD1 and 2 mRNA in the placenta after 21-day fetal placental embolization. **Top**) Northern blot of 11β-HSD1 and 2 mRNA. **Bottom**) Mean ± SEM of the ratios of 11β-HSD1 mRNA/18S rRNA and of 11β-HSD2 mRNA/18S rRNA in the control (n = 6) and embolized (n = 6) groups. $p > 0.05$.

mRNA expression and activity in the fetal kidney should cause an increase in the tissue level of cortisol within the kidney. Through this increase in tissue availability, cortisol could act as aldosterone and bind to the renal mineralocorticoid receptors that are normally protected by the action of 11β-HSD2 [8, 9]. This mechanism could lead to a mild form of mineralocorticoid-excess syndrome occurring in utero and result in chronic fetal hypertension as observed in the current study. Up to 10% of essential hypertensive patients have a prolonged plasma half-life of [^{3}H]cortisol, suggesting 11β-HSD2 relative deficiency [26]. However, these individuals are otherwise normal because they do not have complete deficiency of the enzyme. It should be pointed out that the development of fetal hypertension can also

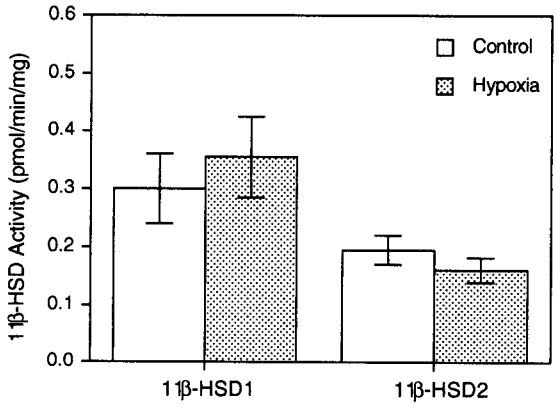

FIG. 6. Changes in 11β-HSD1 and 2 enzyme activity in the placenta after 21-day fetal placental embolization. Each bar represents group mean ± SEM of the level of 11β-HSD1 or 2 activity in the control (n = 6) and embolized (n = 6) groups. $p > 0.05$.

be connected to a host of other mechanisms altering intravascular volume that are unrelated to 11β-HSD types 1 and 2.

This increase in fetal arterial blood pressure could in turn lead to myocardial hypertrophy in response to an increase in afterload that we previously described using this model of placental insufficiency [13]. Interestingly, Veille et al. [27] used M-mode echocardiography to assess heart size and ventricular wall thickness during pregnancy complicated with IUGR and placental insufficiency. When adjusted for gestational age and for fetal weight, the hearts of IUGR fetuses were found to have an increased minor-axis dimension due to free wall hypertrophy but no ventricular dilation. As a result of ventricular wall hypertrophy, IUGR fetuses had a larger heart proportionally to their body weight, a situation similar to the adult hypertensive myocardial hypertrophy [13].

Extensive epidemiological data [28] have recently implicated prenatal events as potential determinants of subsequent hypertension in adulthood and risk of death related to cardiovascular disease. Low birth weight predicted hypertension in childhood, adolescence, and adulthood independently, and better than known adult risk factors such as smoking, obesity, or socioeconomic status [28]. It is therefore possible that placental insufficiency leading to chronic fetal hypoxemia, IUGR, myocardial hypertrophy, and a down-regulation in renal 11β-HSD2 could represent adaptive responses that would become permanent major contributors to the development of cardiovascular diseases in adulthood.

During fetal placental embolization, there was an increase in fetal plasma cortisol on Days 20–21 of the experiment [14]. This increase could be due to an increase in cortisol release from the fetal adrenal. Several factors could be responsible for this increase in fetal adrenal cortisol release, including an increase in immunoreactive ACTH and also prostaglandin E_2 [14] in response to placental embolization. Prostaglandin E_2 is known to stimulate rises in plasma cortisol in the fetal sheep at a time when the adrenal cortex is unresponsive to ACTH [29]. An increase in placental transfer of maternal cortisol is also a possibility. One of the mechanisms by which the placental transfer can be regulated is through the action of placental 11β-HSD enzymes [12]. In the present study, we sought changes in placental 11β-HSD types 1 and 2 mRNA and enzyme activity. Our results demonstrated that levels of both 11β-HSD types 1 and 2 mRNA and activity were highly variable but unchanged following 21 days of fetal placental embolization, indicating that the increased fetal cortisol is unlikely to be the result of a change in transplacental transfer. Since in the syndrome of apparent mineralocorticoid excess (AME), where renal 11β-HSD2 is completely deficient and the circulating levels of cortisol are normal [30], it is also unlikely that a ~50% reduction in renal 11β-HSD2 activity as observed in this study was a significant contributor to the increase in fetal plasma cortisol. However, in AME, the ability of the glucocorticoid to gain access to intracellular mineralocorticoid receptors in the distal nephron is increased dramatically, leading to severe hypertension [6].

It is noteworthy that there was a trend toward an increase in the level of 11β-HSD1 mRNA in the fetal liver. Since fetal plasma cortisol levels are elevated in the embolized fetuses [14], this trend would be consistent with our previous findings that fetal glucocorticoid stimulates fetal hepatic 11β-HSD1 mRNA and activity [22]. Since in that study the fetuses were treated with dexamethasone for 4 days, it is conceivable that the lack of a significant change in fetal hepatic 11β-HSD1 expression may be attributed to the fact that plasma levels of cortisol in the present study did not increase until the last 2 days of the study. It is also interesting to note that the relative abundance of 11β-HSD1 mRNA in the fetal liver was highly variable, especially in the embolized group of fetuses. This variability could be due, at least in part, to variable plasma levels of cortisol seen in those fetuses, because levels of fetal hepatic 11β-HSD1 mRNA were correlated significantly with plasma levels of cortisol.

The present study has provided the first direct evidence that 11β-HSD2 mRNA expression and activity in the fetal sheep kidney are down-regulated by chronic fetal hypoxemia. The decreased enzyme activity within the fetal kidneys should result in an increase in the local tissue level of cortisol, which in turn could contribute to the mechanisms leading to the observed fetal hypertension. Obviously, it will be important to determine the molecular mechanisms responsible for this down-regulation, since it provides a novel mechanism whereby chronic fetal hypoxemia alters fetal development by regulating the bioavailability of glucocorticoids in specific fetal organs through altered local expression of 11β-HSD.

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