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Corticosterone Transfer and Metabolism in the Dually Perfused Rat Placenta: Effect of 11β-hydroxysteroid Dehydrogenase Type 2

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Although rat is the most widely used model of glucocorticoid programming of the fetus, the role of rat placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) in the transplacental pharmacokinetics of the naturally occurring glucocorticoid, corticosterone, has not yet been fully elucidated. In this study, expression of 11β-HSD2 in the rat placenta on two different gestation days (16 and 22) was examined using quantitative RT-PCR and Western blotting, and dually perfused rat term placenta was employed to evaluate its functional capacity to transfer and metabolize corticosterone. Marked decrease in placental expression of 11β-HSD2 toward term was observed on both mRNA and protein levels. In perfusion studies, increasing maternal corticosterone concentration from 3 to 200 nM resulted in the fall of 11β-HSD2 conversion capacity from 64.3 to 16.3%, respectively. Enzyme saturation occurred at about 50 nM substrate concentration. When delivering corticosterone (3 or 100 nM) from the fetal side, a similar decline of 11β-HSD2 conversion capacity was observed (66.5% and 48.5%, respectively). Addition of carbenoxolone (10 or 100 μM), a non-specific 11β-HSD inhibitor, to maternal perfusate decreased conversion capacity from 66.7 to 12.6 or 8.1%, respectively. Similarly potent inhibitory effect was observed in feto-maternal studies. Neither saturation nor inhibition of 11β-HSD2 was associated with transformation of corticosterone in metabolites other than 11-dehydrocorticosterone. These data suggest that 11β-HSD2 is the principal enzyme controlling transplacental passage of corticosterone in rats and is able to eliminate corticosterone in both maternal and fetal circulations.
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INTRODUCTION

It is well established that glucocorticoids influence maturation of fetal organs and the timing of parturition [1–3]. In the fetus, glucocorticoids can be derived from three sources – transplacental transfer from mother, local production in placenta, and secretion of fetal adrenal glands. For much of pregnancy the maternal concentration of biologically active glucocorticoids, cortisol and corticosterone, is higher than in the fetal circulation. This difference is considered to be maintained by placental barrier, in particular by placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) [4,5]. This is an NAD^+-dependent enzyme with K_M in nanomolar range [6] which catalyses the unidirectional conversion (oxidation) of cortisol and corticosterone to cortisone and 11-dehydrocorticosterone, respectively.

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11β-HSD2 has been localized in the syncytiotrophoblast both in human [7–9] and rodent placenta [10,11] in developmentally programmed manner. Animal studies have shown that exposing rats in utero to high levels of dexamethasone or carbenoxolone, a non-specific 11β-HSD inhibitor, reduced birth weight [12] and caused hypertension, hyperinsulinaemia and altered behaviour in the adult offsprings [13–15]. In humans, reduced birth weight was associated with a deficiency of placental 11β-HSD2 [16–18]. Thus, low placental 11β-HSD2 activity and consequent exposure of fetuses to high levels of glucocorticoids of maternal origin seem to lead to disturbances of the intrauterine development.

However, there is a lack of direct evidence of fetal exposure to maternal glucocorticoids in relationship to barrier role of placental 11β-HSD2. In vitro studies revealed placental expression of not only 11β-HSD2 but also of the isoform 1 (11β-HSD1) that predominantly operates as a reductase, converting cortisone and 11-dehydrocorticosterone to cortisol and corticosterone, respectively. In addition, the direction of 11β-HSD activity in vitro is greatly influenced by enzyme stability and assay conditions [19]. Therefore, the relevance of

local metabolism of glucocorticoids to placental function remains uncertain. Only the functional studies with perfused human placenta [20–22] demonstrated that 11β-HSD affects materno-fetal transfer of cortisol.

Despite the fact that rat is the most widely used model of glucocorticoid programming of the fetus [12–15,23], the role of rat placental 11β-HSD2 in the passage of maternal glucocorticoids to the fetal circulation has not yet been fully elucidated. It is also unknown, whether placenta is able to inactivate corticosterone in the fetal circulation and modulate feto-maternal transfer of glucocorticoids, which was suggested three decades ago [24].

To answer these questions, we have employed in situ technique of dually perfused rat term placenta and examined the placental transfer and metabolism of corticosterone in the rat. Our first aim was to investigate materno-fetal and feto-maternal passage of corticosterone and the steroid metabolism associated with this transfer. We have also examined the effect of 11β-HSD inhibitor, carbenoxolone, on the placental transport and metabolism of corticosterone. In addition, comparison of 11β-HSD2 expression at two stages of pregnancy (16 versus 22 days of gestation) has been performed using quantitative RT-PCR and Western blotting techniques.

MATERIALS AND METHODS

Animals

All experiments were approved by the Ethical Committee of the Faculty of Pharmacy (Hradec Králové, Charles University in Prague) and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, 1996; and the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes, Strasbourg, 1986. Pregnant Wistar rats were purchased from Biotest Ltd. (Konárovice, Czech Republic) and were bred in 12/12-h day/night standard conditions with water and pellets ad libitum. Experiments were performed on day 21 of gestation. Fasted rats were anesthetized with pentobarbital (Nembutal; Abbott Laboratories, North Chicago, IL) at a dose of 40 mg/kg administered into the tail vein.

Dual perfusion of the rat placenta

The method of dually perfused rat placenta was used as described previously [25]. Briefly, the placenta was excised and allowed to dive in the heated Ringer saline. A catheter was inserted into the uterine artery proximal to the blood vessel supplying the selected placenta and connected with the peristaltic pump. Krebs' perfusion liquid containing 1% dextran was brought from the maternal reservoir at a rate of 1 ml/min. The uterine vein, including the anastomoses to other fetuses, was ligated behind the perfused placenta and cut so that maternal solution could leave the perfused placenta. The selected fetus was separated from the neighboring ones by ligatures. The umbilical artery was catheterized using 24-gauge catheter and connected with the tubing by which the fetal perfusion liquid from the fetal reservoir was supplied at a rate of 0.5 ml/min. The umbilical vein was catheterized in a similar manner and the selected fetus was removed. Before the start of each experiment, the fetal vein effluent was collected into preweighted glass vials to check a possible leakage of perfusion solutions from the placenta. In the case of leakage, the experiment was terminated. Corticosterone was added to the maternal or fetal reservoir immediately after successful surgery followed by approximately 10 min stabilization period before sample collection started. Maternal and fetal perfusion pressures were monitored continuously throughout the perfusion experiments. Wet weights of the placentae used in experiments were 0.53 ± 0.10 g.

Evaluation of corticosterone metabolism in the rat placenta

In these preliminary experiments, the placenta was loaded with high maternal inflow concentrations of corticosterone (200 nM) to investigate its possible metabolic pathways in the placenta. Fetal effluent samples were collected for 30 min of perfusion and analyzed by high performance liquid chromatography – electrospray ionization mass spectrometry, HPLC/MS [26].

Effect of inflow corticosterone concentrations on the conversion capacity of 11β-HSD2

In materno-fetal studies, corticosterone and [^{3}H]corticosterone as a tracer were brought to the perfused placenta via the uterine artery. Considering physiological values of unbound corticosterone in rat during the last week of gestation to be 55 nM [27], we have employed a series of maternal corticosterone concentrations ranging from considerably lower to higher than physiological values; in particular 3, 6, 23, 50, 100 or 200 nM. In feto-maternal experiments, corticosterone with the radiolabeled tracer was supplied via the umbilical artery in concentrations of 3 or 100 nM. In both experimental designs, the fetal effluent samples were collected in three consecutive10-min intervals for 30 min of perfusion and analyzed for [^{3}H]corticosterone and [^{3}H]11-dehydrocorticosterone by HPLC using online detection of radioactivity. Conversion capacity of 11β-HSD2 and transplacental clearance of corticosterone were calculated for every concentration from all measured intervals as described below.

Effect of carbenoxolone on transplacental passage of corticosterone

Inhibition of 11β-HSD2 was studied using carbenoxolone (CBX), an inhibitor of steroid dehydrogenases, in both materno-fetal and feto-maternal studies.

In experiments examining the materno-fetal passage, corticosterone (3 nM) was brought to the perfused placenta via the catheterized uterine artery immediately after the catheterization. Sample collection started (time 0) after 10 min delay to achieve steady-state conditions. Samples were collected at 10-min intervals from the fetal umbilical vein. After collection

of the first sample (absence of inhibitor), CBX was added to the maternal reservoir to reach a concentration of 10 or 100 μM. After 5 min delay, collection of the second 10-min interval sample started. This experimental design enables direct observations of the effect of CBX on placental metabolism and transport of corticosterone within one perfusion.

For the examination of the feto-maternal passage, fetal solution containing corticosterone (3 or 100 nM) was used to perfuse the selected placenta via the catheter in the fetal umbilical artery immediately after catheterization. Sample collection started 10 min after the installation of the catheter (time 0). In the 10th minute of the experiment, CBX was added to the fetal reservoir to reach a concentration of 10 or 100 μM. Samples were collected in two 10-min intervals as described in materno-fetal experiments.

Calculations

Averaged data from the first two intervals (0–10 and 10–20 min) of placental perfusions were used for the following calculations.

Conversion capacity of 11β-HSD2 was expressed as the ratio of 11-dehydrocorticosterone (metabolite) concentration to the concentration of total corticosteroids (corticosterone and 11-dehydrocorticosterone) in the fetal effluent [20].

Materno-fetal transplacental clearance (Cl_{mf}) of corticosterone normalized to placenta weight was calculated according to Eq. (1):

$$Cl_{mf} = \frac{C_{fv} Q_f}{C_{ma} w_p} \quad (1)$$

where C_{ma} (nM) is the concentration of corticosterone in the maternal reservoir, C_{fv} (nM) is the concentration of total corticosteroids (corticosterone and 11-dehydrocorticosterone) in the umbilical vein effluent, Q_f (ml/min) is the umbilical flow rate and w_p (g) is the wet weight of the placenta.

In feto-maternal studies, the ability of the placenta to remove corticosterone from the fetal circulation was expressed as extraction ratio (ER) using Eq. (2):

$$ER = \frac{(C_{fa} - C_{fv})}{C_{fa}} \quad (2)$$

where C_{fa} (nM) is the concentration of corticosterone in the fetal reservoir entering the perfused placenta via the umbilical artery and C_{fv} (nM) is the concentration of total corticosteroids (corticosterone and 11-dehydrocorticosterone) in the umbilical vein effluent.

Feto-maternal clearance normalized to placenta weight (Cl_{fm}) was calculated according to Eq. (3):

$$Cl_{fm} = \frac{ER Q_f}{w_p} \quad (3)$$

where Q_f (ml/min) is the umbilical flow and w_p (g) is the wet weight of the placenta.

Analysis of corticosterone and 11-dehydrocorticosterone in placental perfusate

Perfusate samples were extracted using C_{18} solid phase extraction cartridges SepPak (Waters, Milford, MA) as described previously [28]. The samples were reconstituted in mobile phase (50 μl methanol) and an aliquot (20 μl) was injected on column for analysis. Steroids were separated and quantified with HPLC using Lichrospher 100 RP-18 column (125 × 4 mm; Merck, Darmastadt, Germany) and a linear methanol–water gradient from 42:58 (v/v) to 62:38 (v/v) at 15 min followed by isocratic washing with 100% methanol for 10 min. The flow rate was 1.0 ml/min and the column temperature was held at 45 °C. Ultraviolet absorbance (multi-wavelength detector Agilent 1100 [formerly Hewlett Packard], Palo Alto, CA) was used for the detection of "cold" steroids whereas radioactivity was quantified using radioactivity detector (Radiomatic 150TR; Packard, Downers Grove, IL) with a flow cell and scintillation cocktail Ultima-Flo M (Perkin Elmer, Boston, MA); the flow rate of scintillation cocktail/mobile phase was 3:1. After subtraction of the background radioactivity, integrated counts within peaks were analyzed by ChemStation program (Agilent). Identity of eluted peaks was confirmed by comparisons with steroid standards purchased from Steraloids Inc. (Newport, RI).

Expression of 11β-HSD2 in the placenta on gestation days 16 and 22

Placentae were collected from five dams on gestation day 16 and from five dams on gestation day 22. Selected placentae were dissected free of endometrium and fetal membranes, snap-frozen in liquid nitrogen and stored at −80 °C for quantitative RT-PCR and Western blotting.

RNA isolation and quantitative RT-PCR analysis

Total RNA was extracted from placenta and kidney as a positive control [6] by the guanidium thiocyanate method. To remove DNA, the isolated RNA was treated with DNAase (Promega, Madison, WI) and the contamination of RNA samples by DNA was detected by PCR followed by electrophoresis on 2% agarose gel as described previously [29].

cDNA was synthesized using about 5 μg of RNA and M-MLV reverse transcriptase reagents (Invitrogen GmbH, Lofer, Austria) in a total volume of 20 μl containing oligo(dT) primer, 0.5 μg; reaction buffer (1×); DTT, 0.01 M; dNTP mix, 0.5 mM; RNaseOUT (recombinant ribonuclease inhibitor), 40 units and M-MLV reverse transcriptase, 200 units. RNA was first heated with oligo(dT) to 70 °C for 10 min and quickly chilled on ice. After cooling, the remaining chemicals were added; the incubation continued 60 min at 37 °C and then was stopped by heating to 95 °C for 5 min.

Two different genes (11β-HSD2 and β-actin as a housekeeping gene) were quantified using LightCycler-FastStart DNA Master SYBR Green I kit (Roche, Mannheim,

Table 1. Sequences of primers used for real-time RT-PCR

Gene	Sequence 5′ → 3′
11β-HSD2	CCGGTTGTGACACTGGTTTTG (fwd) GGGGTATGGCATGTCTCCTG (rev)
β-Actin	CCGTAAAGACCTCTATGCCA (fwd) AAGAAAGGGTGTAAAACGCA (rev)

Germany), LightCycler instrument and primers (VBC-GENOMICS, Wien, Austria). Primers of each amplicon are shown in Table 1. RT-PCR was performed in total volume of 10 µl containing 1 µl of 10-fold diluted cDNA and 5 mM (β-actin) or 4 mM (11β-HSD2) $MgCl_2$; 0.5 µM of each primer and PCR reaction mix (1×). The LightCycler was programmed as follows: pre-incubation and denaturation of the template cDNA for 10 min at 95°C; followed by 45 cycles of amplification: 95°C for 15 s, 55°C for 10 s (β-actin) or 60°C for 5 s (11β-HSD2), 72°C for 15 s (β-actin) or 20 s (11β-HSD2). The melting curve analysis was performed at 95°C for 0 s, 65°C for 60 s and 99°C for 0 s. The temperature slope was always 20°C/s, with the exception of the last step of melting analysis when it was 0.1°C/s. For quantification, we prepared standard curves for each pair of primers from serial dilutions of kidney cDNA containing 11β-HSD2. The results were calculated as relative expression of 11β-HSD2 mRNA to β-actin mRNA.

Western blotting of 11β-HSD2 in the rat placenta

Samples of placenta and kidney as a positive control were homogenized in ice-cold buffer (1:9 w/v) containing 250 mM sucrose, 0.1 mM $MgCl_2$, 100 mM Tris/HCl at pH 8 and protease inhibitor cocktail (1×; Roche Diagnostics GmbH, Mannheim, Germany) using a Polytron homogenizer (Kinematica AG, Littau, Switzerland) and a Ultrasonic processor (Cole-Parmer, Chicago, IL, USA). After centrifugation at 400 × g for 10 min the supernatant was centrifuged at 100,000 × g for 60 min to separate microsomes and cytosol. The pellet was resuspended in ice-cold buffer, sonicated and protein concentration was determined by the Coomassie blue method in both fractions. The samples were then suspended in Laemmli buffer with β-mercaptoethanol (2%) and boiled for 3 min. Samples were stored at −20°C.

Proteins (13 µg of particular or some cytosol fraction) were separated by 10% SDS-PAGE gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA), then transferred to a nitrocellulose membrane using semi-dry system (OMNI, Brno, Czech Republic) and blocked in buffer A (PBS buffer containing 5% non-fat milk and 0.05% Tween-20) at 4°C for 60 min. After washing in buffer A, the membranes were incubated with a sheep anti-rat 11β-HSD2 polyclonal antibody in the buffer A (1:2000 dilution; Chemicon International, Inc., Temecula, CA, USA) at room temperature for 60 min following overnight at 4°C. The blots were washed in buffer A (6-times, 5 min/wash) and subsequently incubated with secondary antibody (rabbit anti-sheep IgG peroxidase conjugated; 1:50,000 dilution; Pierce Biotechnology, Inc., Rockford, IL, USA) for 60 min, washed in PBS containing 0.05% Tween-20 (6×, 5 min/wash), visualized using Super Signal West Femto substrate (Pierce Biotechnology) and detected by Luminescence Analyzing System (LAS-1000, Fuji, Tokyo, Japan). The signal corresponding to 11β-HSD2 was found only in microsomal-enriched pellet but not in cytosol and the secondary antibody did not give any non-specific reaction. For quantification, equal amounts of protein from each sample were used and the chemiluminescent signal of the measured immunoreactivity was in the linear range in terms of protein amounts used in this analysis. The results were calculated as a ratio of chemiluminescent signal to mg of protein.

Statistical analysis

For each group of experiments, the number of animals was $n \geq 4$ (exact n values are given in the figure legends). Student's t-test or ANOVA analysis was used where appropriate to assess statistical significance. Differences of $p < 0.05$ were considered statistically significant.

RESULTS

Corticosterone metabolism in the rat placenta

Experiments with high maternal inflow concentrations of corticosterone (200 nM) revealed that transplacental transfer of corticosterone was associated with transformation of this steroid into 11-dehydrocorticosterone only. No other metabolic products were detected even in the presence of CBX. Corticosterone and 11-dehydrocorticosterone in the perfusate was characterized by their retention times, the mass of the protonated molecules and characteristic fragment ions (Figure 1). Therefore, in subsequent perfusion studies the samples were assayed for corticosterone and 11-dehydrocorticosterone only.

Consistency of perfusion experiments

Comparing the three sample intervals of 30 min perfusions, the enzyme activity appeared to be stable throughout the experiments. On the other hand, transplacental corticosterone clearance was almost identical in the first two intervals (i.e. up to 20 min) but tended to increase in the last interval. This might be a result of impaired placental architecture as described before [30]. Therefore, only the first two intervals were considered in further studies.

Effect of inflow corticosterone concentrations on the conversion capacity of 11β-HSD2

Corticosterone placental metabolism was found to be concentration dependent. In materno-fetal studies, increasing maternal inflow concentration of corticosterone caused the reduction of 11β-HSD2 conversion capacity from 64.3 ± 6.48% at 3 nM

to 16.3 ± 5.34% at 200 nM (Figure 2). Correspondingly, the composition of the fetal effluent changed with rising inflow concentrations, increasing the ratio between unmetabolized corticosterone and 11-dehydrocorticosterone as shown in Figure 3. Fetal concentrations of 11-dehydrocorticosterone seemed to reach a plateau in the three highest doses of maternal corticosterone infused, thus indicating that 11β-HSD2 becomes saturated at corticosterone concentration of approximately 50 nM.

In feto-maternal experiments, two fetal inflow concentrations of corticosterone were tested, in particular 3 and 100 nM. The extraction ratios were 0.92 and 0.85, respectively, indicating rapid removal of fetal corticosterone by the placenta. Similar to materno-fetal experiments, significant decrease in 11β-HSD2 conversion capacity was observed in studies with high corticosterone concentration; the value dropped from 66.5 ± 4.3% to 48.5 ± 3.9% at 3 and 100 nM, respectively.

Effect of carbenoxolone on transplacental passage of corticosterone

Effect of CBX on corticosterone transplacental pharmacokinetics was investigated in both materno-fetal and feto-maternal experiments. In materno-fetal direction, addition of CBX in 10 or 100 μM concentration to the maternal compartment resulted in the decline of 11β-HSD2 conversion capacity from 66.7 to 12.6 or 8.1%, respectively (Figure 4). The ratio of corticosterone to 11-dehydrocorticosterone in the fetal effluent increased 18 times. No significant differences were observed between the two inhibitor concentrations. In the feto-maternal studies, placenta was perfused with 3 or 100 nM corticosterone; addition of CBX (100 μM) caused significant reduction in 11β-HSD2 conversion capacity from 65.3% to 7.71% and from 48.5% to 2.84%, respectively (Figure 5A, B). A 20-fold increase in corticosterone/11-dehydrocorticosterone ratio was recorded in the fetal effluent.

Expression of 11β-HSD2 in the placenta at two different stages of pregnancy

RT-PCR analysis proved the presence of 11β-HSD2 mRNA in the placenta and corresponded with that of a kidney positive control (data not shown). Consistent with 11β-HSD2 mRNA expression, Western blot analysis of microsomes and cytosol demonstrated the presence of 11β-HSD2 protein in both placenta and kidney. The immunoreactive signal was localized to microsomal fractions and was detected as a major band of approximately 40 kDa (Figure 7B), as previously shown for other rat tissues [6]. Lightly staining bands were seen only if extremely sensitive chemiluminescent substrate WestFemto (Pierce) was used; if picogram-level sensitive substrate (West-Pico, Pierce) was used the weak staining in lines 1–3 was not obvious (not shown).

When comparing 11β-HSD2 expression at two stages of pregnancy, marked differences were shown in both quantitative RT-PCR and Western blotting analysis between day 16 and day 22 of gestation. Noticeable, but statistically insignificant drop in 11β-HSD2 transcription was observed (Figure 6). In the case of protein detection, almost 50% reduction was found on day 22 ($p < 0.05$) (Figure 7).

DISCUSSION

11β-Hydroxysteroid dehydrogenase type 2 (11β-HSD2) metabolizes glucocorticoids to their inactive 11-keto metabolites and plays a key role in modulating exposure of fetus to maternal glucocorticoids [31]. Although rats have frequently been used to study the role of placental 11β-HSD2 in mammalian development [12–15,23], the role of this enzyme in the transplacental pharmacokinetics of corticosterone has not yet been fully described in this species. Dual perfusion of the rat placenta is an acknowledged and sensitive experimental technique that has been used in a number of studies to investigate placental physiology and pharmacology [25,30,32–34]. This is the first report that employs the dually perfused rat term placenta to describe materno-fetal and feto-maternal transport and placental metabolism of naturally occurring active glucocorticoid, corticosterone, and regulation of these events by 11β-HSD2.

The presence of the 11β-HSD2 in the rat placenta has been reported elsewhere [29,35] and confirmed in our study on both mRNA and protein levels. 11β-HSD type 1 (11β-HSD1) has also been detected in the rat placenta and appeared to operate as 11-oxoreductase [29,35]. However, our preliminary perfusion experiments specifically designed in an attempt to detect 11β-HSD1 activity in the intact placenta (perfusion with 11-dehydrocorticosterone in a concentration equivalent to K_M) have shown only very limited transplacental transfer delivery of corticosterone to the fetus (data not shown). Together, these data provide evidence that under in vivo conditions at the near-term gestational stage, dehydrogenase activity predominates.

Sampling of the fetal effluent in perfusion experiments was divided into three intervals so that we could assess the integrity of the perfusion and monitor possible changes in enzyme activity and transplacental clearance in each experiment. While 11β-HSD2 activity was found to be stable over the whole perfusion period, transplacental clearance showed a tendency to rise in the last interval (20–30 min) in most experiments. This corresponds with our previous results where transplacental clearance of L-glucose, a marker of paracellular diffusion, was found to grow after about 30 min of perfusion, probably due to impaired placental integrity [30]. Therefore, in this study perfusion experiments did not last longer than 30 min.

Apart from 11β-HSD2, the placenta contains several other steroid metabolizing enzymes such as 3α-, 3β-, 20α- and 20β-hydroxysteroid dehydrogenases [21,36] but their role in corticosteroid metabolism is less clear. Human placental perfusion studies in both open and closed fashion have been employed in the past to study materno-fetal transport and placental metabolism of natural and synthetic glucocorticoids

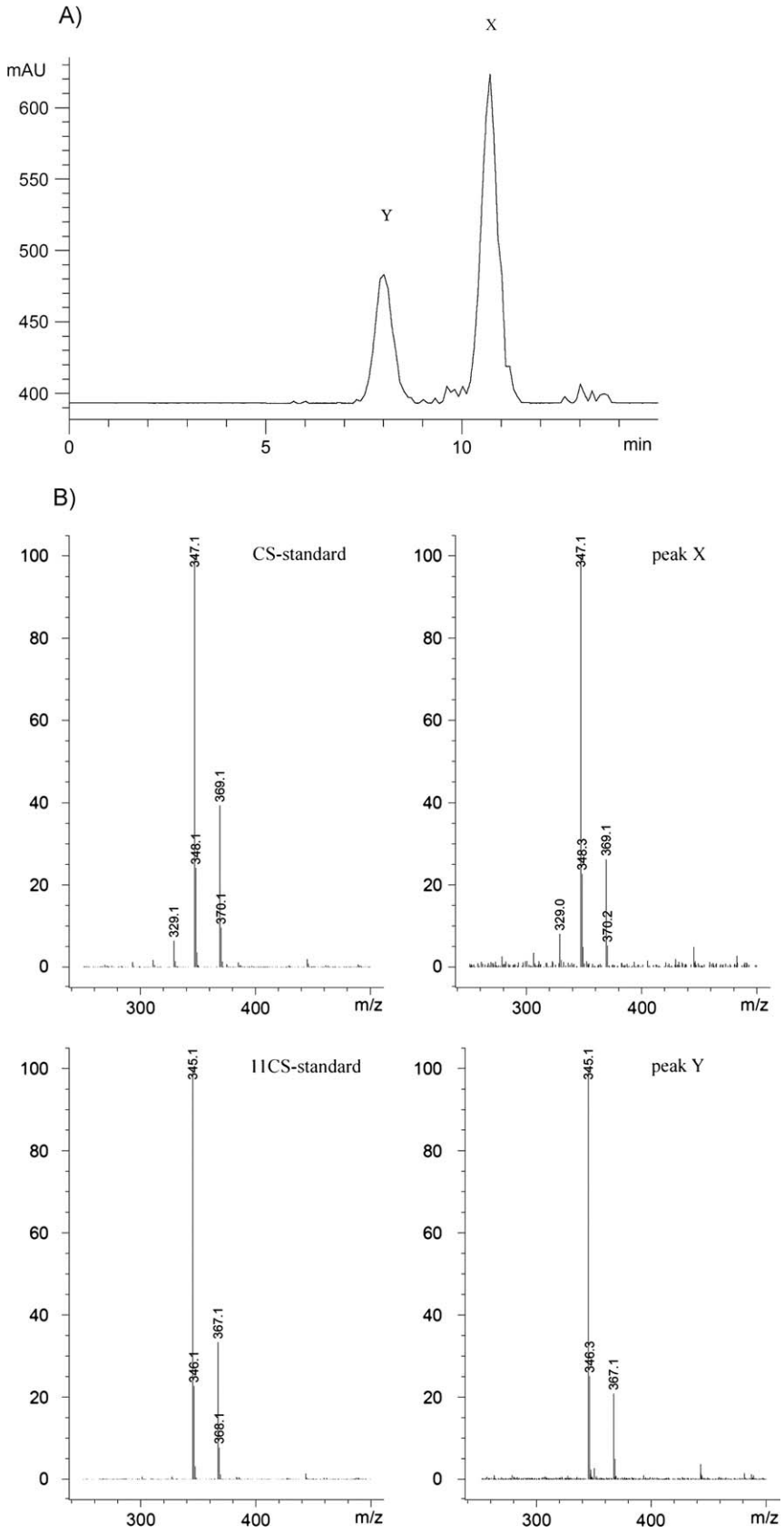

Figure 1. A typical HPLC chromatogram of fetal effluent during placental perfusion of maternal side with perfusate containing corticosterone (A) and the comparison of mass spectra of compounds X and Y with corticosterone and 11-dehydrocorticosterone. CS, corticosterone; 11CS, 11-dehydrocorticosterone; X, Y, steroids found in fetal effluent.

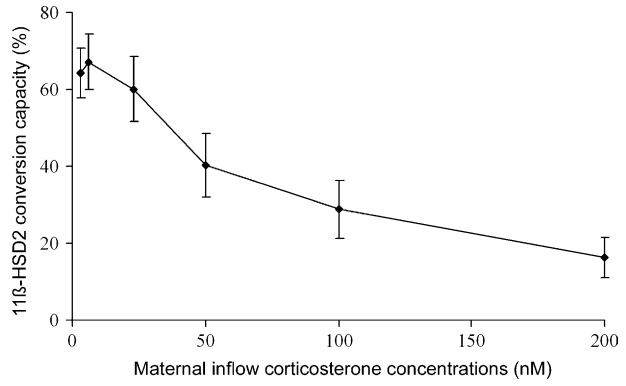

Figure 2. Effect of maternal inflow concentrations of corticosterone on 11β-HSD2 conversion capacity. Corticosterone was infused into the placenta via uterine artery at concentrations of 3, 6, 23, 50, 100, or 200 nM and fetal effluent was sampled and assayed for corticosterone and 11-dehydrocorticosterone. Conversion capacity was calculated as described in Materials and Methods. Data are presented as mean ± SD. Number of experimental animals for each concentration was $n = 5, 6, 4, 7, 5, 5$, respectively.

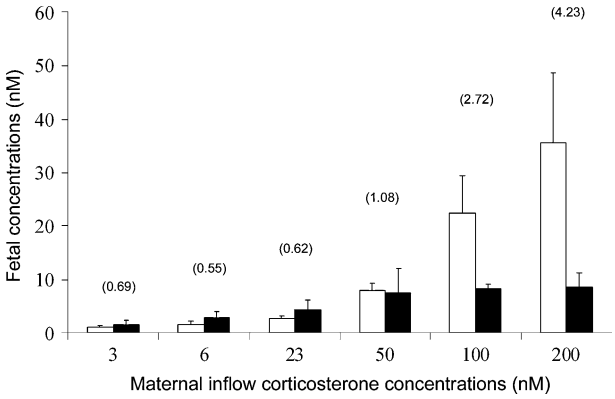

Figure 3. Effect of maternal inflow concentrations of corticosterone on composition of fetal effluent. White and dark columns represent fetal concentrations of corticosterone and 11-dehydrocorticosterone, respectively. Corticosterone was infused into the placenta via uterine artery at concentrations of 3, 6, 23, 50, 100, or 200 nM and fetal effluent was sampled and assayed for corticosterone and 11-dehydrocorticosterone. Production of 11-dehydrocorticosterone seems to reach plateau at 50 nM corticosterone. Numbers in brackets show the ratio between corticosterone and 11-dehydrocorticosterone in the fetal effluent. Data are presented as mean ± SD. Number of experimental animals for each concentration was $n = 5, 6, 4, 7, 5, 5$, respectively.

[20–22,37–40]. While single perfusion setups failed to detect other than 11-keto metabolites [20,22], the studies with recirculated perfusate revealed additional metabolites of cortisol [21] or prednisolone [37,38]. However, the substrate concentrations used in these studies represented pharmacological values equivalent to K_M of 20-hydroxysteroid dehydrogenases [41]. Using open circuit perfusion of the rat placenta in the present study, we have detected only one corticosterone metabolite, 11-dehydrocorticosterone, even with maternal corticosterone concentrations exceeding physiological values of unbound corticosterone (estimated at 55 nM in the last week of gestation [27]). Although biotransformation activity of other enzymes in the rat placenta cannot be excluded, 11β-HSD2 seems to be the principal enzyme in corticosterone metabolism under physiological concentrations.

Examining the saturability of 11β-HSD2 by increasing maternal inflow concentrations of corticosterone, we observed a significant fall in conversion capacity from about 65% at 3 nM to less than 20% at 200 nM. Contradictory results obtained from human studies have been published in the literature recently. While Benediktsson et al. [20] observed almost constant conversion capacity over a range of maternal cortisol concentrations – 92.6, 92.5 and 90.4% at 20, 50 and 200 nM, respectively, other papers reported a decrease in conversion capacity with high cortisol concentrations [21,22]. Our results are in agreement with those of Sun et al. [22] who reported a decrease in 11β-HSD2 conversion capacity from 47.1% (100 nM cortisol) to 15.1% (2 μM). However, comparing rat and human 11β-HSD2 conversion capacity at 100 nM inflow concentration, rat placenta seems to be less effective in metabolizing corticosteroid (28.8% versus 47.1%). This suggests possible interspecies differences in enzyme activity, although more detailed study would be needed to quantify possible variations. Production of 11-dehydrocorticosterone seemed to reach a plateau at maternal corticosterone concentrations of 50 nM indicating enzyme saturation around this substrate concentration.

Up to 75% of active cortisol in the human fetal circulation is derived from the fetal adrenals [42], but the role of hydroxysteroid dehydrogenases in metabolism of fetal glucocorticoids has not been investigated so far. Therefore, we addressed this issue in the present study and examined the role of 11β-HSD2 in feto-maternal passage of corticosterone. Similar to materno-fetal transport, corticosterone was rapidly transported across the placental barrier in the feto-maternal direction at both concentrations tested (3 and 100 nM). High extraction ratios indicated that approximately 90% of incoming fetal corticosterone concentration is removed by the placenta under experimental conditions. Conversion capacity of 11β-HSD2 dropped from 66.5% at the lower concentration to 48.5% at the higher concentration suggesting saturable kinetics. From these results, it seems obvious that placental 11β-HSD2 acts in bidirectional fashion with a similar activity to metabolize both maternal and fetal corticosterone.

CBX, a potent inhibitor of 11β-HSD2, was used in both materno-fetal and feto-maternal studies to investigate its potential to block rat placental 11β-HSD2 and possibly redirect the placental corticosterone metabolism pathway. Both CBX concentrations (10 and 100 μM) caused significant but not complete reduction of 11-dehydrocorticosterone production in both materno-fetal and feto-maternal experiments. Inhibition of 11β-HSD2 did not reveal any alternative metabolite in the fetal effluent confirming the key role of 11β-HSD2 in placental metabolism of corticosterone.

Our model shares the same disadvantage with human placental perfusion, i.e. the perfusion experiment is limited to the very last period of pregnancy. This is especially of an issue in studies dealing with placental 11β-HSD2 whose expression and/or activity have been reported to change over the course of pregnancy in humans, even though contradictory data have

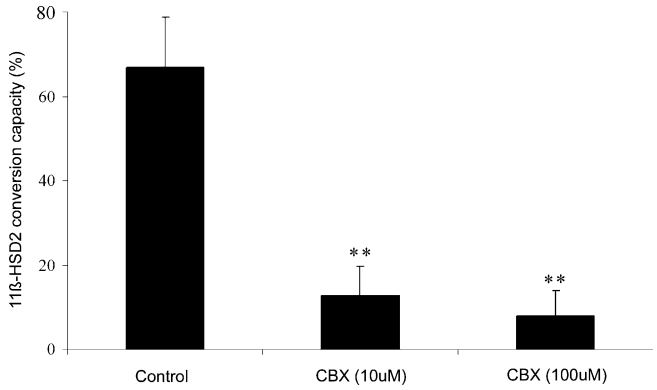

Figure 4. Effect of carbenoxolone (CBX) on 11β-HSD2 conversion capacity in materno-fetal studies. Corticosterone was infused to the placenta from the maternal reservoir at a concentration of 3 nM. After 10 min, CBX in either 10 or 100 μM concentration was added to the maternal perfusion liquid. Fetal effluent was sampled and analyzed for corticosterone and 11-dehydrocorticosterone and conversion capacity calculated as described in Materials and Methods. Asterisks indicate statistically significant effect of CBX ($p < 0.05$). Data are presented as mean ± SD, ($n = 5$).

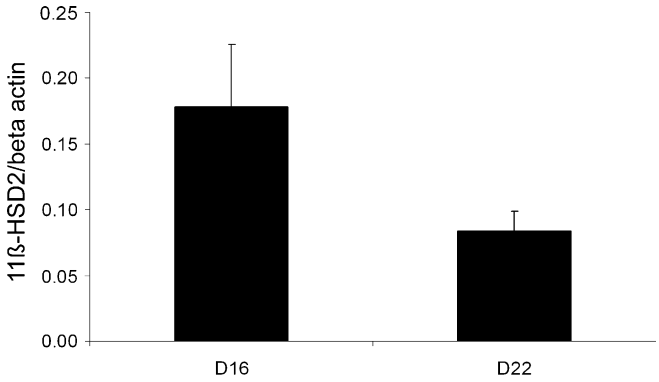

Figure 6. Expression of 11β-HSD2 in the rat placenta on day 16 (D16) and 22 (D22) of gestation analyzed by quantitative RT-PCR. cDNA was detected with SYBRGreen dye and results were calculated as the relative expression of 11β-HSD2 mRNA to β-actin mRNA. Data are presented as mean ± SEM, ($n = 5$).

been presented [16,43–46]. Fall in 11β-HSD2 expression during pregnancy has also been reported in other species, such as baboon [47,48] and guinea pig [49]. To date, there is only limited knowledge of developmental changes of rat placental 11β-HSD2 [35]. Therefore, we employed quantitative RT-PCR and Western blotting techniques to compare 11β-HSD2 expression at two different stages of pregnancy; gestation day 16 was selected as the last day before the onset of fetal corticosterone secretion [50,51] and gestation day 22 as the last day of rat pregnancy and the day of perfusion experiments. In accordance with other mammal species, fall in 11β-HSD2 expression towards the end of pregnancy on both mRNA and protein levels was observed. Nevertheless, more studies are essential to elucidate developmental profiles of both 11β-HSD isoforms during gestational age. Therefore, results obtained from perfusion experiments might have limited physiological relevance to earlier stages of pregnancy and must be treated with caution. On the other hand, animal model presents a significant advantage over the human placental perfusion, i.e. the possibility to pre-treat animals with a compound of interest and to investigate its long-term effect (induction/down-regulation) on placental 11β-HSD2 activity and glucocorticoid metabolism. This approach is not feasible in human studies.

In conclusion, we have confirmed the localization of 11β-HSD2 in the rat placenta and observed decrease in its expression at the end of pregnancy. Using dual perfusion technique, we have described the role of this enzyme in materno-fetal and feto-maternal transport of the naturally occurring glucocorticoid, corticosterone. Conversion capacity of 11β-HSD2 decreased with increasing substrate concentrations and was potently inhibited by CBX. Neither saturation, nor inhibition of 11β-HSD2 caused production of metabolites other than 11-dehydrocorticosterone. In addition, 11β-HSD2 was capable of metabolizing maternal and fetal corticosterone with a similar potency. From our data it seems obvious that 11β-HSD2 is the principal enzyme controlling transplacental passage of corticosterone in rats. We suggest this model be a useful tool for further studies on glucocorticoid transport across the placenta and possible interactions with other compounds.

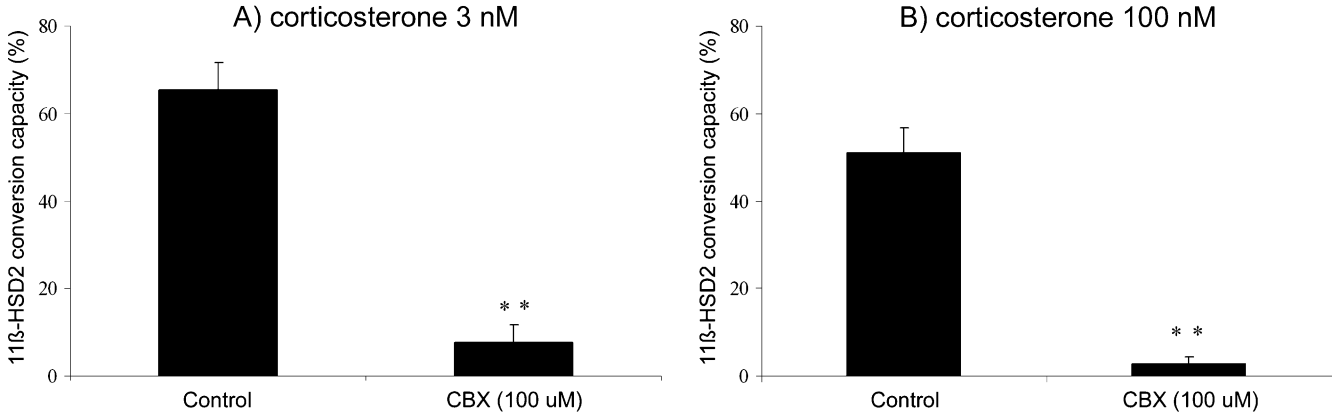

Figure 5. Effect of carbenoxolone (CBX) on 11β-HSD2 conversion capacity in feto-maternal studies. Corticosterone was infused to the placenta from the fetal reservoir at concentrations of 3 nM (A) or 100 nM (B). After 10 min, CBX (100 μM) was added to the perfusion liquid. Fetal effluent was sampled and analyzed for corticosterone and 11-dehydrocorticosterone. Conversion capacity was calculated as described in Materials and Methods. Asterisks indicate statistically significant effect of CBX ($p < 0.05$). Data are presented as mean ± SD, ($n = 5$).

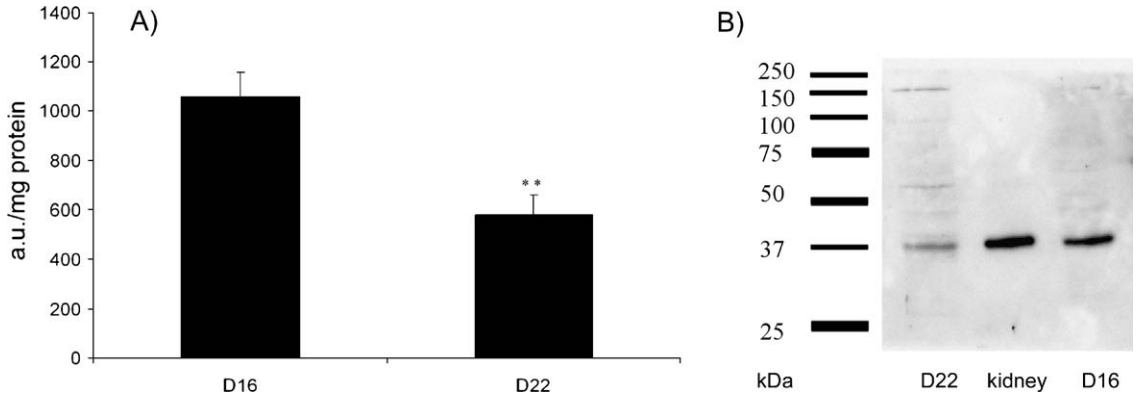

Figure 7. Expression of 11β-HSD2 in the rat placenta on day 16 (D16) and 22 (D22) of gestation analyzed by Western blotting. (A) For quantification, see Materials and Methods. Asterisks indicate statistically significant ($p < 0.05$) difference in 11β-HSD2 content between gestation days 16 and 22. Data are presented as mean ± SEM ($n = 5$). (B) A typical example of protein separation and detection by Western blotting.

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