Species differences of 11beta-hydroxysteroid dehydrogenase type 2 function in human and rat term placenta determined via LC-MS/MS

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1. Introduction

The concept of fetal programming describes the association of a stimulus or insult during a critical phase of in utero organ development with persistent structural/functional changes after birth. In humans, fetal programming can result in low birth weight and the subsequent development of insulin resistance, type 2 diabetes and cardiovascular disease [1]. Evidence from animal studies by us [2–4] and others [5] suggests that manipulation of the fetal environment can account for pathophysiological changes in the adult. Glucocorticoids are highly influential on fetal organ development [6,7]. While this knowledge is clinically applied for the induction of lung maturation [8], overexposure to these hormones during pregnancy reduces birth weight and can be detrimental to fetal development in both animals and humans [9–11].

In mammals, the placental enzyme 11beta-hydroxysteroid dehydrogenase type 2 (11β-HSD2) shields the fetus from maternal glucocorticoid excess [12]. Located in placental compartments that act as interfaces of feto-maternal exchange - the syncytiotrophoblast in human placenta and the labyrinth layer in the rat placenta

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[13–15] - 11β-HSD2 catalyzes the conversion of active glucocorticoids (cortisol in humans, corticosterone in rats) to their inactive 11-keto metabolites (cortisone and 11-dehydrocorticosterone, respectively) [14,16,17]. As a consequence, only up to 20% of maternal cortisol passes to the human fetus [18,19]. In contrast, the placental enzyme 11β–HSD1 converts inactive glucocorticoids to cortisol/corticosterone [14,20].

In humans, reduced placental 11β-HSD2 mRNA levels are found in pregnancies complicated by intrauterine growth restriction (IUGR) [21]. Rats exposed to dexamethasone (poorly catalyzed by 11β-HSD2) or to 11β-HSD inhibitors during the last third of pregnancy, are of low birth weight and develop hypertension and glucose intolerance in adulthood [22–25]. Furthermore, recent studies in animals and humans suggest a negative influence of glucocorticoid-induced fetal programming on offspring neurodevelopment and behavior [26,27]. Interestingly, prenatal stress was found to epigenetically regulate 11β-HSD2 gene expression in the rat placenta and brain [27].

So far, studies have assessed placental 11β-HSD2 activity indirectly via placental perfusion studies or via measurement of the respective hormone levels at the maternal and fetal side, mostly using immunoassays [16,28]. Recently, we have introduced liquid chromatography tandem mass-spectrometry (LC-MS/MS) as a reliable method for the combined quantification of steroids and corticotropin-releasing hormone (CRH) in human placental tissue [29]. CRH is an important regulator of placental 11β-HSD2 expression in humans [30], where the peptide hormone regulates the timing of birth via its interaction with progesterone [31]. Moreover CRH indirectly drives maturation of fetal organ systems via induction of adrenocorticotropic hormone (ACTH) release and subsequent fetal glucocorticoid production. This feedback loop is regulated by the transplacental transport of biologically active glucocorticoids, which is limited by 11β-HSD2 [32].

While only a few mass-spectrometric studies (GC-MS/MS) exist on the analysis of tissue (neuro-)steroids in the rat brain [33–35], the LC-MS/MS method has not been used in rat placental tissue, so far. Moreover, as indirect measurements of 11β-HSD2 substrate- and product-glucocorticoids in the placenta are only surrogates for its enzymatic activity, we set out to determine its direct enzymatic turnover in vitro via microsome preparation. Because the rodent glucocorticoid-excess model is frequently used to address questions of human fetal programming, we were interested in the placental 11β-HSD2 activity of the different species.

Typical assays of enzyme activity are difficult, since they commonly utilize radiolabeled steroid substrates [36,37] followed by separation techniques such as thin layer chromatography to remove unreacted substrate and liquid scintillation counting. Such approaches are time-consuming and impractical for high-throughput screening. Moreover handling and disposal of radioactive material require special licensing for their handling [38,39]. To address this problem, we have developed a sensitive LC-MS/MS method for assessing enzyme activity and show its utility to quantify enzyme activity for a variety of steroid substrates. The aim of our study is the introduction of the LC-MS/MS method as a novel, sensitive technique for the comparison of human and rat 11β-HSD-activities in placental tissue at term, offering the possibility of direct and indirect determination of glucocorticoid turnover rates along with the ability to study multiple steroids simultaneously.

2. Materials and Methods

2.1. Animals and surgical procedures

All procedures performed on animals were in line with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The *EU Directive 2010/63/EU* for animal experiments was followed. The experimental protocol was approved by the appropriate Institutional and Governmental Review Boards (Regierung von Mittelfranken, AZ #54-2531.31-12/06). Placental tissue was obtained from healthy rats at E21.5, as previously described in detail [40,41]. Briefly, time-mated pregnant Wistar rats were ordered form Charles River (Sulzfeld, Germany). Animals were housed under standard conditions with free access to standard chow (no. 1320; Altromin, Lage, Germany) and tap water. On day E21.5 of pregnancy, all dams were anesthetized by isoflurane inhalation and underwent Cesarean section. Rat brain hemispheres were obtained from adult rats, as a reference for CRH after sacrifice.

2.2. Sample collection and preparation

Rat placenta and brain were collected as described above. All collected tissues and tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until further usage. Healthy human placental tissue at term was obtained and processed immediately after birth, as previously described [42]. In short, after removal of decidua and fetal membranes placental tissue (~8 g per sample) was collected from three different sites with regard to their proximity to the umbilical cord to minimize sampling site influences. Samples were snap-frozen in liquid-nitrogen and stored at −80 °C until further use. For the LC-MS/MS measurement of tissue steroids along with CRH we used total rat placenta ($n = 6$, range 157–269 mg), total brain hemispheres ($n = 4$, range 299–532 mg) and fractions from each of the three samples from 5 human placentas (range 84–445 mg). For the measurement of 11β-HSD enzymatic activity, total rat placentas and a ~2.5 g fraction from each of the three samples from 5 human placentas was used.

Steroid and CRH measurements were conducted in rat and human placentae at term (range of gestational age 37.5–40.3 weeks, mean maternal age 35.3 years, all cesarean sections, 3 from male, 2 from female newborns). These time-points were chosen for a better comparison of our findings between species and the literature [13,14,43]. LC-MS/MS measurements were carried out using rat total placenta ($n = 6$), rat brain hemispheres ($n = 4$) and samples of human placenta ($n = 5$). Per 0.5 g of tissue, 1.02 ml of ethanol containing 20 µl/ml proteinase-inhibitor cocktail (cOMPLETE, Roche Diagnostics Deutschland GmbH, Penzberg, Germany) were added. Tissues were homogenized at 4 °C using a Precellys Ceramic Kit on a Precellys® 24 tissue grinder equipped with a Cryolys-module for liquid-nitrogen cooling (Peqlab, Erlangen, Germany). Conditions were 2 × 30 s at 224 × g for rat tissue and 6 × 30 s at 224 × g for human tissue with an inter-cycle pause of 40 s. Subsequently, samples were ultra-sonificated on ice (UW2070, Bandelin Electronic, Berlin, Germany) (settings: cycle 5, power 50%, 40 s). The homogenized samples were transferred into Eppendorf LoBind-tubes (Fisher Scientific GmbH, Schwerte, Germany). After centrifugation for 10 min (23,000 × g, 4 °C), supernatant was used for further analysis.

2.3. Microsomal preparation

Microsomes were prepared from total placentae of Wistar rats ($n = 6$) and humans ($n = 5$, with 3 samples from different sites of the placenta, which makes a total of 15 samples). All subsequent steps were performed at 0–4 °C. Tissues were homogenized in 0.01 M sodium phosphate buffer (pH 7.4) containing 0.25 M sucrose (Precellys® 24-Dual homogenizer, Peqlab, Erlangen, Germany). Homogenates were ultrasonificated, followed by filtration through shredder spin columns (QIAshredder homogenizer, QIAgen GmbH, Hilden, Germany) at 21,000 × g for 2 min. For further extraction,

lysates were differentially centrifuged using the method of Lakshmi and Monder [44]. Sequential centrifugation of the supernatants was performed (Centrifuge Universal 320R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany), each for 30 min at 750 × g and 20,000 × g. The last supernatant was ultracentrifuged (Optima™ L-80XP with SW 41 Ti rotor, Beckman Coulter Inc., Munich, Germany) at 105,000 × g for 60 min, washed with sucrose-phosphate buffer and centrifuged again with the same setting. Pellets were re-suspended in 0.1 M sodium phosphate buffer (pH 7.4) and stored at −70 °C until further use. For use in protein assays, microsomes were diluted to the concentration of 50 μg/mL with 0.1 M sodium phosphate buffer of the appropriate pH for either oxidation (pH 8.0) or reduction (pH 6.5) activity. Protein quantification was performed before every incubation experiment on a COBAS INTEGRA® 800 (Roche Diagnostics) by the turbidimetric method using benzethonium chloride for protein precipitation.

For enzymatic assays, incubation experiments were carried out in a Thermomixer comfort 5355 (Eppendorf AG, Wesseling-Berzdorf, Germany) that had been preheated to 37 °C. 11β-HSD2 catalyzed oxidation at C11 of cortisol or corticosterone was determined by quantifying the conversion to cortisone or 11-dehydrocorticosterone at pH 8.0 in the presence of β-NAD as co-substrate. Reduction catalyzed by 11β-HSD1 of cortisone to cortisol or corticosterone to 11-dehydrocorticosterone was determined at pH 6.5 using β-NADPH as co-substrate. Since pH and co-substrate were chosen based on the enzyme of interest, particular conversion rates of both isoforms of 11β-HSD could be determined separately. The substrates cortisol, cortisone, corticosterone and 11-dehydrocorticosterone were purchased from Sigma Aldrich (Sigma−Aldrich Chemie GmbH, Taufkirchen, Germany). The respective stock solutions (2.5 mM) were further diluted (total dilution 1/1000, v/v) with sodium phosphate buffer (100 mM, pH 8.0) for cortisol and corticosterone and sodium phosphate buffer (100 mM, pH 6.5) for corticosterone and 11-dehydrocorticosterone. 150 μL microsome suspension (~50 mg/L) were incubated at 37 °C with 50.0 μL substrate (2.50 μM) and 50 μL cosubstrate (50 mM) for a period of 90 min. Every 15 min equal amounts of reaction mixtures were precipitated in a prepared solution of zinc sulfate (25 g/L in methanol/water, 1/1, v/v) and internal standard (containing cortisol-d4, corticosterone-d8, 17-OH-progesterone and progesterone-d9). The samples were centrifuged at 23,000 × g for 10 min 120 μL of each resulting supernatant were transferred into a 96 well microtiterplate (Greiner MTP, polypropylene, Greiner Bio-One, Frickenhausen, Germany), where it was diluted with an equal volume of high-purity water. The plate was kept at 15 °C in the autosampler (CTC PAL - LC System, CTC Analytics, Zwingen, Switzerland) for further LC-MS/MS analysis (Triple-Quadrupol Mass spectrometer, API 4000 QTrap, Applied Biosystems, MDS Sciex, Darmstadt, Germany). Negative controls were generated using a solution of 150 μl sodium-phophate buffer 0.1 M instead of 150 μl microsomes, while substrate and cosubstrate were added in the same concentration used for the samples. Hence, negative controls served as internal controls of substrate concentrations. LC-MS/MS data analysis was performed using Analyst Software (Version 1.6.2, Applied Biosystems/MDS SCIEX, Darmstadt, Germany).

3. LC-MS/MS

LC-MS/MS was performed applying the method established in human placenta, as previously described by us in detail [29,45].

3.1. Statistical analysis

Data processing and graphic presentation were performed with Microsoft Excel 2010 (Microsoft, Bellevue, USA) and GraphPad Prism Version 4.0c (GraphPad Software, San Diego, CA, USA). Data are presented as mean ± standard error of the mean (SEM), unless otherwise stated.

3.2. Ethics

All participants gave their written informed consent with the approval by the Ethics Committee of the University of Erlangen-Nuremberg. All procedures were carried out in accordance with *The Code of Ethics of the World Medical Association* (Declaration of Helsinki) for experiments involving humans.

4. Results

4.1. Species-specific expression of CRH and steroids in human and rat term placenta

Table 1 a gives an overview of the measured CRH and glucocorticoid levels. Supplementary Table 1 additionally provides the levels of 11-deoxycortisol, 11-dehydrocorticosterone and the determined sex steroids. In contrast to the human placenta (Fig. 1a) and rat brain (used as a positive control for rat CRH, Fig. 1e) we did not detect CRH in the rat placenta (Fig. 1d). Interestingly, levels of CRH in the human placenta (Fig. 1a) were in the range of CRH levels obtained in rat brain hemispheres (Fig. 1e). Cortisol (F) and cortisone (E), as well as corticosterone (B) and 11-dehydrocorticosterone (A) were concomitantly expressed in the human term placenta (Fig. 1a) with both E/F- and A/B-ratios >1. Levels of corticosterone (B) in rat placenta exceeded the respective levels in the human placenta and the rat brain (Table 1 a). In contrast to human placenta (Fig. 1a), A/B was <1 in both rat placenta and brain hemispheres (Fig. 1d & e). Tissue 11-deoxycortisol expression was only detectable in human placenta, while below the limit of quantification (LLQ) in rat placenta and brain hemispheres (Supplementary Table 1). The tissue content of 11-deoxycorticosterone was similar in human and rat placentas (Supplementary Table 1s). With regard to sex steroids, rat placenta showed little progesterone and 17-OH-progesterone expression in contrast to human placenta, while tissue content of androstenedione and testosterone were at comparable levels (Supplementary Table 1). The steroid and CRH findings in rat placenta were additionally verified by mechanical vapor recompression of three pooled homogenates (data not shown).

4.2. Species-specific glucocorticoid turnover by placental 11β-HSD enzymes in vitro

Table 1 b gives an overview of 11β-HSD1 and 2 enzymatic activities in rat and human term placentas. Rat brain was not examined. The activity of 11β-HSD1 in human placental microsomes, as indicated by cortisone (E) and 11-dehydrocorticosterone (A) conversion rates (Fig. 1 b and c, respectively; Table 1 b) was low. Overall, the enzymatic turnover rate (ratio of 11β-HSD2/11β-HSD1) in human placenta was higher when cortisone (E)/cortisol (F) instead of corticosterone (B) /11-dehydrocorticosterone (A) were used as substrates (Fig. 1 b and c, respectively; Table 1 b), while the opposite effect was observed using rat microsomes (Fig. 1 f and g; Table 1 b). The 11β-HSD1 activity of human placental microsomes was ~1/50th (E and F) of the respective 11β-HSD2 turnover rate (Table 1 b), while rat microsomal 11β-HSD1 activity was ~2-fold (A and B) higher than the respective 11β-HSD2 activity (Table 1 b). Microsomal 11β-HSD2 turnover in human placenta (cortisol conversion) was ~44-times higher than in rat placenta (corticosterone conversion), while human 11β-HSD1 activity (cortisone

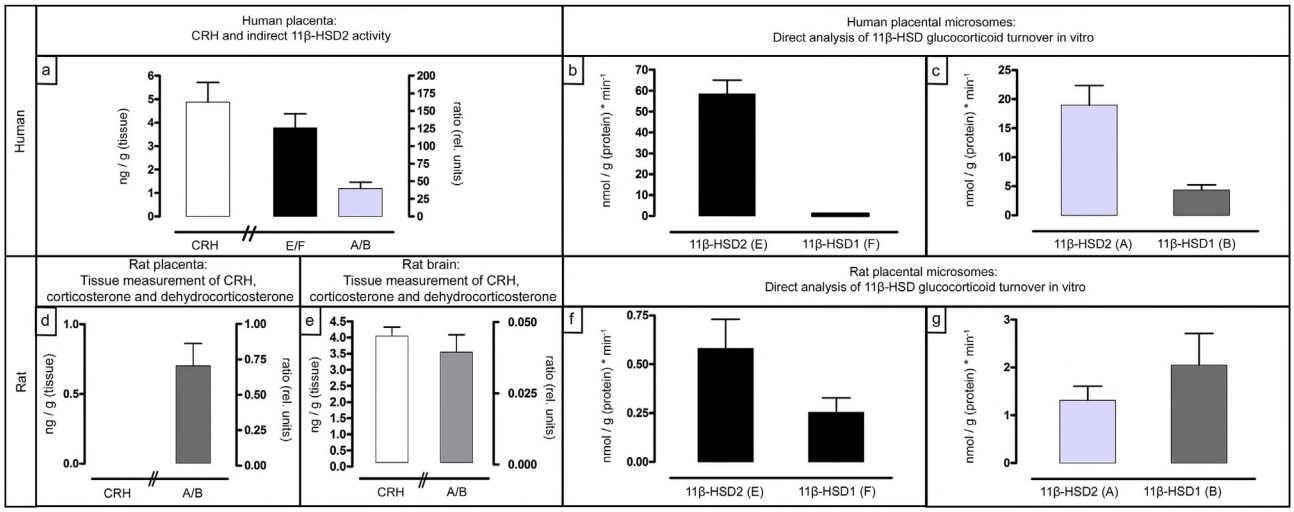

Fig. 1. Analysis of glucocorticoids and CRH in human placenta (a–c), as well as in rat placenta (d, f, g) and brain hemispheres (e) via LC-MS/MS: (a d, e) Determination of indirect 11β-HSD-activity: LC/MS–MS measurement of active-to-inactive glucocorticoid ratios and CRH (ng/g tissue). **//-** separation of y-axis (left-right) reference. (b, c, f, g) Determination of direct 11β-HSD-activity: In vitro LC/MS–MS measurement of microsomal cortisone (E) and cortisol (F), as well as 11-dehydrocorticosterone (A) and corticosterone (B) turnover rates (nmol/g (protein) x min^{-1}). Abbreviations: A- 11-dehydrocorticosterone, B- corticosterone, E-cortisone, F- cortisol, 11β-HSD- 11beta-hydroxysteroid dehydrogenase (enzymatic product in parenthesis).

Table 1
Overview of LC/MS–MS glucocorticoid and CRH measurements in human/rat placentae and rat brain hemispheres: a) Results of LC/MS- MS detection of tissue hormones. b) In vitro analysis of microsomal placental glucocorticoid turnover. Abbreviations: A- 11-dehydrocorticosterone, B- corticosterone, E-cortisone, F- cortisol, 11β- HSD- 11beta-hydroxysteroid dehydrogenase (enzymatic product in parenthesis), LLQ-lower limit of quantification, n.d.-not determined, SEM–standard error of the mean.

	Human placenta (n = 5) mean ± SEM	Rat placenta (n = 6) mean ± SEM	Rat brain hemispheres (n = 4) mean ± SEM
a)			
CRH (ng/g tissue)			
CRH	4.87 ± 0.84	< LLQ	4.01 ± 0.29
Glucocorticoids (nmol/g tissue)			
cortisol (F)	0.01 ± 0.001	< LLQ	< LLQ
cortisone (E)	0.72 ± 0.03	< LLQ	< LLQ
E/F	125.9 ± 19.99	–	–
corticosterone (B)	0.003 ± 0.001	0.20 ± 0.04	0.06 ± 0.002
11-dehydrocorticosterone (A)	0.09 ± 0.01	0.11 ± 0.021	0.002 ± 0.0002
A/B	39.67 ± 9.27	0.70 ± 0.16	0.04 ± 0.006
b)			
11βHSD-1 and 2 glucocorticoid turnover (nmol/g (protein)* min ˆ -1)			
11β-HSD2 (E)*	58.43 ± 6.59	0.58 ± 0.15	n.d.
11β-HSD1 (F)*	1.07 ± 0.22	0.25 ± 0.07	n.d.
11 β - HSD2/11 β - HSD1	185.9 ± 76.79	2.92 ± 0.78	n.d.
11β-HSD2 (A)*	18.96 ± 3.40	1.32 ± 0.29	n.d.
11β-HSD1 (B)*	4.36 ± 0.86	2.05 ± 0.66	n.d.
11 β - HSD2/11 β - HSD1	7.84 ± 2.97	0.92 ± 0.30	n.d.

* enzyme (product)

conversion) was ~1/2 of the respective rat enzyme (11-dehydrocorticosterone conversion) (Table 1 b). Noteworthy, cortisone (E) and cortisol (F) were poorer substrates of rat 11β-HSD-enzymes when compared to corticosterone (B) and 11-dehydrocorticosterone (A) turnover, and vice versa (Fig. 1 f & g; Table 1 b).

5. Discussion

Our study describes a new and sensitive non-radioactive method for carrying out 11β-HSD activity assays using liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS). Our new, non-radioactive, LC-MS/MS-based method of measuring enzyme activity obviates the need of radiolabeled substrates with following separation of the unreacted substrate and should increase the speed, precision, and reliability of activity measurements without sacrificing the sensitivity of radiodetection. It is suitable for high throughput analysis with the measurement at several time points. Moreover the assay can be easily adapted to different steroid substrates and products and allows the simultaneous measurement of substrate and product concentrations.

We measured tissue steroids and the peptide hormone CRH in human placenta and rat placenta and brain via LC-MS/MS, providing novel reference values for this species. Our main finding was that the ratio of inactive-to-active glucocorticoids (i.e. E/F-ratio in human placenta vs. A/B-ratio in rat placenta and rat brain) showed opposing results. In the human placenta inactive glucocorticoid levels were higher than in rat placentae and brain. In contrast, these tissues showed higher levels of active glucocorticoids. Moreover, CRH was undetectable in rat placenta, while

present in rat brain and the human placenta. Supportive of the findings from LC/MS–MS hormone measurement in placental tissue, our in vitro LC/MS–MS analysis of placental microsomal 11β-HSD activity indicated a higher activity of 11β-HSD2 activity in humans, yet a higher 11β-HSD1 turnover-rate in rats.

Our findings are in line with other studies by Waddell et al. [14] and Burton et al. [13], who examined the pattern of placental 11β-HSD2 expression during pregnancy and its bioactivity in the rat. They were able to identify species differences with respect to the pattern of placental 11β-HSD expression in the rat and the baboon (a model for human pregnancy) [43]: With advancing gestational age, the placental labyrinth zone of the rat shows a reduction of 11β-HSD2 expression paralleled by an increase in 11β-HSD1 expression (converting inactive 11-dehydrocorticosterone/cortisone into active corticosterone/cortisol). This finding indicates a reduction in the placental glucocorticoid barrier of the rat near term, while in contrast the placental barrier function of the baboon enhances in late pregnancy [46,47]. Our study is limited by the fact, that total rat placenta was subjected to LC-MS/MS analysis. Hence, our findings reflect overall rat placental steroid metabolism, as we did not differentiate between placental basal and labyrinth zone HSD-activities. Waddell et al. [14] found the gene expression of 11β-HSD2 in rats markedly diminished in trophoblast cells of the labyrinth zone from days E16 onwards, while maintained in the basal zone. Conversely, barely any 11β-HSD1 expression was detected in the labyrinth zone on day E16, but then significantly increased to day E22 [14]. The reason for this finding is still elusive, however, it was hypothesized that the observed species difference could have evolutionary evolved based on species-specific strategies for estrogen synthesis [14]. While in the rat the maternal estrogen surge near term is of ovarian origin [48], the baboon utilizes the placenta for estrogen synthesis mainly from fetal adrenal androgens (besides some de novo synthesis) [49]. The induction of fetal adrenal androgen production in the baboon is triggered by pituitary adrenocorticotropic hormone (ACTH) [43], which in turn is negatively controlled by the amount of transplacental passage of active glucocorticoids (regulated by 11β-HSD2). An ineffective placental glucocorticoid metabolism in late pregnancy could eventually result in a compromised synthesis of placental estrogen, which is vital for the promotion of changes in myometrium and cervix, which facilitate labor and delivery [43,50]. Third trimester human placental tissue levels of CRH, cortisol (F) and cortisone (E), as well as progesterone detected in our study, were in line with our previous LC-MS/MS findings in this tissue at term [29]. Future LC-MS/MS studies are needed for the evaluation of placental 11β-HSD2 function in early human and rat pregnancies, via the measurement of tissue steroids by LC-MS/MS. It also has to be noted that both our human and rat tissue samples were of male and female origin. This non-selective approach might have masked possible gender-specific differences in 11β-HSD activity levels [37]. We did not detect a measurable placental CRH peak in the rat by LC-MS/MS, while CRH was present in the human placenta and the rat brain. In line with our findings, Robinson et al. [51] demonstrated that CRH mRNA is species-specifically expressed in human, gorilla and rhesus monkey placentae, while expressed at a very low level, if at all, in rat placenta using northern blotting and radioimmunoassays of chromatographed fractions of rat placental extracts. In humans, CRH is an important regulator of placental progesterone production [52]. Although the rat placenta does not produce CRH, progesterone is detectable. This might point to regulators other than CRH in this species, as progesterone does have important effects on placental function in rats, most notably with respect to placental growth [53]. Moreover, progesterone inhibits 11β-HSD activity and is synthesized locally within the basal zone of the rat placenta [14].

Humans and many other higher mammalian species show an adrenal secretion of both cortisol and corticosterone, while some rodents, birds and other species secrete only corticosterone [54]. The role of this dual secretion in humans is not fully understood by now. We found cortisol and corticosterone in human placenta. While corticosterone was long considered to be a secondary glucocorticoid in humans, there is growing evidence that corticosterone may have additional roles and a physiology on its own (reviewed by Ref. [55]). Interestingly, the K_m of corticosterone is much lower for both 11β-HSD2 (10-fold) and 11β-HSD1 (25-fold) dehydrogenase activity, compared to that for cortisol. Similarly, the K_m of 11-dehydrocorticosterone for 11β-HSD1 reductase is below the respective K_m of cortisone [55–57]. Hence, compounds derived from corticosterone metabolism might act as effective competitive inhibitors of 11β-HSD activity [58]. This effect is observed in Addisonian patients where corticosterone significantly induces sodium retention and potassium secretion contributing to an increased blood pressure via inhibiting the rate of deactivation of cortisol by 11β-HSD isoforms [55]. The role of dehydroxylated derivatives for the feto-maternal unit in pregnancy and their cross-talk with 11β-HSD-function is yet to be examined.

Taken together, we were able to characterize the indirect and direct placental 11β-HSD function and the placental steroid profile in rats. Interestingly, we found relevant species-specific differences in comparison to human placenta, which have to be taken into account when using this model to address questions regarding human glucocorticoid-excess and subsequent fetal programming.

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2015.11.009.

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