—Full Paper—

Involvement of 20 α -Hydroxysteroid Dehydrogenase in the Maintenance of Pregnancy in Mice

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Abstract. The enzyme 20 α -hydroxysteroid dehydrogenase (20 α -HSD) catabolizes progesterone into a biologically inactive steroid, 20 α -dihydroprogesterone (20 α -OHP). In the corpora lutea of rats and mice, 20 α -HSD is considered to be involved in functional luteolysis. It is also distributed in other tissues including the placenta, endometrial epithelia and fetal skin, although the roles it plays in these tissues remain to be elucidated. In the present study, we investigated the role of 20 α -HSD in the maintenance of pregnancy using mice with targeted disruption of the 20 α -HSD gene. We first confirmed that the number of pups was significantly smaller in 20 α -HSD^{-/-} pairs than in 20 α -HSD^{+/+} pairs. We then mated 20 α -HSD^{+/+} males and females so that each pregnant female produced 20 α -HSD^{+/+}, 20 α -HSD^{+/-} and 20 α -HSD^{-/-} offspring. The genotype ratio of the offspring did not match the Mendel's law of inheritance, and the numbers of 20 α -HSD^{+/+} and 20 α -HSD^{-/-} offspring were smaller than expected values. Although the genotype ratio of fetuses on days 13, 15 and 18 of pregnancy matched the Mendel's law, the total number of fetuses on day 18 was significantly smaller than that on day 13, suggesting that fetal loss occurred during late pregnancy. Next, we transferred 20 α -HSD^{+/+} embryos to 20 α -HSD^{+/+} or 20 α -HSD^{-/-} females and found that the number of offspring was significantly smaller in 20 α -HSD^{-/-} dams than in 20 α -HSD^{+/+} dams. Expression of 20 α -HSD mRNA in the fetus, placenta and uterus progressively increased from day 11 to 18 of pregnancy. In addition, concentrations of progesterone were significantly higher in the 20 α -HSD^{-/-} fetuses than in the 20 α -HSD^{+/+} fetuses, while those of 20 α -OHP were lower in the 20 α -HSD^{-/-} fetuses than in the 20 α -HSD^{+/+} fetuses. These results suggest that both maternal and fetal 20 α -HSD play a role in maintaining normal pregnancy at least partially by reducing progesterone concentrations in fetuses.

Key words: Fetus, 20 α -hydroxysteroid dehydrogenase, Placenta, Pregnancy, Progesterone

(J. Reprod. Dev. 54: 408–412, 2008)

The steroid-metabolizing enzyme 20 α -hydroxysteroid dehydrogenase (20 α -HSD) catalyzes the reaction that converts progesterone to its biologically inactive metabolite, 20 α -dihydroprogesterone (20 α -OHP). Expression of 20 α -HSD in the corpus luteum is supposed to play a crucial role in recurrence of short cycles through promotion of functional luteolysis in rats and mice [1–3]. During pregnancy when high levels of progesterone are required, 20 α -HSD activity in the corpus luteum is suppressed by prolactin [4, 5] and placental lactogens [6] in early and mid-late pregnancy, respectively, in these rodent species. Due to the detrimental effect of 20 α -HSD on luteal progesterone secretion, the corpus luteum of pregnancy does not express 20 α -HSD until 24 h before parturition [7]. It has been demonstrated that the rapid appearance of luteal 20 α -HSD activity in the corpus luteum is due to the increase in 20 α -HSD gene expression and not to activation of an already existent enzyme [8]. Previously, 20 α -HSD gene-deficient strains of mice have been generated, and mice deficient in 20 α -HSD sustain high progesterone levels and exhibit delayed parturition [3, 9] as well as prolonged continuous diestrous days during the estrous cycle and pseudopregnancy [3].

Other than the corpus luteum, 20 α -HSD is also distributed in a

variety of tissues including the placenta [10–12], endometrial epithelia and fetal skin [13]. Although the roles it plays in these tissues remain to be elucidated, the importance of 20 α -HSD in fetal survival and/or maintenance of pregnancy have been suggested. For instance, we have previously reported localization of 20 α -HSD mRNA in endometrial epithelial cells, maternal placental endothelial cells and fetal epidermal cells in the mid to late stages of pregnancy [13]. It has also been previously reported that both the mRNA and enzyme activity of 20 α -HSD were detected in the placenta and uterus during mid to late pregnancy and that progesterone concentrations in fetal serum and amniotic fluid remained extremely low throughout pregnancy in goats [14]. In addition, the number of pups in another study, especially live pups, from 20 α -HSD^{-/-} pairs was markedly smaller than that from 20 α -HSD^{+/+} pairs [3], suggesting the physiological importance of 20 α -HSD in normal fetal development and/or successful parturition.

Despite the indispensable roles of progesterone in reproductive functions, it is interesting to know that progesterone exerts detrimental effects on several cell functions. For example, progesterone has inhibitory effects on the proliferation of several cell types [15]. It has been shown that a high concentration of progesterone inhibits the proliferation of embryonic thymocytes [16]. In addition, injection of a large quantity of progesterone into the amniotic sac has been shown to cause fetal death in mice [17]. Together, these

observations raise the notion that 20 α -HSD expressed in the placenta, pregnant uterus and fetal tissue may be involved in protecting developing fetuses from high levels of progesterone, which is a prerequisite for maintenance of pregnancy by inhibiting uterine contraction. In this study, we investigated whether maternal and/or fetal 20 α -HSD are indeed involved in the maintenance of pregnancy using 20 α -HSD-deficient mice we generated in a previous study [3].

Materials and Methods

Experimental animals

The 20 α -HSD^{+/-} mice [3] were backcrossed to C57BL/6J strain mice for more than nine generations. They were then intercrossed with each other to obtain littermates of each genotype for the present experiments. The mice were kept under controlled lighting conditions with a 12 h light/12 h dark cycle (lights on 0700–1900 h) at a temperature of 23 C. Food and water were available *ad libitum*. Each female was housed with a male, and the day vaginal plug formation was observed was designated as day 0 of pregnancy. The experiments were conducted according to the Guidelines for the Care and Use of Laboratory Animals, Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Genotyping of mice

All experimental animals were genotyped by PCR using genomic DNA as a template. Genomic DNA was extracted from mice tails on day 26 after birth. The forward primer sequences to determine mutant type and wild type were neoPGK-F, 5'-ATC TGC ACG AGA CTA GTG AGA CGT-3', and int4/5-F, 5'-CAG GAT CAT CTC CAG TTG TCT ACG-3', respectively. The reverse primer sequence was E5 Revs-2, 5'-ATG CAG TCA TGG TCT CTC ACT AGG-3'. PCR was performed using 3 min for denaturation at 94 C followed by 35 cycles at 94 C for 30 sec, 62 C for 1 min and 72 C for 1 min, with a final extension at 72 C for 10 min in a RED Extract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO, USA). PCR products were electrophoresed on 1% agarose (SeaAG, Vero Beach, FL, USA) gel, and the gels were stained with ethidium bromide solution and visualized with ultra violet light.

Embryo transfer

Superovulation was induced in C57BL/6 wild-type females by injecting 5 IU equine chorionic gonadotropin (eCG) followed by 5 IU human chorionic gonadotropin (hCG) 48 h later, and the females were then mated with wild-type males. Embryos were collected from oviducts 16 h after hCG injection, and fifty embryos were transferred into the oviducts of each pseudopregnant recipient 20 α -HSD^{+/+} or 20 α -HSD^{-/-} mouse. The numbers of live fetuses and newborns were counted on day 17 of pregnancy and the day of birth, respectively.

Preparation of tissue samples and total RNA

The mice were sacrificed by cervical dislocation between 0900 and 1200 h on days 11, 13 and 18 of pregnancy. For total RNA extraction, the fetus, placenta, uterus and ovary were separated, snap-frozen in liquid nitrogen and stored at -80 C until use. Total

RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Briefly, tissue samples were homogenized in 5 volumes of TRIzol reagent, incubated at room temperature for 5 min, vortexed with 1/5 volumes of chloroform and centrifuged to collect the aqueous phase. Total RNA was precipitated with isopropyl alcohol and dissolved in RNase-free water.

Reverse transcription-polymerase chain reaction (RT-PCR)

For RT-PCR analysis, each total RNA sample (1–2 μ g) was reverse transcribed into cDNA at 42 C for 60 min using oligo (dT) and SuperScript II (Invitrogen). Two microliters of the cDNA was then subjected to PCR. Each 20 α -HSD and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) cDNA was amplified in 50 μ l reactions using 5 U of TaKaRa Taq (Takara Bio, Ohtsu, Japan) in PCR buffer with a final concentration of 0.2 mM dNTP and 0.2 μ M each primers. For 20 μ -HSD, the forward primer was 5'-TTT GAC ACA GTG GAT CTC TC-3' and the reverse primer was 5'-TAG CCA AGA GTT TCA ATG AGG-3'. These primers amplified a 376 bp PCR product. The PCR cycle profile was as follows: 1 cycle at 94 C for 5 min followed by 22 cycles at 94 C for 1 min, 60 C for 30 sec, 72 C for 1 min and a final cycle at 72 C for 10 min. For GAPDH, the forward primer was 5'-GAT TGT TGC CAT CAA GGA CCC CTT-3' and the reverse primer was 5'-ACT CAG CAC CAG CAT CAC CCC ATT-3'. These primers amplified a 177 bp PCR product. The PCR cycle profile was as follows: 1 cycle at 94 C for 3 min followed by 20 cycles at 95 C for 30 sec, 60 C for 30 sec, 72 C for 1 min and a final cycle at 72 C for 10 min.

Determination of progesterone and 20 α -OHP

Whole fetuses obtained on day 17 of pregnancy were weighed, minced, and homogenized in 5 volumes (v/w) of phosphate-buffered saline (PBS). This homogenate preparation procedure was performed at 4 C. The samples for radioimmunoassay (RIA) were extracted from the fetal homogenates with diethyl ether. The concentrations of progesterone and 20 α -OHP were measured by RIA using specific antibodies as described previously [18]. Briefly, the samples were incubated with radiolabeled progesterone or 20 α -OHP and the respective antibodies for 14 h at 4 C. Free and bound steroids were separated with dextran-coated charcoal. After centrifugation, the supernatants were analyzed by scintillation spectrometry. Concentrations were calculated according to the respective standard curves.

Statistics

Statistical analysis was conducted using ANOVA followed by Student's *t*-test or Tukey HSD test and using the χ^2 -test. Differences at *P* < 0.05 were considered to be statistically significant.

Results

Number of pups from 20 α -HSD-deficient mice

First, 20 α -HSD^{-/-} females were crossed with 20 α -HSD^{-/-} males, and the number of offspring was compared with that from 20 α -HSD^{+/+} females crossed with 20 α -HSD^{+/+} males. The number of offspring from 20 α -HSD^{-/-} pairs (1.0 $\pm$ 0.4, mean $\pm$ SE, *n*=8) was

Fig. 1. The numbers of live fetuses of each genotype (open column, 20α -HSD^{+/+}; grey column, 20α -HSD^{+/-}; closed column, 20α -HSD^{-/-}) per litter on days 13 (A), 15 (B) and 18 (C) of pregnancy from 20α -HSD^{+/-} pairs. The data represents means $\pm$ SEM (n=5).

Fig. 2. The number of pups on the day of birth obtained following transfer of 20α -HSD^{+/+} embryos to 20α -HSD^{+/+} females (open column, n=8) or 20α -HSD^{-/-} females (closed column, n=8). The number of fetuses on day 17 of pregnancy is also presented (gray column, n=3). The data represents means $\pm$ SEM. *P<0.05 vs. 20α -HSD^{+/+}.

significantly smaller than that from 20α -HSD^{+/+} pairs (9.8 ± 0.4 , n=8). Next, we mated 20α -HSD^{+/-} males and females so that each pregnant female produced 20α -HSD^{+/+}, 20α -HSD^{+/-} and 20α -HSD^{-/-} offspring. The total numbers of pups of each genotype from the 22 dams were 50 for 20α -HSD^{+/+}, 63 for 20α -HSD^{+/-} and 18 for 20α -HSD^{-/-}, respectively. The mean litter size was 6.0 ± 0.4 (n=22). The ratio of each genotype (20α -HSD^{+/+}: 20α -HSD^{+/-}: 20α -HSD^{-/-}) was 1:1.3:0.4, which did not match the expected ratio (1:2:1) based on Mendel's laws of inheritance ($\chi^2=15.82$, P<0.001). To investigate when the loss of 20α -HSD^{+/-} and 20α -HSD^{-/-} fetuses occurred during pregnancy, the ratios of each genotype of live fetuses were examined on days 13, 15 and 18 of pregnancy. Although dead fetuses were occasionally observed, the number of dead fetuses was not included in this study. On all the days examined, the ratio of 20α -HSD^{+/+}: 20α -HSD^{+/-}: 20α -HSD^{-/-}

Fig. 3. Expression of 20α -HSD (upper) and GAPDH (lower) mRNA in fetuses, uteri, placentae and ovaries from 20α -HSD^{+/+} mice on days 11, 13 and 18 of pregnancy.

Fig. 4. Progesterone and 20α -OHP concentrations of 20α -HSD^{+/+} (open column) and 20α -HSD^{-/-} fetuses (closed column) on day 17 of pregnancy. The data represents means $\pm$ SEM (n=4). *P<0.05 vs. 20α -HSD^{+/+}.

fetuses was not significantly different from the expected ratio (Fig. 1). The total numbers of fetuses on days 13, 15 and 18 were 9.8 ± 0.4 , 9.2 ± 0.6 and 6.8 ± 0.9 (n=5 for each day), respectively, and the number of fetuses on day 18 was significantly smaller than that on day 13 of pregnancy.

Number of offspring obtained following embryo transfer

The influence of maternal 20α -HSD on fetal survival was investigated by embryo transfer of wild type embryos to 20α -HSD^{-/-} mice. As shown in Fig. 2, the number of pups obtained from 20α -HSD^{-/-} dams on the day of parturition was significantly smaller than that obtained from 20α -HSD^{+/+} dams. The significant decrease in fetal number was already evident on day 17 of pregnancy.

Expression of 20α -HSD mRNA in fetuses and reproductive tissues

The expressions of 20α -HSD mRNA in the fetus, uterus, placenta and ovary were determined on days 11, 13 and 18 of pregnancy by RT-PCR. As illustrated in Fig. 3, 20α -HSD mRNA was already discernible on day 11 in the fetus, uterus and placenta, and gradually increased thereafter during pregnancy. In the ovary, however, strong expression of 20α -HSD mRNA was only observed just before parturition (day 18 of pregnancy).

Progesterone and 20 α -OHP levels in fetuses

To determine the role of extraovarian 20 α -HSD in modulating progesterone concentrations in the fetus, we measured the progesterone and 20 α -OHP concentrations in fetal homogenates on day 17 of pregnancy. As shown Fig. 4, the progesterone concentrations were significantly higher in the 20 α -HSD^{-/-} fetuses than in the 20 α -HSD^{+/+} fetuses, while the 20 α -OHP levels were lower in the 20 α -HSD^{-/-} fetuses than in the 20 α -HSD^{+/+} fetuses.

Discussion

In the present study, the number of offspring from 20 α -HSD^{-/-} pairs was significantly smaller than that from 20 α -HSD^{+/+} pairs, confirming our previous observation [3]. To determine whether maternal or fetal 20 α -HSD participates in maintaining normal pregnancy, we mated 20 α -HSD^{+/-} males and females and found that the genotype ratio of offspring delivered from 20 α -HSD^{+/-} pairs did not match the Mendelian ratio. The mean litter size was 6.0 ± 0.4 , which was significantly smaller than that of wild-type pairs (9.8 ± 0.4), and the numbers of 20 α -HSD^{+/-} and 20 α -HSD^{-/-} offspring were smaller than the expected values. These observations indicate that fetal 20 α -HSD plays at least a partial role in preventing fetal loss. Next, we investigated when fetal loss occurred by genotyping fetuses on days of 13, 15 and 18 of pregnancy. Although the numbers of 20 α -HSD^{+/-} and 20 α -HSD^{-/-} fetuses tended to decrease along with the number of days, the genotype ratios of the fetuses on all the days examined were not significantly different from the expected ratio (20 α -HSD^{+/+}:20 α -HSD^{+/-}:20 α -HSD^{-/-}=1:2:1). The total number of fetuses on day 18 of pregnancy was significantly smaller than that on day 13, indicating that fetal loss occurred during late pregnancy. It is therefore suggested that fetal 20 α -HSD is an important enzyme for maintaining successful pregnancy during the late phase.

In this study, we also investigated the contribution of maternal 20 α -HSD in maintaining pregnancy by transfer of 20 α -HSD^{+/+} embryos to 20 α -HSD^{+/+} and 20 α -HSD^{-/-} dams. We found that the number of pups delivered was significantly smaller in the 20 α -HSD^{-/-} dams than in the 20 α -HSD^{+/+} dams. When examined on day 17 of pregnancy, the number of fetuses was already significantly decreased and no dead fetuses were observed, indicating that fetal loss occurred in a relatively early phase of pregnancy. This suggests that maternal 20 α -HSD, probably uterine 20 α -HSD, is also involved in maintaining successful pregnancy during earlier phases. The possibility, however, that uterine 20 α -HSD plays a role in successful implantation also cannot be ruled out in the present study.

Strong expression of 20 α -HSD mRNA in the ovary was only detected at term (day 18), while 20 α -HSD mRNA expression was already detected on day 11 of pregnancy in the fetus, uterus and placenta, and the expression levels gradually increased with the progress of pregnancy. These observations support our previous histological study in mice using an in situ hybridization method for detection of 20 α -HSD mRNA [13]. In addition, we have observed that 20 α -HSD mRNA is expressed in the placenta and uterus during mid to late pregnancy in goats [14]. It has been also shown that placental 20 α -HSD increases 5-fold between mid and late preg-

nancy in humans [10, 19, 20]. Since fetal 20 α -HSD seems to play roles in fetal survival during mid to late pregnancy as mentioned above, the 20 α -HSD expressed in the fetus and placenta may be involved in protecting the developing fetus from the detrimental effect of progesterone during these phases of pregnancy. On the other hand, uterine 20 α -HSD may play roles in successful implantation and/or proliferation of endometrial epithelial cells during early to mid pregnancy, since maternal 20 α -HSD seems to be more important during earlier phases of pregnancy than fetal 20 α -HSD as mentioned above. Since progesterone is known to inhibit estrogen-induced proliferation of endometrial epithelial cells [21, 22], uterine 20 α -HSD during the late phase of pregnancy may also contribute to proliferation of these cells during late pregnancy, thereby enabling physical extension of the uterine lumen of the pregnant uterus. Thus, although it appears paradoxical that the enzyme that inactivates progesterone is expressed in the pregnant uterus, contraction of which needs to be suppressed by high levels of progesterone during pregnancy, uterine 20 α -HSD appears to have roles throughout pregnancy.

Although the progesterone concentrations in maternal serum are high during pregnancy, those in the amniotic fluid are very low in rats [11] and goats [14]. Since it was difficult to collect amniotic fluid from the mice, we determined the progesterone and 20 α -OHP levels in homogenates of whole fetuses on day 17 of pregnancy in the present study. We found that the progesterone concentrations were significantly higher in the 20 α -HSD^{-/-} fetuses than in the 20 α -HSD^{+/+} fetuses, while the 20 α -OHP levels were low in the 20 α -HSD^{-/-} fetuses, and thus the progesterone/20 α -OHP ratio was much higher in the 20 α -HSD^{-/-} fetuses than in the 20 α -HSD^{+/+} fetuses. This observation provides direct evidence regarding the physiological significance of 20 α -HSD in lowering progesterone levels in fetuses. The presence of 20 α -HSD has also been demonstrated in the placentae of primates, including baboons [23], rhesus monkeys [23] and humans [10, 19, 20], and a decrease in the fetal concentrations of progesterone and/or an increase in the fetal concentrations of 20 α -OHP during late pregnancy has previously been reported in these primate species [24–26]. Because progesterone secretion into the maternal compartment remains elevated until after parturition in primates, it has been postulated that local withdrawal of progesterone within the fetal compartment may minimize the detrimental effects of progesterone [27]. These reports are consistent with the notion that increased activity of 20 α -HSD in the placenta during late pregnancy is important for fetal development and/or successful parturition.

In conclusion, the results of the experiments in the present study suggest that an increase in the fetal progesterone concentration causes fetal loss during mid to late pregnancy probably due to its direct cytotoxic effects on proliferating cells within the developing fetus. We also demonstrated that both maternal and fetal 20 α -HSD plays important roles in fetal survival by preventing the detrimental effects of progesterone on fetuses. Although the importance of 20 α -HSD in ovarian tissue for functional luteolysis is rather specific to rodent species such as rats and mice, the physiological significance of 20 α -HSD in extraovarian tissues for fetal development and parturition may be common among mammalian species including primates.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (18658113) from the Japan Society for the Promotion of Science.

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