Ontogeny of estrogen sulfatase activity in ovine fetal hypothalamus, hippocampus, and brain stem

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Purinton, Scott C., Howard Newman, Maria I. Castro, and Charles E. Wood. Ontogeny of estrogen sulfatase activity in ovine fetal hypothalamus, hippocampus, and brain stem. Am. J. Physiol. 276 (Regulatory Integrative Comp. Physiol. 45): R1647–R1652, 1999.—Ovine parturition is initiated by increases in fetal hypothalamus-pituitary-adrenal (HPA) axis activity, which in turn increase placental estrogen biosynthesis and ultimately increase uterine contractility. In addition to the action in the uterus, estrogens augment fetal ACTH secretion. In late gestation, estrone sulfate is more abundant in fetal plasma than is unconjugated estrone. We studied hypothalami, hippocampi, and brain stem tissue from fetal, neonatal, and adult sheep to test the hypothesis that the ovine brain contains estrogen sulfatase activity. We found that the activity in the hippocampus was significantly increased in late-gestation fetuses compared with both younger and older animals. No significant change in either hypothalamus or brain stem was revealed; however, the activity in all brain areas was high. Immunohistochemistry revealed the presence of estrogen sulfatase in the paraventricular nucleus of the hypothalamus, the nucleus of the solitary tract, and the rostral ventrolateral medulla. We conclude that ovine fetal hypothalamus, hippocampus, and brain stem contain estrogen sulfatase activity and that the activity in the hippocampus is developmentally regulated.

steroid; estrone; development; sheep; labor; parturition; central nervous system

IN OVINE PREGNANCY, developmental changes in the synthesis of estrogens and progesterone are important for the initiation, maintenance, and spontaneous termination of the pregnancy. Our interests have focused on the mechanisms controlling spontaneous parturition. In the ovine fetus, parturition is initiated by an increase in the activity of the fetal hypothalamus-pituitary-adrenal (HPA) axis (12). The resultant increase in fetal plasma cortisol induces the activity of cytochrome P-450c11 in the placenta (14). This enzyme has both 17α-hydroxylase and 17,20-lyase activity; induction of this enzyme allows proportionately more estrogen and proportionately less progesterone biosynthesis. The increase in the so-called “estrogen-to-progesterone” ratio, in plasma and locally within the uterine tissues, allows increased uterine contractility (12). It is the increased uterine tone that initiates labor and delivery of the fetus.

We have recently demonstrated that physiological increases in fetal plasma estrogen concentrations greatly augment fetal ACTH secretion (20). This effect of estrogen can be demonstrated on both basal and hypotension-stimulated fetal ACTH secretion. Although estrogen has a potentially important effect on fetal ACTH secretion, it is well known that fetal plasma concentrations of unconjugated estrogens increase only after the beginning of the increase in fetal plasma ACTH and cortisol (16, 23). Conjugated estrogens, mostly estrone sulfate, circulate in high concentrations compared with unconjugated estrogens (2, 24). The concentration of estrone sulfate increases before the increase in fetal HPA axis activity (16). However, conjugated steroids cannot bind to nuclear receptors unless deconjugated (5). It is possible that estrone sulfate is converted to estrone locally within the fetal brain and that the circulating estrone sulfate acts as a reservoir for estrone acting within the fetal brain.

Estrogen sulfatase activity has been reported in brain tissue from adult sheep (9, 15), rats (3, 7), primates (10), and humans (17). Activity of this enzyme has not been investigated in fetal sheep. We hypothesized that estrogen sulfatase activity could be demonstrated in hypothalamus, hippocampus, and brain stem of fetal sheep and that the activity changes as a function of gestational age. We further hypothesized that estrogen sulfatase would be present throughout the final trimester of fetal development, as well as in the postnatal animal, in brain regions relevant for HPA axis control. The experiments reported in this paper were designed to test these hypotheses.

MATERIALS AND METHODS

Enzyme activity. We studied fetuses (86–147 days gestation, term = 147 days), four lambs (3–4 wk old), and four adult nonpregnant ewes to determine estrone sulfatase activity. The sheep were killed with an intravenous overdose of pentobarbital sodium. Gestational ages of the fetal sheep were calculated from known breeding dates. Whole brains were rapidly removed, dissected into discrete regions, and quickly frozen on dry ice or in a slurry of dry ice and acetone. All tissues were stored at −20 or −40°C until studied.

Hypothalami, brain stems, and hippocampi were then processed to determine estrone sulfatase activity. Each tissue sample was homogenized in medium 199 (Sigma, St. Louis, MO) containing 25 mM HEPES. Homogenization was performed using a Polytron homogenizer (Tekmar, Cincinnati, OH). The concentration of each tissue in the homogenate was 0.5 g tissue in 5 ml medium.

Tissues were centrifuged at 1,200 rpm for 5 min; supernatant was then collected and assayed immediately. A sample of each homogenate was assayed for protein concentration according to the method of Bradford (1) with a commercially available assay kit (Bio-Rad Laboratories, Hercules, CA).

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Homogenate (0.1 ml) was aliquoted in duplicate into borosilicate tubes (16 × 150 mm) containing 0.8 ml of a mixture of [6,7-3H]estrone sulfate (DuPont-NEN, Wilmington, DE) and unlabeled estrone sulfate (E1SO4; Sigma). All reactions were run at 37°C. Reactions were terminated by immediate cooling on ice, addition of 5 volumes of ethyl acetate:hexane (3:2), and vigorous mixing for 30 s. The aqueous phase was frozen by submersion of the reaction tube into a dry ice and acetone slurry. Subsequently, the organic phase containing the [3H]estrone was decanted into 13 × 75-mm borosilicate glass tubes and dried under a gentle stream of room air. Dried extracts were reconstituted in scintillant (Cytoscint; ICN, Costa Mesa, CA) and counted in a scintillation counter (LKB, Gaithersburg, MD).

Enzyme activities at different developmental ages and in different tissues were measured using a substrate concentration of 3 µM and [3H]estrone sulfate specific activity of ~0.67 µCi/nmol. For this experiment, reactions were allowed to run for 5 min. Under these conditions, <20% of the substrate was converted to [3H]estrone.

Comparison of relative activities at different developmental ages was achieved with one-way ANOVA. A posteriori comparison of individual mean values was performed with Newman-Keuls multiple range test (26). Comparison of two means was performed with Student’s t-test (27). All statistical computations were performed with SigmaStat (Jandel Scientific, San Rafael, CA).

Western blotting. Hypothalami and brain stems were harvested from fetuses, lambs, and adults of known gestational and postnatal ages. The number and ages of animals varied slightly between hypothalami and brain stem, but 11–12 fetuses, 3–4 lambs, and 2 adults were used per tissue type. These tissues were originally obtained and homogenized for other studies (19). Unfortunately, hippocampi from these animals were not available. All tissue was homogenized in reducing buffer and boiled for 5 min. The samples were centrifuged to remove particulate matter, and supernatant was recovered. Protein concentrations were obtained with the use of the Bradford technique (1). Western blots were performed with a mini-Protein electrophoresis system (Bio-Rad) on 10% precast polyacrylamide gels purchased from Bio-Rad Laboratories. Samples were diluted so that an equal amount of protein was loaded per lane (20 µg for brain stem and 40 µg for hypothalami). The protein was then transferred to a nitrocellulose membrane and probed for estrogen sulfatase with a custom-made rabbit polyclonal antibody (Alpha Diagnostic, San Antonio, TX). The peptide sequence used from the human sulfatase gene, amino acids 294–309, was NH2-FSFKDFAGKSQHGVYC-COOH (21). Primary antibody was diluted to a concentration of 1:1,000 in antibody diluent (1% BSA in PBS with 0.05% Tween 20). Visualization of the protein-antibody complex was accomplished with a chemiluminescence detection system (Renaissance; DuPont-NEN, Boston, MA) and analyzed by densitometry (Bio-Rad). Antibody specificity was confirmed by preabsorption of the primary antibody with a peptide (1 µg/ml), also supplied by Alpha Diagnostic. Developmental changes were calculated with multiple linear regression to control for differences between gel running conditions (SigmaStat).

Immunohistochemistry. Fetal brains were perfusion-fixed with 4% paraformaldehyde, dissected, and cut into gross tissue regions (hypothalamus, midbrain, pons, medulla, spinal cord, etc.). Tissue was processed for embedding by dehydration with progressively increasing concentrations of ethanol followed by xylene. All tissue was embedded in paraffin and cut into 10-µm sections with a Zeiss microtome. Sections were mounted on poly-L-lysine slides, deparaffinized with xylene, and rehydrated in decreasing concentrations of ethanol. Immunohistochemistry and visualization were made possible with a Histostain-SP kit from Zymed and a metal-enhanced diaminobenzidine (Pierce, Rockford, IL). Sections were stained for estrogen sulfatase with the same custom-made antibody used for Western blotting. Primary antibody was diluted to a concentration of 1:500 in antibody diluent (1% BSA in PBS 0.01% with Triton X-100). Specific staining was confirmed by dilution tests; staining decreased as primary antibody was further diluted. Specific staining was absent after primary antibody was replaced with 10% normal goat serum. All slides were dehydrated before being mounted on coverslips with Permount (Fisher Scientific, Pittsburgh, PA).

RESULTS

Enzyme activity. Estrogen sulfatase activity was measurable in ovine fetal, neonatal, and adult hypothalamus, hippocampus, and brain stem (Fig. 1). The activity in the hippocampus was significantly increased in late-gestation fetuses compared with younger fetuses, lambs, and adult ewes as tested by ANOVA and Newman-Keuls multiple range test (n = 3 or 4/group; P < 0.01). The activity in hypothalamus appeared to decrease in more mature animals, but this was not statistically significant, possibly caused in part by insufficient numbers in each group. The activity in brain stem was highly variable and overall did not change as a function of age.

As shown in Fig. 2, the activity of the estrogen sulfatase within these brain regions is very high, even compared with ovine myometrium. When compared by Student’s t-test, the activity in ovine adult hypothalamus was significantly different from the activity in ovine myometrium (n = 3/group; P < 0.001).

Western blotting. For Western blotting, tissues were distributed among gels so that each gel contained a subset of the developmental ages studied. For brain stem, 12 fetuses, 3 lambs, and 2 adults were used. The developmental ages were as follows: fetuses 90–110 days (n = 3), 119–129 days (n = 4), 134–138 days (n = 3), and 140–147 days old (n = 2); lambs 1–21 days old (n = 3); and adult ewes (n = 2). Western blots revealed three distinct bands at 66, 45, and 30 kDa. The 66-kDa band is shown in Fig. 3 and corresponds to the correct molecular mass of 65,492 Da for estrogen sulfatase (22). As shown by Fig. 3, there are slight molecular mass variations of the 66-kDa band for ovine brain stem. Preabsorption of the enzyme revealed the 66-kDa band to be specific, whereas the lower molecular mass bands were shown to be nonspecific. Multiple linear regression did not reveal the 66-kDa band to vary between groups. Figure 4A shows group means ± SE measured in relative optical density across developmental age for the 66-kDa band present in brain stem.

For hypothalamic tissue, 12 fetuses, 4 lambs, and 2 adults were used. The developmental ages were as follows: fetuses 86–110 days (n = 2), 119–129 days (n = 4), 134–138 days (n = 3), and 140–147 days old (n = 3); lambs 1–21 days old (n = 4); and adults (n = 2). Western blots again revealed three distinct bands at 66, 45, and 30 kDa. Again, preabsorption of the enzyme revealed only the 66-kDa band to be specific. Western
blots showing the 66-kDa band for ovine hypothalamus as well as brain stem are shown in Fig. 3. The 66-kDa band was not statistically different between groups by multiple linear regression. Figure 4B shows group means ± SE measured in relative optical density across developmental age for the 66-kDa band present in hypothalami.

Immunohistochemistry. Specific staining for estrogen sulfatase was widespread throughout the hypothalamus and brain stem of all developmental and postdevelopmental groups. Immunohistochemical results from regions important for HPA axis control are shown in Fig. 5. Specific neuronal staining was seen in the paraventricular nucleus (PVN) of the hypothalamus (Fig. 5, A and B), the nucleus of the solitary tract (NTS) of the medulla (Fig. 5, C and D), the rostral ventrolateral medulla (RVLM) (Fig. 5E), and the raphe nucleus (Fig. 5F).

![Fig. 1. Estrone sulfatase activity in ovine hippocampus (A), hypothalamus (B), and brain stem (C) for fetuses 119–129, 134–138, and 140–147 days old, lambs, and adult ewes. For all reactions, velocity is expressed as picomoles estrone formed per minute per milligram protein. Substrate concentration in all reactions was 3 µM. Data are means ± SE (n = 3 or 4 sheep/group). Activity in hippocampus was significantly increased in late-gestational fetuses compared with younger fetuses, lambs, and adult ewes (n = 3 or 4/group, P < 0.01).

![Fig. 2. Estrone sulfatase activity in ovine myometrium (n = 3 samples) and hypothalamus (n = 3 samples). Activity is expressed as picomoles estrone formed per minute per 10 mg tissue wet weight. Substrate concentration in all reactions was 3 µM.]

![Fig. 3. Western blots showing 66-kDa band of estrogen sulfatase for ovine hypothalami and brain stems. Number below each band designates developmental age in days. L, lamb; A, adult ewe.](http://dx.doi.org/10.1152/ajpregu.00000.00000)
DISCUSSION

The results of this study demonstrate that there is significant estrogen sulfatase activity in ovine fetal hypothalamus, hippocampus, and brain stem and that there are statistically significant ontogenetic changes in activity of this enzyme in the hippocampus. We have previously demonstrated that estrogens in fetal plasma increase both basal and stimulated fetal plasma ACTH secretion. The present results suggest a mechanism by which the most abundant form of estrogen in ovine fetal plasma, estrone sulfate, might be made available to areas within the fetal brain known to be involved in the control of the fetal HPA axis.

Mathew and Balasubramanian (15) and Lakshmi and Balasubramanian (9) have previously demonstrated estrogen sulfatase activity in adult sheep brain tissue. Other investigators have demonstrated this enzymatic activity in adult brain tissue from rats (3, 7), mice (6), nonhuman primates (10), and humans (17). Hobkirk et al. (6) demonstrated that the enzyme activity is transiently increased postnatally in the brain of the mouse. Although the development of brain estrogen sulfatase activity has not been studied in sheep, the development of activity in mice suggests the possibility that this might be an important developmental process in the perinatal period.

In the present study, we found an unequal distribution of estrogen sulfatase activity in the brain regions studied, and we found that the developmental changes in activity were not identical among the regions. Among
the areas that we studied, we found highest activity in the hippocampus and lower but still substantial activity in the hypothalamus and brain stem. Western blotting in the hypothalamus and brain stem confirmed the enzyme activity results. Thus estrogen sulfatase was present throughout development in both hypothalamus and brain stem, but it did not change significantly between groups. The presence of multiple bands (66, 45, and 30 kDa) is not surprising because the primary antibody used was polyclonal. The 66-kDa band best represents the enzyme that has a molecular mass of 65,492 Da (22) and was the only band that was specific as tested by preabsorption. This band was shown to be nonsignificant across developmental age (Fig. 4), which agrees with the results of the enzyme kinetics. The 66-kDa band for brain stem exhibited slight variations in molecular mass as shown by Fig. 3. Although no previous evidence exists in the literature for such an observation, a probable explanation may exist through posttranslational modification. That is, events such as phosphorylation or glycosylation could be responsible for slight variations in molecular mass.

Using a histochemical technique, Kawano and Aikawa (7) found that sulfatase activity is highest in pineal gland, choroid plexus, and pars distalis of the pituitary in adult rats. We investigated the activity in hypothalamus, brain stem, and hippocampus because these areas are known to contain nuclei involved in integration, afferent signal relay, or negative feedback inhibition within the HPA axis (4, 8, 13, 25). The presence of activity in any of these areas could be important for the deconjugation of sulfated estrogens in the blood perfusing the brain. Rosenfeld et al. (18) in 1980 reported that the majority of estrogen produced by the ovine placenta is sulfon conjugated and thus protected because sulfatase is not present. Our data suggest otherwise inasmuch as sulfon conjugates in the fetal compartment may have specific regional roles. The effect of estrogen on both basal and hypotension-stimulated concentrations of ACTH could be the result of an action of estrogen on the PVN in the hypothalamus, the hippocampus (which mediates some of the negative feedback actions of corticosteroids on ACTH secretion), the NTS (which relays neural traffic from visceral afferents), or any part of the pathways leading from the NTS to the PVN (e.g., the RVLM). Estrogen receptors have been demonstrated in the NTS and hippocampus (11). Although estrogen receptors within the hypothalamus are most concentrated in the arcuate nucleus, estrogen receptors have been demonstrated in the PVN (11, 21). The results of the present experiments identify the cellular location of the sulfatase activity, which is consistent with these centers for HPA axis control. We found widespread staining throughout nuclei and fiber tracts of the hypothalamus and brain stem. Neuronal staining was much more concentrated than fiber tract staining; however, both were observable. Specifically, we found intense neuronal staining in the PVN (Fig. 5, A and B), the NTS (Fig. 5, C and D), the RVLM (Fig. 5E), and the dorsal raphe nucleus (Fig. 5F).

**Perspectives**

We propose that parturition in the sheep and possibly other species involves an interaction among several variables whose net result is the activation of the HPA axis. In sheep, increases in fetal plasma cortisol concentration induce placental synthesis of estrogens. In nonhuman primates and humans, increases in fetal plasma ACTH stimulate fetal adrenal secretion of dehydroepiandrosterone, which is then converted to estrogen by the placenta. We have recently reported that physiological increases in fetal plasma estrogen concentrations stimulate fetal ACTH secretion (19) and that physiological increases in fetal plasma androgen concentrations decrease the sensitivity of the fetal hypothalamic pituitary unit to negative feedback inhibition by cortisol (19). Therefore, the increases in fetal plasma estrogen and androgen concentrations, themselves in part a function of fetal HPA axis activity, further augment fetal ACTH secretion. We hypothesize that parturition results from the onset of a hypothalamic drive to ACTH secretion, with interaction between adrenal, placenta, and hypothalamus producing a positive feedback cycle, which ultimately concludes with the separation of placenta from the fetal HPA axis (parturition). The present study suggests that the influence of estrogens on HPA axis activity could be expressed earlier than would be predicted on the basis of changes in plasma concentrations of unconjugated forms.

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