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Research Article

High Expression of Adrenal Cortisol Synthases Is Acquired After Intrauterine Inflammation in Periviable Sheep Fetuses

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Abstract

Context: Intrauterine inflammation, a representative stressor for the fetus, has been shown to alter the hypothalamus–pituitary–adrenal (HPA) axis reactivity in preterm fetuses and increase postnatal cortisol production. However, the mechanism of this alteration has not yet been elucidated.

Objective: We aimed to clarify the effects of endotoxin-induced intrauterine inflammation on the HPA axis of periviable sheep fetuses.

Methods: Fetal sheep (0.63 term) were divided into 2 groups: (1) the endotoxin group, in which the endotoxin was injected into the amniotic fluid; and (2) the control group, in which the saline solution was injected instead. A corticotropin-releasing hormone (CRH) challenge test was performed on the third day after injection to evaluate the cortisol-producing capacity of each group. Gene expression levels in the fetal adrenal glands of each group were analyzed by RNA-seq.

Results: The cortisol levels were significantly higher in the endotoxin group than in the control group after CRH challenge ($P = .02$). There were no significant differences in the responsiveness of adrenocorticotropin and cortisone between the 2 groups. Gene expression levels of the following enzymes involved in cortisol synthesis were significantly elevated in the endotoxin group: cytochrome P450 family (CYP) 11 subfamily A member 1 ($\log_2$FC 1.75), CYP 17 subfamily A member 1 ($\log_2$FC 3.41), 3β-hydroxysteroid dehydrogenase type I ($\log_2$FC 1.13), steroidogenic acute regulatory protein ($\log_2$FC 1.09), and CYP 21 ($\log_2$FC 0.89).

Conclusion: Periviable fetuses exposed to inflammation in utero have altered the responsiveness of the HPA axis with increased expression of enzymes involved in cortisol synthesis in the adrenal gland.

Key Words: intrauterine inflammation, RNA sequence, cortisol, corticotropin-releasing hormone, preterm fetus, adrenal gland

Abbreviations: ACTH, adrenocorticotropin; AUC, area under the curve; CRH, corticotropin-releasing hormone; CV, coefficient of variation; CYP, cytochrome P450; CYP11A1, cytochrome P450 family 11 subfamily A member 1; CYP17A1, cytochrome P450 family 17 subfamily A member 1; CYP21, cytochrome P450 family 21; DG, days of gestation; G-CSF, granulocyte colony-stimulating factor; HPA, hypothalamus–pituitary–adrenal; HSD3B1, 3β-hydroxysteroid dehydrogenase type I; MCP-1, monocyte chemoattractant protein 1; STAR, steroidogenic acute regulatory protein.

Adaptation to stress, either physical or psychological, is essential for survival. One of the biological responses is the activation of the hypothalamus–pituitary–adrenal (HPA) axis. Stress promptly activates the HPA axis and induces various defensive responses. Because the fetal HPA axis is immature, it relies heavily upon maternal and placental inputs, functioning more so as an endocrine network than a linear axis [1]. During the periviable period, the fetal adrenal glands have low enzyme activity in glucocorticoid synthesis, such as 3β-hydroxysteroid dehydrogenase. Therefore, it has been suggested that the fetus adapts to stress by receiving cortisol from the mother and placenta [2].

The HPA axis function is altered by past experiences of stress and can increase or decrease over time. Studies have shown that this alteration is also caused by stress during fetal life [3-6]. Fetal stressors include hypoxia, undernutrition, drugs administered to the mother, and intrauterine inflammation [7-11]. Intrauterine inflammation is one of the most common types of fetal stressors, accounting for approximately 60% of preterm births [12], and is associated with severe sequelae such as brain white matter damage [13-18], chronic lung disease [19], and retinopathy of prematurity [20-22] in preterm infants.

In clinical studies, it has been reported that human infants born preterm due to complications from in utero chorioamnionitis are more likely to experience elevated plasma cortisol levels after birth than infants born preterm without these complications [23, 24]. In animal experiments using sheep, it has been reported that fetuses exposed to maternal infections during late gestation have altered reactivity of the HPA axis, and

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these changes continue into adulthood [25], which may increase the risk of developing diseases in adult animals. Today, an increasing number of very premature infants are born at periviable gestation, but, to date, it has not been known whether exposure to intrauterine inflammation during the periviable period alters the reactivity of the fetal HPA axis.

Therefore, we aimed to investigate the effect of intrauterine inflammation on the fetal HPA axis during periviable period, and induced intrauterine inflammation by injecting endotoxin into the amniotic fluid during this period [26, 27]. After the normalization of cortisol levels in the fetuses [28], we performed a corticotropin-releasing hormone (CRH) challenge test on them to evaluate the responsiveness of their HPA axis. Based on the results of the CRH challenge test, we focused on the alteration of cortisol production in the fetal adrenal gland and compared it with in the control group.

Materials and Methods

This study was approved by the Animal Care and Use Committee of the Tohoku University Graduate School of Medicine, Sendai, Japan (no. 2015MdA-209, 2016 MdA-294), and was carried out between November 2015 and March 2018.

Animal Preparation

A total of 30 Suffolk ewes in pregnancies with known mating dates underwent aseptic surgery at 91 to 95 days of gestation (DG); full term in ewes is 147 DG. The CRH challenge test was performed on 20 fetuses, and the adrenal glands of the remaining 10 fetuses were used for RNA-seq without CRH challenge test. The preparations and protocol that were used in the present experiment were largely the same as those used in our previous study [26, 27, 29-31]. In brief, the ewes were anesthetized with 1.5% isoflurane (Isoflurane for animals; Merck & Co., Inc., Rahway, NJ, USA) and intravenous thiamylal sodium (Isozol; Nichi-Iko Pharmaceutical Co., Ltd. Toyama, Japan). After laparotomy and hysterotomy were performed on the pregnant ewes, polyvinyl vascular catheters (SMAC plus; Cardinal Health K.K., Tokyo, Japan) were inserted into the fetal carotid artery and jugular vein, maternal jugular vein, and the fetal amniotic cavity (Fig. 1). All catheters were filled with heparinized saline (heparin sodium injection 5000 units/5 mL; Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) and were exteriorized through an incision in the ewes' flanks. After the surgical sites were sutured closed, the ewes were housed in individual pens in animal holding rooms with a 12:00 hour/12:00 hour light–dark cycle and fed throughout the day. A recovery period of 2 days was allowed before the start of the experiments; during this time, antibiotics (Cefotax; Nichi-Iko Pharmaceutical Co., Ltd. Toyama, Japan) were administered to the mother (700 mg), fetus (200 mg), and amniotic cavity (100 mg) twice daily for 5 days to prevent postsurgical infection.

Induction of Intrauterine Inflammation

In a previous study [29], we developed an animal model of necrotizing funisitis and chorioamnionitis (severe in utero inflammations of the umbilical cord and fetal membrane, respectively) using the intravenous administration of granulocyte colony-stimulating factor (G-CSF) and the intraamniotic administration of endotoxin in sheep fetuses. In this experiment, intrauterine inflammation was induced according to the following protocol, the same as previously reported.

At 93-97 DG, 48 hours after surgery, ewes with single fetuses were randomly assigned into 2 treatment groups as follows: (1) the endotoxin group (n = 11), in which fetuses received an intraamniotic injection of 5 mg of endotoxin (*Escherichia coli* 055:B5 endotoxin; Sigma-Aldrich Co., St Louis, MO, USA) solubilized in 2 mL of saline once after 4 days of surgery. In addition, the fetuses were infused with 40 μg of G-CSF (Neutrogin; Chugai Pharmaceuticals Co. Ltd., Tokyo, Japan) solubilized in 2 mL of saline that was administered into the left jugular vein daily for 2 to 6 days after surgery, to increase the number of leukocytes in the fetoplacental circulation [26, 27, 29]. (2) The control group (n = 9), in which fetuses received only saline injections into both the jugular vein and the amniotic fluid.

Measurement of Physiological Parameters

Fetal heart rate and arterial and amniotic pressure were continuously monitored and recorded using the PowerLab (ADInstruments, Dunedin, New Zealand) throughout the study. All fetal arterial pressure values were corrected for the amniotic fluid pressure. The white blood cell counts (Celltac MEK-6550; Nihon Kohden Co., Tokyo, Japan) were measured in blood samples (0.5 mL) taken from the fetal carotid artery every 12 hours during the experiment.

CRH challenge Test

At 72 hours after the endotoxin infusion on 98-102 DG, 1 μg of CRH (Ovine CRF; Peptide Institute, Inc., Tokyo, Japan) solubilized in 1 mL of saline was injected into the fetal left jugular vein in 20 fetal sheep at 10:00 hours (control: n = 9, endotoxin: n = 11). Blood samples (2.5 mL) were collected into heparinized tubes (Insepack II-D; Tokuyama Sekisui Co., Ltd., Yamaguchi, Japan) from the fetal carotid artery at 0, 15, 30, 45, 60, 90, and 120 minutes after CRH administration. All blood samples were centrifuged at 3000*g* for 5 minutes, and harvested plasma was stored at −80 °C until assayed. Immediately after the CRH challenge test, the fetuses were delivered by cesarean section and euthanized by administration of an intravenous bolus of pentobarbital (50 mg/kg) (Somnopentyl; Kyoritsu Seiyaku Co., Tokyo, Japan).

Laboratory Analyses

Enzyme-linked immunosorbent assays

Inflammatory protein concentrations for monocyte chemoattractant protein 1 (MCP-1) in fetal plasma samples were measured using commercial kits (Catalog # VS0083B-002, RRID:AB_2924218) from Kingfisher Biotech (St Paul, MN, USA), with washing performed on a plate washer (IW-8, Intelispeed washer; Biosan, Riga, Latvia) as previously described [32]. Standards (calibration curve $R^2 > 0.99$) were assayed in triplicate (average coefficient of variation [CV], 7.8%) and samples were assayed in duplicate. The assay limit of detection was <4 pg/mL. Next, 100 μL of each standard or sample was incubated overnight (16 hours) at 4 °C. Assays were performed in accordance with the manufacturer's instructions, with absorbance at 450 nm read on the Model 680 Microplate Reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

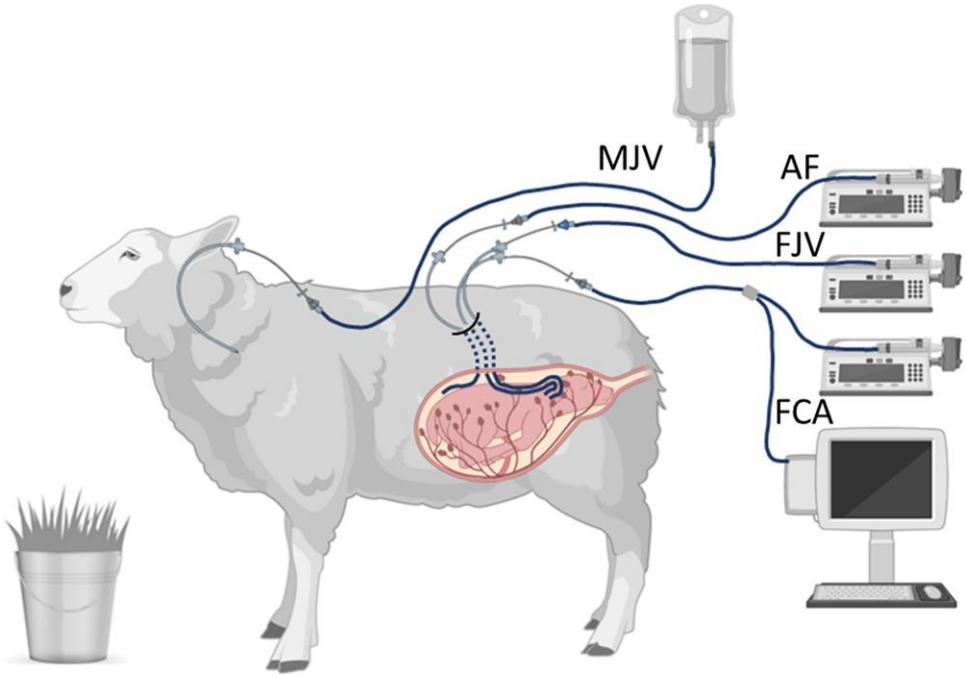

Figure 1. The schematic of the experimental setup. MJV, maternal jugular vein; AF, amniotic fluid; FJV, fetal jugular vein; FCA, fetal carotid artery.

Adrenocorticotropin immunoradiometric assay

Fetal plasma adrenocorticotropin (ACTH) concentrations were measured using the ACTH immunoradiometric assay kit (IM2030; Beckman Coulter, Inc. Brea, CA, USA) according to the manufacturer's instructions. The sensitivity of the assay was 0.31 pg/mL, and the intra-assay CV for ACTH was 10.0% and the interassay CV was 10.8%.

Liquid chromatography–mass spectrometry analysis of cortisol and cortisone

Reference standards were purchased from Cerilliant Co. (TX, USA) and IsoSciences LLC (PA, USA) and used to prepare calibration standards and QC samples. Bovine serum albumin (5%) (BSA; Sigma-Aldrich Co., St Louis, MO, USA) was used as a surrogate matrix. The nominal concentrations of the calibration standards and QC samples used in the study are elsewhere (Table S1 [33]). Stable-isotope–labeled steroid hormones were purchased from Biocrates Life Sciences AG (Innsbruck, Austria), Cerilliant Co., and IsoSciences LLC to be used as internal standard (IS). Aliquots (100 μL) of plasma samples were pretreated using a solid phase extraction plate (Oasis MAX; Waters Co., MA, USA) according to the manufacturer's generic method with a minor modification. The extracts obtained by MAX were used to analyze cortisol and cortisone, respectively. Liquid chromatography–tandem mass spectrometry analysis was performed on a Shimadzu Nexera X2 system (Shimadzu Co., Kyoto, Japan) coupled with a QTRAP 5500 mass spectrometer (Sciex, MA, USA). Chromatographic separation was achieved on a reverse-phased high-performance liquid chromatography column under high-pressure gradient elution. The mass spectrometer was operated in negative or positive electrospray ionization mode and multiple reaction monitoring mode. The multiple reaction monitoring transitions are shown elsewhere (Table S2 [33]). Data analysis was performed using Analyst 1.6.2 (Sciex). The calibration curves were created based on the relationship between the peak area ratios of each of the analytes to ISs and the nominal concentrations in calibration standards, using the linear least-squares method with a weighting factor of 1/×2. Concentrations of each steroid hormone in plasma samples were determined from their respective calibration curves.

RNA extraction and RNA-seq

Past studies have shown that CRH can directly stimulate fetal adrenal cells to produce cortisol and dehydroepiandrosterone sulfate [34, 35]. Therefore, CRH itself may affect the expression of steroid synthase. To eliminate the effect of the CRH challenge on RNA expression, the adrenal glands of the fetal sheep that did not undergo CRH challenge were used for RNA-seq. Therefore, the 10 ewes (5 animals per group) were prepared using the same protocol as described above and the fetuses were delivered by cesarean section. The fetal adrenal glands were then collected, snap-frozen immediately in liquid nitrogen, and stored at −80 °C until analysis.

Rhelixa Inc. (Tokyo, Japan) provided the RNA-seq data. RNA was extracted from the fetal adrenal gland using ISOSPIN Cell & Tissue RNA (catalog number 314-08211; Nippon Gene Co., Ltd, Tokyo, Japan) according to the manufacturer's instructions. To select only high-quality RNA, the RNA integrity number was characterized with the TapeStation 4150 system (Agilent Technologies, Wilmington, DE, USA) and samples with an RNA integrity number of at least 7.0 were used. According to the evaluation of the NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA), samples with A260/A280 of around 2.0 and A260/A230 of 2.0 or higher were used. Libraries were made using the NEBNext Ultra II Directional RNA Library Prep Kit (catalog number E7760; New England Biolabs, Inc., MA, USA). Paired-end sequence reads of 150 bp length were generated using the Illumina NovaSeq 6000. The sequencing data were converted into FASTQ data for the RNA-seq analysis.

Computational analysis

RaNA-seq software was used to perform FASTQ preprocessing (fastp 0.19.4) and quantification (salmon 0.9.1) of the samples [36]. Differentially expressed genes (DEGs) were detected by DEseq2 R package and Waid's test. The Kyoto Encyclopedia of Genes and Genomes database was used for the detailed study of the results obtained. To perform functional enrichment analysis, normalized data were filtered according to the following criteria: *P*adj ≤.05 and fold change of 1.5 ($\log_2$FC ≤−0.585; $\log_2$FC ≥0.585).

Statistical Analyses

Statistical analyses were performed using IBM SPSS Statistics, Version 25.0.0 (IBM Corporation, Armonk, NY, USA). The Shapiro–Wilk test was used to assess for normality. In the comparison of the fetal data, between-group differences in the parametric data were tested for significance with Student's t tests, whereas Mann-Whitney U tests were used for nonparametric data. A χ^2 test was used to test the differences in nominal values between the 2 groups. The Dunnett test was performed to test for significant changes in the physiological parameters from the baseline values during the 72 hours after endotoxin infusion. Differences in basal hormone levels between the 2 groups were analyzed by Student's t tests. The responses of ACTH, cortisol, and cortisone in the CRH challenge were summarized using their respective area under the curve (AUC), which were calculated with GraphPad Prism 9 (GraphPad Software Inc., San Diego, CA, USA), based on the trapezoid rule. AUC differences were tested using the Student's t test. Statistical significance was set $P = .05$. All values are expressed as the mean ± SEM.

Results

Continuous monitoring of parameters showed that all fetuses (20 fetuses with CRH challenge and 10 fetuses without CRH challenge) were in good condition, with no occasions when the mean arterial pressure was <35 mmHg or the heart rate was <100 bpm throughout the experiment. There were no significant differences in sex ratio, fetal body weight at autopsy, and adrenal gland weight among the 4 groups (Table 1).

Endotoxin Administration

Figure 1 shows the changes in blood test values and physiological parameters in the endotoxin (n = 11) and control (n = 9) groups after endotoxin and saline administration, respectively.

Significant changes in MCP-1 concentration ($P = .007$), fetal heart rate ($P = .023$), and white blood cell counts ($P = .035$) were observed over the 72-hour period after endotoxin injection. The MCP-1 concentration in the endotoxin group increased significantly at 12 hours and 24 hours after the endotoxin infusion compared with the baseline value (Fig. 2A). The fetal heart rate increased significantly from 6 to 9 hours (Fig. 2B) and the white blood cell counts increased significantly at 48 and 72 hours (Fig. 2C) after endotoxin infusion in the endotoxin group.

ACTH, Cortisol, and Cortisone Response to CRH Challenge Test

The plasma cortisol levels of fetuses in the endotoxin group decreased to the same levels as those in the control group before the CRH challenge test (control: 0.76 ± 0.53 ng/mL; endotoxin: 1.23 ± 0.74 ng/mL, $P = .13$). The dose of CRH administered per kg body weight in the CRH challenge test did not differ significantly between the control and endotoxin groups (control: 1.24 ± 0.65 μg/kg; endotoxin: 1.10 ± 0.35 μg/kg, $P = 1.00$) (Table 1). Figure 2 shows the response of ACTH, cortisol, and cortisone to the CRH challenge test. The ACTH and cortisone response to the CRH challenge test evaluated using the AUC did not differ significantly between the control and endotoxin groups. *P* values were 0.28 and 0.15 for ACTH (Fig. 3A and 3D) and cortisone (Fig. 3C and 3F), respectively. In contrast, the cortisol response to the CRH challenge test expressed as AUC in the endotoxin group was significantly greater than that in the control group ($P = .002$) (Fig. 3B and 3E).

Transcriptome Changes of Steroidogenic Enzymes in Fetal Adrenal Glands

Total reads obtained for each library ranged between 21 and 30 million, and between 75% and 80% for the properly matched fragments. In total, 350 genes were identified as significant DEGs (*P*adj <.05), of which 258 genes were

Table 1. Comparison of basic fetal characteristics between endotoxin and control groups

	CRH challenge test		RNA expression			
Variables	Endotoxin (n = 11)	Control (n = 9)	Endotoxin (n = 5)	Control (n = 5)	Statistical test	*P* value
GA at endotoxin administration (day)	98.1 ± 1.6	97.6 ± 1.6	99.0 ± 0.0	99.0 ± 0.0	ANOVA	.085
GA at CRH challenge/autopsy (day)	101.1 ± 1.6	100.6 ± 1.6	102.0 ± 0.0	102.0 ± 0.0	ANOVA	.085
Sex (male/female)	4/5	4/7	2/3	2/3	χ2 test	.446
BW (kg)	1.13 ± 0.22	1.21 ± 0.31	1.34 ± 0.17	1.44 ± 0.20	ANOVA	.112
Adrenal gland weight (g)	0.13 ± 0.04	0.13 ± 0.02	0.14 ± 0.04	0.15 ± 0.03	ANOVA	.661
BW-corrected adrenal gland weight (g/kg)	0.12 ± 0.05	0.12 ± 0.04	0.10 ± 0.03	0.11 ± 0.02	ANOVA	.970
BW-corrected CRH dose (μg/kg)	1.10 ± 0.35	1.24 ± 0.65	N.D.	N.D.	Mann–Whitney U	1.000

The 20 fetuses underwent the CRH challenge test and the adrenal glands of the remaining 10 fetuses were used for RNA-seq without the CRH challenge test.
Values are expressed as mean ± SD. $P < .05$ is considered as significant difference.
Abbreviations: ANOVA, 1-way analysis of variance; BW, body weight; CRH, corticotropin-releasing hormone; GA, gestational age.

upregulated and 92 genes were downregulated (Fig. 4A). Functional enrichment analysis showed 4 steroid hormone biosynthesis pathways in the top 10 upregulated metabolic pathways (Fig. 4B). Transcripts encoding steroidogenic enzymes higher in the endotoxin group included cytochrome P450 family 11 subfamily A member 1 (CYP11A1) (log_2FC 1.75), cytochrome P450 family 17 subfamily A member 1 (CYP17A1) (log_2FC 3.41), 3β-hydroxysteroid dehydrogenase type I (HSD3B1) (log_2FC 1.13), steroidogenic acute regulatory protein (STAR) (log_2FC 1.09), and cytochrome P450 family 21 (CYP21) (log_2FC 0.89) (Fig. 4C and 4D and Table 2). The RNA-seq data discussed in this publication have been deposited in National Center for Biotechnology Information's Gene Expression Omnibus [37].

Discussion

The primary findings of this study were as follows: (1) After the normalization of cortisol levels in the fetuses exposed to intrauterine inflammation, the CRH challenge test showed that plasma cortisol levels were significantly increased in the endotoxin group compared with the control group, even though there was no significant difference in plasma ACTH levels (Fig. 3). (2) RNA-seq performed on adrenal glands with increased cortisol production confirmed that CYP11A1, CYP17A1, HSD3B1, STAR, and CYP21, enzymes involved in cortisol synthesis, were significantly upregulated in the endotoxin group (Fig. 4, Table 2).

For the endotoxin group, fetal heart rate and white blood cell count were significantly increased compared with those in the control group in this study (Fig. 2). In addition, fetal plasma MCP-1 levels [38] were predominantly elevated at 12 hours after endotoxin administration (Fig. 2). In the present study, we used the protocol described previously, wherein the endotoxin was injected into the amniotic fluid and G-CSF was administered to the fetuses to expose them to intrauterine inflammatory stress [26, 27, 29]. The previous protocol induced a high rate of necrotizing funisitis caused by a fetus's own immune response, and similar changes were induced in this study. These results confirm that the fetuses in the endotoxin group are definitely exposed to inflammation in utero, as in the previous inflammatory load experiment.

The plasma cortisol levels of fetuses in the endotoxin group normalized to the same levels as those in the control group at 3 days after endotoxin administration as in the previous experiment [28], and a CRH challenge test was performed on fetuses in each group. When a CRH dose with no significant difference per body weight was administered (Table 1), plasma cortisol levels increased significantly in the endotoxin group compared with those in the control group (Fig. 3). This indicated that the fetal adrenal gland's cortisol-producing capacity was enhanced after intrauterine exposure to inflammation. In late pregnancy, 11β-hydroxysteroid dehydrogenase type 2 becomes predominant in the placenta and cortisol is converted to cortisone, which is inactivated. Since this experiment was performed in midgestation and cortisone levels were not significantly altered, 11β-hydroxysteroid dehydrogenase type 2 was not suggested to be involved in cortisol levels in both groups.

In the CRH challenge test, plasma cortisol levels were significantly elevated in the endotoxin group compared with the control group, although there was no significant difference in plasma ACTH levels. We hypothesized that cortisol production capacity in the fetal adrenal gland was altered, and analyzed gene expression in the adrenal gland by RNA-seq. On comparing the RNA expression levels between the endotoxin and control groups, CYP11A1, CYP17A1, HSD3B1, STAR, and CYP21, which are enzymes involved in cortisol synthesis, were significantly upregulated in the endotoxin group (Fig. 4 and Table 2). This indicates that the increased expression of adrenal glucocorticoid synthetic enzymes in the endotoxin group is involved in the maintenance of high cortisol production capacity. In this study, we analyzed gene expression in the adrenal glands of fetuses exposed to the intrauterine inflammation on 99 DG. These results indicate that inflammatory stress may alter the expression of steroid synthase enzymes in the very preterm fetus at periviable period and affect subsequent steroidogenesis.

Cortisol is known to be transferred from the mother to the fetus [39]. Therefore, it cannot be completely ruled out that

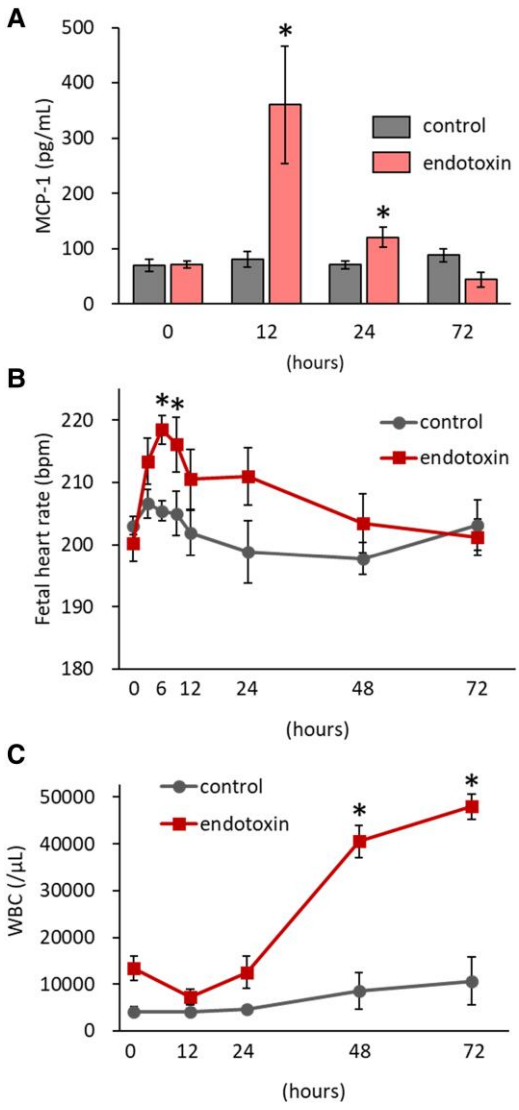

Figure 2. Change in inflammatory protein concentrations and physiologic parameters over 72 hours after endotoxin infusion in sheep fetuses. (A) Inflammatory protein concentrations for MCP-1; (B) fetal heart rate; (C) white blood cells. All data are expressed as the mean ± SEM. Asterisks indicate $P < .05$ (Dunnett test), compared with the value before endotoxin infusion.

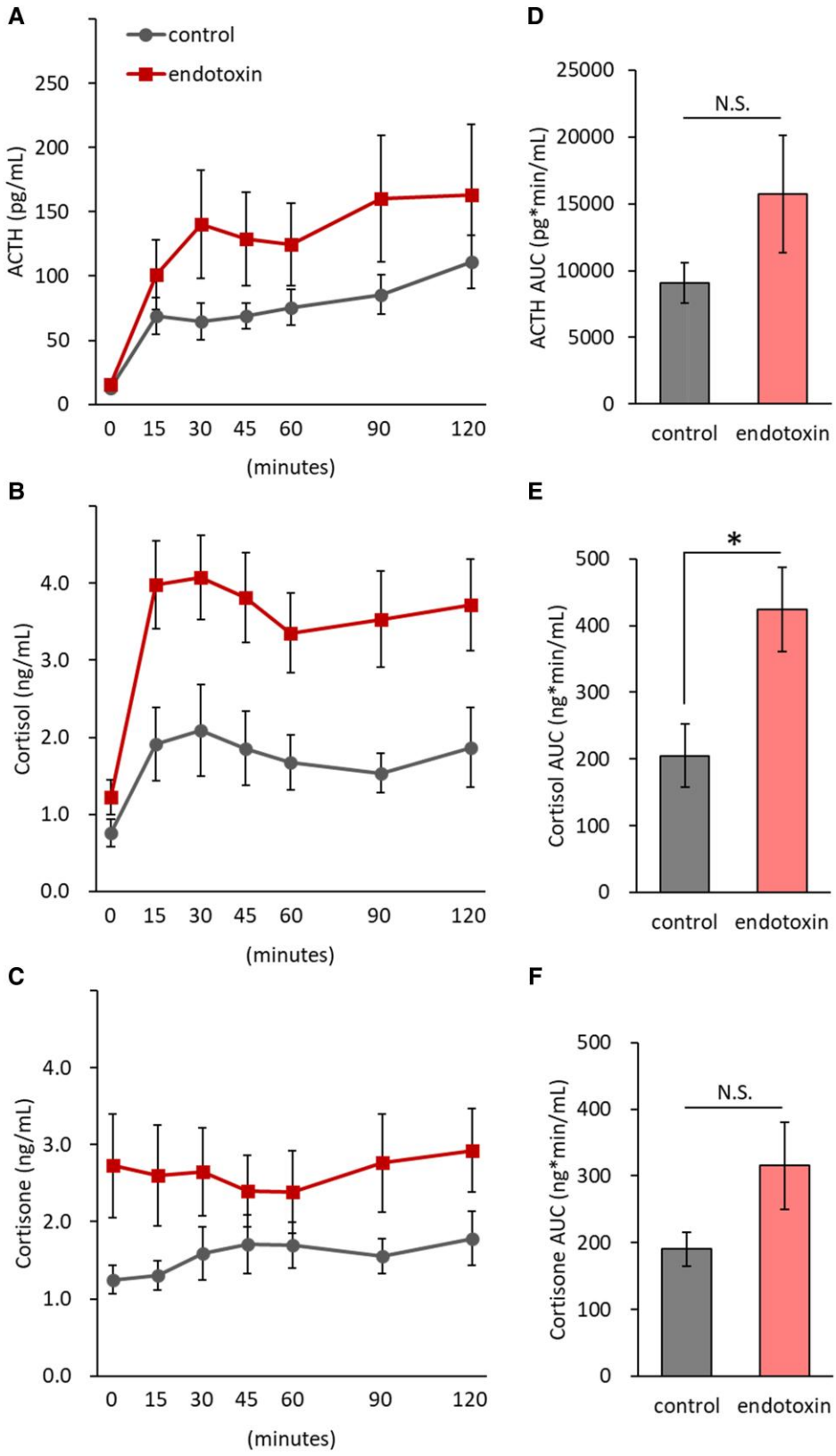

Figure 3. Adrenocorticotropic hormone (ACTH), cortisol, and cortisone responses to corticotropin-releasing hormone (CRH challenge test) in sheep fetuses. Changes in ACTH (A), cortisol (B), and cortisone (C) values over 120 minutes after administration of CRH and their AUCs depicted as histograms in D, E, and F, respectively. All data are expressed as the mean ± SEM; $*P < .05$; control vs endotoxin.

maternal synthesis of cortisol in response to CRH administered to the fetus may have influenced the results of this study. However, CRH is not expected to pass through the placenta due to its molecular weight. Furthermore, the amount of CRH administered to the fetus in this study was less than 1/50 of the amount that would be administered to the mother

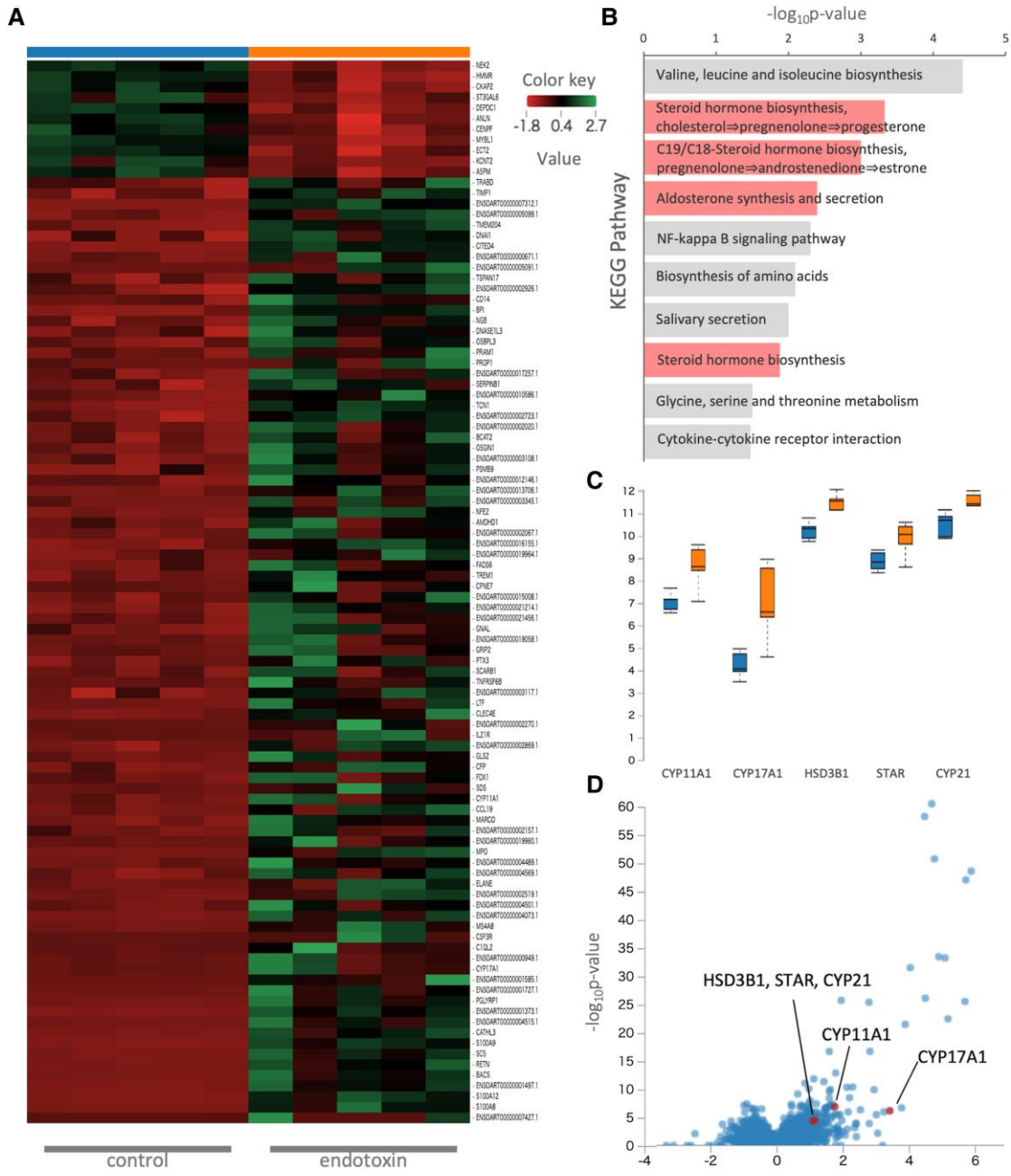

Figure 4. (A) Heatmap showing the adrenal gland expression of the top 100 differentially expressed genes (DEGs) ranked by magnitude of change between groups. In top 100 DEGs, 86 genes were upregulated, shown in red, and 14 genes were downregulated, shown in green. (B) Metabolic pathways identified from the functional enrichment analysis, which found 4 steroid hormone biosynthesis pathways (red bar) in the top 10 upregulated metabolic pathways in the endotoxin group. (C) Boxplot shows the expression value distribution (normalized as Transcripts Per Million) of selected genes in each group (blue bar, control group (n = 5); Orange bar; endotoxin group (n = 5)). (D) Volcano plot of DEGs. Steroid synthase genes with significant differences are shown in red. CYP11A1, cytochrome P450 family 11 subfamily A member 1; CYP17A1, cytochrome P450 family 17 subfamily A member 1; HSD3B1, 3β-hydroxysteroid dehydrogenase type I; STAR, steroidogenic acute regulatory protein; CYP21, cytochrome P450 family 21.

in a CRH challenge. Therefore, the influence of maternal ACTH and cortisol on the results of this study does not seem to be significant.

Several studies have been conducted on pregnant sheep to evaluate the fetal HPA axis in various gestational environments using the CRH loading test [40, 41]. We selected sheep to provide fetal samples (blood and adrenal glands) for evaluation. The human fetal adrenal gland in midgestation is mainly composed of a fetal zone in which HSD3B expression is suppressed. From 23 to 4 weeks of gestation, the transitional zone, in which HSD3B is expressed, occupies a large portion of the cortex, and the cortisol-producing capacity of the fetus increases [42-45]. In contrast, in fetal sheep, unlike humans and primates, there is no such localization of fetal and transitional zones. Therefore, in this study, it was not possible to histologically examine the sites where the changes in enzyme expression mentioned above occur in primates. An additional limitation of importance is the inflammatory stimuli employed. Common pathogens causing chorioamnionitis are bacteria such as Group B *Streptococcus* (GBS), *Escherichia coli*, and *Mycoplasma* spp. In this study, we used endotoxin, an element of the extracellular membrane

Table 2. Fold changes in steroid hormone biosynthesis enzymes in the endotoxin group

Gene name	Log_2FC	*P*adj
CYP11A1	1.75	8.6×10^{-5}
CYP17A1	3.41	3.1×10^{-4}
HSD3B1	1.13	.007
STAR	1.09	.008
CYP21	0.89	.031

Logarithmic fold change of the steroid hormone biosynthesis enzymes gene expression between the endotoxin and control group.
Abbreviations: CYP11A1, cytochrome P450 family 11 subfamily A member 1; CYP17A1, cytochrome P450 family 17 subfamily A member 1; CYP21, cytochrome P450 family 21; HSD3B1, 3β-hydroxysteroid dehydrogenase type I; STAR, steroidogenic acute regulatory protein;

of Gram-negative bacteria that induces inflammation via toll-like receptor 4. Endotoxin was used because it can produce a standardized inflammatory response and can histologically induce inflammation in the amnion. Therefore, it does not faithfully reproduce infections caused by other live bacteria such as GBS.

It is also unclear whether the increase in cortisol synthase expression induced by intrauterine inflammation is a transient change or a change that persists into the neonatal period and early childhood. Furthermore, since fetal stress in utero varies, the changes in the reactivity of the HPA axis in response to various stressors such as hypoxia, undernutrition, maternal alcohol and drug consumption, and prenatal glucocorticoid administration [46-50] need to be investigated in the future.

In the present study, we found that the reactivity of the HPA axis is altered in fetuses exposed to intrauterine inflammation, even at the immature stage of periviable gestation. It was also revealed that high expression of adrenal glucocorticoid synthase is involved in the enhanced cortisol production capacity.

Today, it has been suggested that stress during late pregnancy can also affect the health and development of the child [51]. In this study, we found that infection stress can affect subsequent adrenal function not only in late pregnancy but even during the periviable period. If these changes are long-lasting, new preventive strategies for child health promotion and disease prevention may be developed in the future based on the effects of stress in midpregnancy.

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Disclosures

All authors declare that this study was conducted free of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data Availability Statement

Original data generated and analyzed during this study are included in this published article or in the data repositories listed in References.

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