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Sex Differences in the Ovine Fetal Cortisol Response to Stress

DINO A. GIUSSANI, ANDREW J. W. FLETCHER, AND DAVID S. GARDNER

Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3EG, United Kingdom

ABSTRACT: This study tested the hypothesis that the sexually dimorphic adrenocortical response to stress is already established before birth. Chronically instrumented late gestation pregnant sheep carrying 16 male and 15 female age-matched singleton fetuses were subjected to an acute episode of hypoxic stress. Maternal and fetal blood gases, adrenocorticotrophic hormone (ACTH), and cortisol were measured. In addition, six male and six female fetuses received the ACTH analog, Synacthen, and plasma cortisol was measured. During hypoxic stress, the increment in plasma cortisol was 2-fold greater in male *versus* females fetuses (30.6 ± 3.2 *versus* 14.3 ± 2.0 ng/mL; $p < 0.001$) mediated, in part, by greater adrenocortical sensitivity to ACTH. The data support the hypothesis tested and show that sex-specific differences in the cortisol stress response are present before birth with the output of cortisol being much greater in male than in female fetuses. **(*Pediatr Res* 69: 118–122, 2011)**

During threatening situations, the stress system coordinates adaptive responses that adjust homeostatic mechanisms to increase the individual's chance of survival. The hypothalamic-pituitary-adrenal (HPA) axis constitutes one of the main efferent limbs of the stress system, and measurement of circulating plasma adrenocorticotrophic hormone (ACTH) and cortisol concentrations are established measures of the level of psychological, social, and/or physiological stress (1). An adequate capacity for secretion of adrenal glucocorticoids is essential for fetal development and maintenance of an independent life (2,3); the adrenal glands are thus highly perfused with blood and richly innervated. Adrenocortical secretion of glucocorticoids is often used as an example of classical endocrinology, with the principal control provided by the release of ACTH from the distant anterior pituitary and with excess cortisol production switching off the stimulatory signal through negative feedback.

It is widely acknowledged that there are differences in the response to stress between the sexes, but the reason underlying these differences is unclear. The literature is often contradictory due, in part, to variations in the type of stress imposed, the particular species being studied, and the age of the subject. For example, in adult rats, females have greater stress responses (4,5); however, in humans, it is young men and old women who show an increased stress response relative to old men and young women (6). In addition, when compared with age-matched women, men showed consistently higher plasma cortisol responses to stress in four independent studies (7). It is also accepted that sex hormones can alter the magnitude of the stress response, with androgens being suppressive and estrogens being stimulatory to the HPA axis (8,9) and that manipulation of gonadal steroids soon after birth can have activational and organizational effects on the function of the stress axis later in life (9). Development of the fetal HPA axis is exquisitely controlled and critical for the appropriate maturation of multiple vital organs and systems before term (10). Preventing the action of glucocorticoids in the fetus by knock out of the glucocorticoid receptor is invariably embryonically lethal (>90%) (2). By contrast, fetal exposure to excess glucocorticoids, either as a result of maternal stress or maternal treatment with steroids to accelerate fetal lung maturation, has been shown to induce changes on the offspring's stress axis (11,12) in a sex-specific manner (13–15). In addition, it has been reported that a sexually dimorphic response to exogenous ACTH seems to develop postnatally (16) and that this can be affected by adult lifestyle (17–20). To date, what remains completely unknown is whether there is a difference between the sexes in the HPA axis response to stress that is already established before birth and, thereby, that is independent of exposure to maternal antenatal exogenous steroid therapy, changes in sex hormones throughout the postnatal life course, and/or environmental influences determined by lifestyle.

In this study, we have used the chronically instrumented sheep preparation to compare the circulating plasma concentrations of ACTH and cortisol in age-matched singleton male and female late gestation ovine fetuses during basal conditions and during an episode of acute hypoxic stress, a well-characterized stressor relevant to fetal life (21–23). To mechanistically explore potential differences between the sexes in the adrenocortical sensitivity to ACTH, a separate cohort of age-matched singleton male and female sheep fetuses were challenged with a bolus of synthetic ACTH and the plasma cortisol response determined.

MATERIALS AND METHODS

Animals. All procedures were performed under the UK Animals (Scientific Procedures) Act 1986 and were approved by the Ethical Review Committee of the University of Cambridge. Under general anesthesia, 43 (22 male and 21 female) Welsh Mountain singleton sheep fetuses and their mothers

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Correspondence: Dino A. Giussani, Ph.D., Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge CB2 3EG, United Kingdom; e-mail: dag26@cam.ac.uk

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D.S.G. is currently at The School of Veterinary Medicine and Science, University of Nottingham, Sutton Bonington Campus, Loughborough LE12 5RA, United Kingdom.

Abbreviations: HPA, hypothalamic-pituitary-adrenal; **pH_a,** arterial pH; **$Paco_2$,** arterial partial pressure of carbon dioxide; **Pao_2,** arterial partial pressure of oxygen

were surgically prepared for long-term recording with vascular and amniotic catheters at 125 ± 1 d of gestation (term is ~145 d) as previously described (24,25). During recovery, ewes were housed in individual pens in rooms with a 12 h/12 h light/dark cycle where they had free access to hay and water and were fed concentrates twice daily. Antibiotics were administered daily to the ewe i.m. and the fetus i.v. and into the amniotic cavity. Five days after surgery, 31 of the fetuses (16 male and 15 female) were exposed to a 3 h protocol consisting of 1 h of normoxia, 1 h of hypoxic stress, and 1 h of recovery at the same time of the day. Acute hypoxia in the fetus was induced by maternal inhalational of a hypoxic mixture, using well-established protocols (24,25).

Hormone measurements. Maternal and fetal arterial blood samples were taken at 15 and 45 min of each experimental period for measurement of blood gases, pH, and the plasma concentrations of ACTH and cortisol. In the remaining 12 fetuses (6 male and 6 female), arterial blood samples were taken for measurement of plasma ACTH and cortisol concentrations during basal conditions and after i.v. treatment with exogenous ACTH (2.5 μg; Synacthen; Ciba Pharmaceuticals, United Kingdom). The dose of Synacthen used was based on previous studies from this laboratory (26,27). Hormone measurements were performed within 4 mo from plasma collection by the same investigators using the same established procedures, which were validated for use with ovine plasma (26,27).

Statistics. All values are expressed as mean + SEM unless otherwise indicated. The residuals for all measured variables were first assessed for equality of variance across fitted values. Data for plasma ACTH were highly positively skewed and, therefore, were analyzed using a generalized linear mixed model regression procedure incorporating a gamma distribution and logarithm-link function (Genstat v12; VSNi, United Kingdom). Sex, time, and sex × time were included as fixed effects with each fetus included as a random effect in the model. The estimated means (back transformed) are presented together with estimated error at each time point. Linear regression analysis of paired logACTH and plasma cortisol for each sex were conducted using Graphpad Prism 5.0. For all comparisons, statistical significance was accepted when $p < 0.05$.

RESULTS

Blood gases during hypoxia. During baseline conditions, there were no differences in arterial blood gases or pH between ewes bearing male or female fetuses or between the male or female fetuses themselves (Table 1). Acute hypoxia reduced the maternal and fetal arterial partial pressure of oxygen (Pao_2) to similar levels in pregnancies bearing either male or female fetuses (Table 1). By the end of the hypoxic challenge, female fetuses had become more acidemic [lower arterial pH (pH_a)] than males (Table 1) despite maintenance of arterial partial pressure of carbon dioxide ($Paco_2$) at similar levels.

ACTH and cortisol during hypoxia. Basal maternal plasma ACTH and cortisol were similar in ewes carrying male or female fetuses and remained unchanged throughout the hypoxic challenge (Table 2). Fetal plasma ACTH and cortisol concentrations were also not different from each other during baseline (male ACTH, 38.5 ± 2.9 pg/mL and male cortisol, 23.4 ± 2.3 ng/mL; female ACTH, 32.3 ± 3.1 pg/mL and female cortisol, 22.2 ± 3.6 ng/mL). Fetal hypoxia resulted in a significant increase in ACTH in both groups of fetuses, with no effect of sex (Fig. 1*A*). In contrast, the increase in fetal cortisol triggered by fetal hypoxia was markedly greater in male compared with female fetuses ($p < 0.05$, Fig. 1*B*). Linear regression analyses of paired plasma ACTH and cortisol concentrations throughout the hypoxia protocol indicated that the slope of the linear relationship was twice as steep in male (10.77 ± 0.63; $r^2 = 0.98$) compared with female (3.43 ± 0.18; $r^2 = 0.99$) fetuses ($p < 0.001$, Fig. 1*C*).

ACTH challenge. After an exogenous bolus of synthetic ACTH, fetal plasma ACTH increased significantly in all fetuses, but the increment was significantly greater ($F = 5.0$, $p = 0.003$) in females compared with males (peak plasma ACTH; females, 522 ± 130 *versus* males, 246 ± 55 pg/mL; Fig. 2*A*). Synthetic ACTH significantly increased fetal plasma cortisol with the increment being variable but not significantly different between male and female fetuses (Fig. 2*B*). When the measured fetal plasma cortisol was considered relative to the

Table 1. *Maternal and fetal arterial blood gases and pH*

	Normoxia		Hypoxia		Recovery	
	N15	N45	H15	H45	R15	R45
Maternal blood gases						
pH_a						
Male	7.45 ± 0.01	7.44 ± 0.01	7.49 ± 0.01	7.49 ± 0.01	7.48 ± 0.01	7.47 ± 0.01
Female	7.44 ± 0.01	7.46 ± 0.01	7.49 ± 0.01	7.49 ± 0.01	7.48 ± 0.01	7.48 ± 0.01
$Paco_2$						
Male	34.1 ± 0.8	35.2 ± 0.6	35.1 ± 0.9	35.8 ± 0.9	33.8 ± 0.8	32.5 ± 0.5
Female	34.3 ± 0.9	34.5 ± 0.8	35.4 ± 0.5	36.2 ± 0.6	34.0 ± 0.2	33.4 ± 0.7
Pao_2						
Male	98.2 ± 1.2	99.4 ± 1.3	40.8 ± 1.1*	37.6 ± 1.5*	99.9 ± 1.2	98.3 ± 1.1
Female	99.3 ± 1.1	98.4 ± 1.4	40.2 ± 1.2*	37.1 ± 1.4*	99.5 ± 2.0	99.6 ± 1.1
Fetal blood gases						
pH_a						
Male	7.33 ± 0.01	7.33 ± 0.01	7.32 ± 0.01	7.27 ± 0.01*	7.27 ± 0.01*	7.30 ± 0.01
Female	7.32 ± 0.01	7.33 ± 0.01	7.29 ± 0.01	7.22 ± 0.03*†	7.20 ± 0.02*†	7.25 ± 0.02
$Paco_2$						
Male	52.7 ± 0.6	55.6 ± 0.7	52.4 ± 0.8	52.5 ± 0.7	51.1 ± 0.6	51.4 ± 0.8
Female	52.4 ± 1.2	52.3 ± 1.2	53.0 ± 1.2	52.8 ± 1.4	51.4 ± 0.9	50.9 ± 0.9
Pao_2						
Male	21.4 ± 0.5	21.0 ± 0.5	11.8 ± 0.6*	11.6 ± 0.5*	22.2 ± 0.7	20.2 ± 0.7
Female	22.2 ± 0.8	21.6 ± 0.7	11.6 ± 0.7*	11.5 ± 0.4*	22.1 ± 0.8	20.6 ± 0.7

Values are mean ± SEM at 15 (N15) and 45 (N45) min of normoxia, 15 (H15) and 45 (H45) min of hypoxia, and 15 (R15) and 45 (R45) min of recovery for 16 male and 15 female fetuses. Significant differences ($p < 0.05$).

* Differences by post hoc analysis indicating a significant main effect of hypoxia.

† Differences by post hoc analysis indicating a significant main effect of sex (two-way repeated measures ANOVA + Tukey test).

Table 2. *Maternal plasma ACTH and cortisol*

	Normoxia		Hypoxia		Recovery	
	N15	N45	H15	H45	R15	R45
ACTH (pg/mL)						
Male	28.5 ± 5.1	27.5 ± 4.8	31.9 ± 5.4	31.4 ± 4.9	30.2 ± 4.0	25.9 ± 7.6
Female	30.0 ± 4.7	29.5 ± 5.2	30.5 ± 6.0	30.3 ± 7.8	30.5 ± 5.2	27.8 ± 8.0
Cortisol (ng/mL)						
Male	35.2 ± 4.7	38.3 ± 3.8	41.2 ± 4.3	39.4 ± 6.5	37.6 ± 7.4	35.4 ± 6.4
Female	37.4 ± 6.5	34.2 ± 4.9	39.5 ± 5.6	39.2 ± 6.3	38.5 ± 5.3	35.9 ± 7.2

Values are mean ± SEM at 15 (N15) and 45 (N45) min of normoxia, 15 (H15) and 45 (H45) min of hypoxia, and 15 (R15) and 45 (R45) min of recovery for 16 male-bearing and 15 female-bearing ewes.

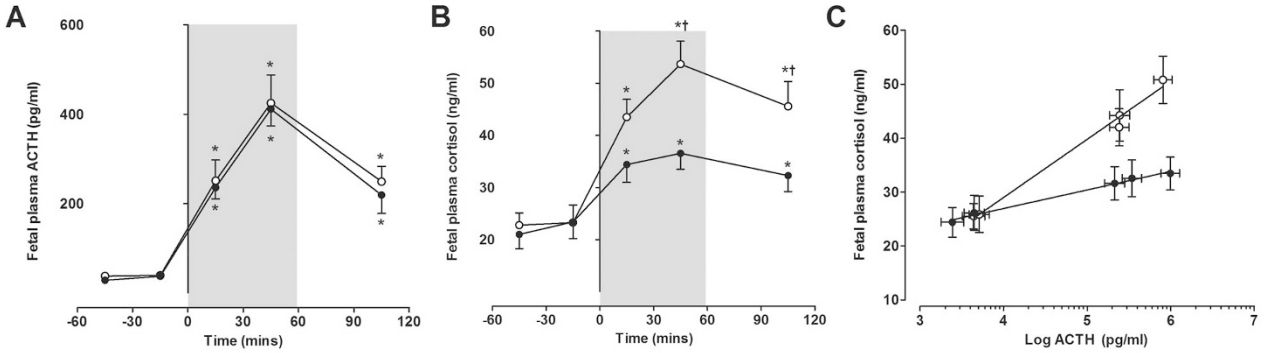

Figure 1. Male and female fetal HPA axis function during hypoxic stress. Values are mean ± SEM for plasma ACTH (*A*) and cortisol (*B*) concentrations at 15 (N15) and 45 (N45) min of normoxia, 15 (H15) and 45 (H45) min of hypoxia (*shading*), and 15 (R15) and 45 (R45) min of recovery in 16 male (○) and 15 female (●) fetuses during the experimental protocol. (*C*) Relation between values for ACTH and cortisol in male and female fetuses. Significant differences ($p < 0.05$): *, differences by post hoc analysis indicating a significant main effect of time compared with normoxia; †, differences by post hoc analysis indicating a significant main effect of sex. Analysis of slopes and intercepts revealed a significant main effect of sex on the slopes, but not the intercept, of the ACTH and cortisol relationship.

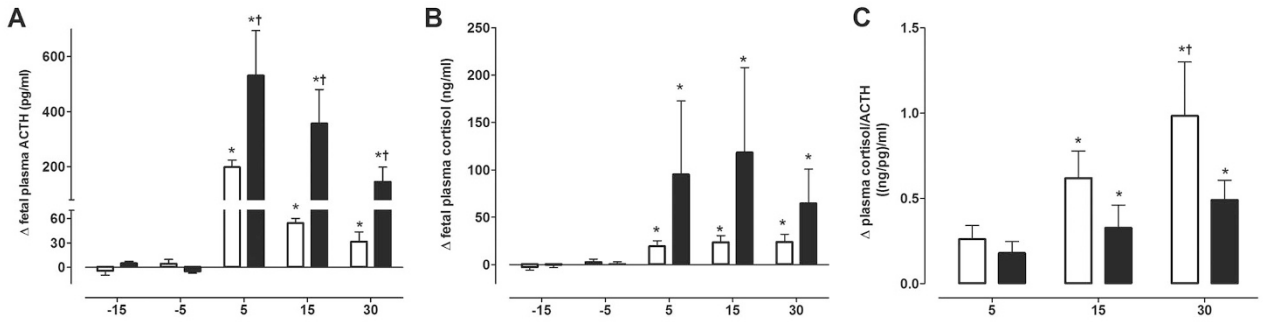

Figure 2. Adrenal cortical response to synthetic ACTH in the male and female fetuses. Values are mean ± SEM for the change from baseline in plasma ACTH (*A*) and cortisol (*B*) concentrations before (−15 and −5 min) and at 5, 15, and 30 min after i.v. treatment with exogenous ACTH (2.5 μg bolus; Synacthen) in six male (□) and six female (■) fetuses. (*C*) Values are the mean ± SEM increment from baseline in the ratio of plasma cortisol to plasma ACTH. Significant differences ($p < 0.05$): *, differences by post hoc analysis indicating a significant main effect of time compared with baseline; †, differences by post hoc analysis indicating a significant main effect of sex.

concentration of measured fetal plasma ACTH (cortisol/ACTH ratio), male fetuses compared with female fetuses showed a significantly greater increment in plasma cortisol at 30 min after the ACTH bolus administration ($F = 3.27$, $p = 0.02$; Fig. 2*C*).

DISCUSSION

Fetal hypoxia is one of the major challenges that the unborn child may face during gestation (28,29). Reductions in fetal oxygenation may occur in healthy pregnancies during compressions of the umbilical cord (30), in high-altitude pregnancy (31), or during spinal or epidural anesthesia (32). In complicated pregnancies, fetal hypoxia is common and may develop as a result of maternal diabetes, preeclampsia, Rhesus sensitization, maternal infection, sickle cell anemia, chronic substance abuse, asthma, and/or smoking (33). Much of our basic scientific and clinical knowledge on the fetal physiological and endocrine responses to hypoxic stress has been obtained from seminal studies using the chronically instrumented, unanaesthetized fetal sheep preparation. The ovine fetal HPA axis responds to hypoxic stress in late gestation with increases in arterial plasma concentrations of ACTH and adrenocortical output of cortisol (34–36). The data in this study show that the male fetus responds to the same magni-

tude and duration of hypoxia with a much greater increment in plasma cortisol, despite a similar increment in plasma ACTH during the acute episode of stress in both sexes. Indeed, this sex difference exists despite a greater fall in pH_a in the female fetus during hypoxia, which itself is known to exacerbate the plasma cortisol response (37). To isolate one particular element of this response, we specifically stimulated the adrenal glands of fetuses under normoxic conditions to determine whether adrenal glands from male fetuses were more responsive to exogenous ACTH. Although the data were more variable than those collected during hypoxia, treatment of the fetus with exogenous ACTH led to an enhanced cortisol response per mole of ACTH in male *versus* female fetuses. Combined, these data show a sexually dimorphic fetal cortisol response to intrauterine hypoxic stress and that a part of the greater plasma cortisol response in the male compared with female fetus is mechanistically linked to sex-specific changes in adrenocortical sensitivity to ACTH.

Several factors can affect adrenocortical sensitivity. Only relatively recently has the importance of the innervation of the gland on adrenocortical secretion been determined. Thus, Edwards and Jones (38) using conscious, hypophysectomized calves showed that stimulation of the splanchnic input to the adrenal gland doubled, whereas splanchnic denervation halved (39), the output of cortisol in response to an exogenous infusion of ACTH. In the fetus, similar neural mechanisms operate in the control of stimulated adrenocortical secretion, because functional innervation of the ovine fetal adrenal gland is present by the final third of gestation (40,41), and Myers *et al.* (42) showed that splanchnic nerve section in the ovine fetus had no effect on basal plasma cortisol concentration but significantly attenuated the cortisol increment during acute hypotensive stress. Other mechanisms that may affect adrenocortical sensitivity in the fetus include the actions of neuropeptides such as vasoactive intestinal peptide, corticotropin releasing hormone (CRH), and the eicosanoid prostaglandin E2 (PGE2), because all have been shown to promote steroidogenesis, even in the absence of changes in circulating ACTH (43). It is also possible that the density of ACTH receptors in the fetal adrenal may differ between the sexes. However, to date, there have been no reports, even in the adult, on sex-specific differences in adrenocortical responsiveness resulting from alterations in sympathetic control, or in adrenocortical sensitivity to neuropeptides or eicosanoids, or in the expression of ACTH receptors within the adrenal gland, warranting further investigation in all of these areas of interest.

The metabolic responses to acute hypoxia in the late gestation fetus involve an increase in the circulating concentrations of glucose and lactate (44). The fetal hyperglycemia results from decreased glucose uptake and utilization by peripheral tissues (45) and an increase in hepatic glucose production (46). Fetal lactic acidemia results from anaerobic metabolism of glucose in hypoxic fetal tissues, particularly in the carcass where blood flow and oxygen delivery are markedly declined. Increased sympathetic outflow inhibits insulin release from the fetal pancreas, thereby decreasing glucose uptake and utilization by the fetal tissues (45). As hypoxia progresses, catecholamines (47) and neuropeptide Y (NPY; 48) are released into the fetal circulation and act to maintain the peripheral vasoconstrictor response. In addition, catecholamines are also known to mobilize and release glucose from glycogen stores in the fetal liver (49). The greater acidemic response to hypoxia in the female compared with the male fetus may therefore represent a greater adrenergic and/or peripheral constrictor response to the challenge, warranting focus on these outcome variables in future studies.

In adulthood, it is established that hypercortisolemia is associated with increased morbidity and mortality (50) and that cardiovascular morbidity and mortality are much higher in men than similarly aged premenopausal women (51). Ovarian hormones have long been thought to explain the higher resistance of females to cardiovascular stress because estrogen replacement therapy can reduce postmenopausal morbidity by ~50% (51). Our study is the first to show that a sex-specific adrenocortical response to stress is already established during the fetal period, a time when the major source of the high circulating concentrations of estrogen in plasma of male or female fetuses is the placenta. A component of the well-described increased susceptibility to stress in men may therefore be predetermined even before birth, independent of exposure to maternal antenatal exogenous steroid therapy, changes in sex hormones throughout the postnatal life course, or environmental influences determined by lifestyle.

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