Adrenalectomy alters regulation of blood pressure and endothelial nitric oxide synthase in sheep: modulation by estradiol

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Li F, Wood CE, Keller-Wood M. Adrenalectomy alters regulation of blood pressure and endothelial nitric oxide synthase in sheep: modulation by estradiol. Am J Physiol Regul Integr Comp Physiol 293: R257–R266, 2007. First published April 25, 2007; doi:10.1152/ajpregu.00082.2007.—Hypoaldrenocorticism produces more severe hypotension during the peripartal period in pregnant ewes and women. We hypothesized that estradiol increases the severity of hypotension after withdrawal of corticosteroids and that this results from combined effects of adrenalectomy and estradiol to increase endothelial nitric oxide synthase (eNOS). In study I, blood pressure and eNOS mRNA and protein in aorta, uterine, renal, and mesenteric arteries were measured in intact ewes or adrenalectomized ewes 18–20 h after cessation of infusion of cortisol and aldosterone; half of each group ewes were treated with estradiol. In study II, adrenalectomized ewes were similarly studied 22–28 h after withdrawal of corticosteroids. Estradiol treatment in both studies significantly increased eNOS mRNA and protein in uterine artery, whereas corticosteroid withdrawal decreased expression of eNOS mRNA and protein in uterine artery. In both studies, adrenalectomy and steroid withdrawal decreased mean arterial pressure. In study II, four of six adrenalectomized ewes not treated with estradiol showed dramatic phasic variations in blood pressure and heart rate with a period of ~20 s, developing within 22–28 h after corticosteroid withdrawal. Although there was no effect of estradiol on blood pressure in study I, in study II, ewes treated with estradiol did not develop this pattern. Estradiol also slowed both the decline in plasma sodium and the rise in plasma potassium after corticosteroid withdrawal. These results disprove the hypothesis that estradiol increases the severity of hypotension during hypoaldrenocorticism. However, the study reveals an important effect of corticosteroid withdrawal on blood pressure, consistent with corticosteroid modulation of baroreflex responsiveness.

hypocorticoid; blood pressure; hypotension; cortisol

DURING PREGNANCY, BOTH ESTROGEN and cortisol concentrations normally increase. In previous studies, we have demonstrated that the increase in maternal cortisol is important in maternal volume expansion in late pregnancy (11) and that experimentally induced reduction in maternal cortisol has implications for both maternal and fetal physiology (12, 13). In pregnant ewes, reduction in maternal cortisol increases the incidence of maternal and fetal death at term (17) and maternal death in the immediate postpartum period, indicating the importance of the increase in maternal cortisol in late gestation. Addison’s disease can be successfully managed in pregnant women but requires increased replacement doses of glucocorticoid during times of stress (1, 39). There have been several case reports of hypoadrenal crisis in women after delivery (8, 28, 30, 38), and therefore it is recommended that women with Addison’s disease be treated with higher glucocorticoid doses at delivery and in the immediate postpartum period (35, 41, 45). These clinical reports and our observation in sheep suggest that increased glucocorticoid action may be critical for normal cardiovascular homeostasis, particularly at the end of pregnancy.

In nonpregnant human and animal subjects, corticosteroids have been shown to have an important role in control of blood pressure. Addison’s disease, hypoaldrenocorticism, is characterized by severe hypotension and hypovolemia, accompanied by hyponatremia and hyperkalemia. In human subjects, hypotension leads to death if corticosteroids are not administered (23). We have developed a model of hypoaldrenocorticism in sheep, a model that reproduces the known consequences of hypoaldrenocorticism in human beings (29). Although the effects of cortisol are not completely understood, corticosteroid action is thought to be important within the central nervous system (CNS), in the kidney, as well as directly on vascular responsiveness to vasoconstrictors (23). There is some evidence that cortisol may affect vasoconstriction by an inhibitory effect on endothelial NO generation in vessels (32, 48, 49). Glucocorticoids have also been shown to inhibit inducible NOS in tissues and neural NOS in neurons (6, 19, 22, 31, 40).

The increase in plasma estradiol in pregnancy is thought to mediate the decrease in mean arterial pressure and peripheral resistance in pregnancy through affecting the decrease in uterine vascular resistance (33, 43). During ovine pregnancy, uterine vascular responses to ephedrine (20) are decreased, and uterine artery endothelial nitric oxide synthase (eNOS) mRNA is increased (26). Estradiol also increases eNOS abundance in the uterine artery of nonpregnant ewes, suggesting that the reduced uterine resistance and decreased response to vasoconstrictors during pregnancy is caused by estrogen stimulation of eNOS expression and NO generation in the uterine endothelium (34, 43).

Because pregnant ewes and pregnant women appear to be the most vulnerable to Addisonian crisis very late in gestation, when the estrogen-to-progesterone ratio is greatest, we hypothesized that the vascular effects of estrogen were critical in producing the hypotensive crisis. We hypothesized that the increased cortisol and aldosterone levels, characteristic of human and ovine pregnancy, may be a balance to the vasodilatory effects of increased estradiol and, in particular, to the stimulatory effect of estradiol on endothelial NOS. We therefore tested whether the effects of corticosteroid...
DYSREGULATION OF BLOOD PRESSURE AND eNOS AFTER ADRENALECTOMY

MATERIALS AND METHODS

Experimental Procedures

Two studies were performed. The first study focused on relatively short-term (18–20 h) reduction in plasma corticosteroid concentrations. The second study focused on a longer time course (28 h) of reduction in plasma corticosteroids.

**Study I.** For this study, 22 adult ewes of mixed Western breeds were studied. The ewes were assigned to one of four groups at the time of surgery: adrenal-intact (ADI; n = 6), adrenal-intact and treated with estradiol (ADI+E2; n = 4), adrenalectomized (ADX; n = 6), or adrenalectomized and treated with estradiol (ADX+E2; n = 6).

Surgery was performed as previously described (18). Ewes were fasted ~24 h before surgery. All ewes were subjected to bilateral adrenalectomy and bilateral ovariohysterectomy, and catheterization of both femoral arteries and veins, as previously described (18). Catheters were filled with heparin (1,000 U/ml; Elkins-Sinn, Cherry Hill, NJ), plugged with sterile nails, and routed to the flank. Ewes were treated with antibiotics (750 mg of ampicillin; Polypoxy; 750 mg im bid; Fort Dodge Animal Health, Fort Dodge, IA) for 5 days after surgery. The body temperature of each ewe was measured daily and before each experiment to monitor the health of the animal.

After surgery, animals in the ADX groups were continuously treated with cortisol and aldosterone for 9 days by infusion via a femoral venous catheter. Aldosterone (Steraloids, Wilton, NH) was infused at the rate of 3 μg·kg⁻¹·day⁻¹. Cortisol hemisuccinate was infused at 1 mg·kg⁻¹·day⁻¹ for the first day postoperatively, and then at 0.5 mg·kg⁻¹·day⁻¹ for the second postoperative day. Thereafter, cortisol was infused at 0.3 mg·kg⁻¹·day⁻¹. Estradiol was infused intravenously at 4 μg·kg⁻¹·day⁻¹ (17β-estradiol; Sigma-Aldrich, St. Louis, MO) following an intravenous bolus of 17β-estradiol of 5 μg/kg. This treatment regimen has been reported to produce uterine vascular vasodilation (24, 25, 43). In ADX ewes, the hypoadrenal state was produced by stopping the cortisol and aldosterone infusion on postoperative day 9. Estradiol infusion was continued throughout the remainder of the experiment.

The experimental design for study I is depicted in Fig. 1. In study I, plasma sodium, potassium, and protein concentration were measured at 2-h intervals beginning 8 h after the end of the infusion of cortisol and aldosterone. The ewes were carefully monitored until the plasma potassium concentration exceeded 6.0 meq/l, as defined in our protocol approved by the University of Florida Institutional Animal Care and Use Committee (IACUC). These levels were reached 18–20 h after the end of the infusion of cortisol and aldosterone. Basal mean arterial pressure (MAP) and heart rate (HR) were recorded, and blood samples were collected for plasma hormone, electrolyte, protein, and PCV determinations at 0 h and 12–20 h. Blood pressure and HR were measured using pressure transducers and a National Instruments analog-to-digital conversion board (LabView, National Instruments, Austin, TX). HR and blood pressure were sampled for 10-min periods every hour.

Pressor responsiveness was tested as the blood pressure response to phenylephrine (3 μg/kg bolus) 18–20 h after stopping infusion of corticosteroids in vivo. Following the return of blood pressure (about 20 min), a bolus of nitro-1-arginine methyl ester (L-NAME) (Sigma; 10 mg/kg iv) was administered, and after 5 min, the same bolus dose of phenylephrine was repeated. Vascular responsiveness was calculated as the MAP in the first minute after infusion of phenylephrine minus MAP for the 30 s before infusion of phenylephrine. Blood pressure and heart rate were measured throughout the test period.

Study II. To determine the effects of estradiol in more hypotensive ewes, a further study was performed in which blood pressure was monitored from 20–28 h after stopping the infusion of cortisol and aldosterone. Twelve adult ewes of mixed Western breeds were studied; all animals were adrenalectomized and assigned to either ADX (n = 6) or ADX+E2 (n = 6) groups. These ewes were studied in pairs consisting of one estradiol-treated and one non-estradiol-treated ewe. The experimental design for study II is depicted in Fig. 1. Before stopping the steroid infusion, basal blood pressure was measured, and blood samples were collected. Blood pressure and plasma electrolytes were also measured 12 h after stopping infusion of corticosteroids to assure animals were not hypotensive. Beginning 20 h after stopping corticosteroids infusion, plasma glucose, sodium, potassium, and protein concentration were measured at 2-h intervals, and blood samples also were collected for further hormone assays. Tests of vascular reactivity were performed using the same protocol as in study I and were designed to be performed at 28 h. In the cases in which ewes showed signs of severe, marked variations in blood pressure and respiratory frequency, suggesting loss of normal blood pressure control mechanisms, the protocol for testing vascular reactivity was performed, and the animals were killed before 28 h. This termination was consistent with our IACUC approval to complete the study if arterial pressure were reduced to levels less than 60 mmHg. This pattern was noted in four of six non-estradiol-treated adrenalectomized ewes: one at 22 h, one at 24 h, one at 26 h, and one at 28 h; the pattern was absent in the paired estradiol-treated adrenalectomized ewes at these times. The tests of vascular reactivity were performed in both the non-estradiol-treated ewe and their paired estrogen-treated ewe at the same time, and both ewes were subsequently killed to obtain tissues. The other two non-estradiol-treated ewes and their estradiol-treated paired ewes did not show this marked blood pressure variability and were studied at 28 h.
Blood Collection and Handling

All blood samples for hormone analysis were collected in centrifuge tubes containing EDTA (0.015 M Na$_2$ EDTA; Sigma) and centrifuged at 3,000 g at 4°C, and plasma aliquots were frozen at −20°C until time of assay. The total volume of blood sampled was ≈50 ml for the experimental day, which should not have an effect on circulating hormones and/or arterial blood pressures.

Samples for determination of plasma Na$^+$ and K$^+$ concentration were taken in heparinized syringes. Plasma Na$^+$ and K$^+$ concentration were determined by ion-specific electrodes (ABL 77 blood gas analyzer; Radiometer, Copenhagen, Denmark). Packed cell volume (PCV) was measured with a microcapillary centrifuge and reader (Damon/IEC Division, Needham Heights, MA) to the nearest 0.5%. Plasma protein was measured with a refractometer (Fisher Scientific, Waltham, MA) and read to 0.1 mg/100 ml of plasma.

Plasma estradiol concentration was measured using an ELISA kit (Oxford Biomedical, Oxford, MI), following estradiol extraction with ethyl ether. Recovery of estradiol was calculated using the addition of estradiol to an aliquot of plasma; all estradiol values were corrected for recovery. Plasma cortisol was measured using a radioimmunoassay developed in our laboratory (47), and plasma aldosterone was measured using a modification of a commercial assay kit (Diagnostic Products), as previously described (11).

Necropsy

At the end of the experiment, all animals were anesthetized with pentobarbital sodium. After achieving surgical-depth anesthesia, the uterine artery, a section of mesenteric artery, both renal arteries, and the aorta were sequentially collected as quickly as possible and placed in Krebs solution (Sigma). Animals were then euthanized by an overdose of pentobarbital, and other tissues were collected and rapidly frozen in liquid nitrogen and then stored at −80°C for further protein and mRNA determinations.

Endothelial cells were isolated from the whole blood vessels immediately after collection, as described by Rupnow et al. (34) and Vagnoni et al. (43). Throughout the dissection, the vessels were kept on ice in Krebs buffer. The blood was washed off, the adventitia was removed, the vessels were opened with scissors, and endothelial cells were scraped using a round end spatula and collected in the lysis buffer and TRIZol for protein and mRNA determinations, respectively. The remaining vessel (without endothelia) and pieces of whole vessels without dissection of endothelia were also collected. RNA was immediately extracted using TRIZol (Life Technologies, Gaithersburg, MD) and frozen at −80°C for later analysis.

In the first series of experiments, mRNA for eNOS mRNA determination was successfully isolated from endothelial cells collected from uterine artery and aorta; however, the mRNA collected from renal and mesenteric artery endothelium was degraded and unable to be used. In the second series, we were unable to obtain eNOS mRNA from the endothelial samples in many of the animals. We believe this is because the very low blood pressure in these ewes resulted in very poor perfusion, especially once the pentobarbital sodium was administered. In the second series, therefore, we report only whole blood vessel eNOS expression.

Western Blot Analysis

Tissue eNOS protein levels in endothelia and whole vessel were estimated using a semiquantitative Western blot analysis procedure using an antibody specific for eNOS (Transduction Laboratories, Lexington, KY). Protein was loaded on 7.5% polyacrylamide, Tris-HCl gel (Bio-Rad, San Rafael, CA) and electrophoresed at 200 V for 1 h. Molecular weight markers and positive controls (human endothelial cell) were also loaded on each gel. In preliminary experiments, for eNOS, endothelial cell and vascular smooth muscle cell homogenates from the vessels collected in study I were tested to assure that endothelia had been obtained by the dissection procedure. Protein was visualized using the enhanced chemiluminescence reagent (Amersham, Piscataway, NJ). The density of bands was quantified by Quantity One densitometric analysis software (Bio-Rad, San Rafael, CA). The results of the densitometry were expressed as relative optical density (OD) units and normalized to the β-actin signal on the same blot.

Two-Step Real-Time RT-PCR

Messenger RNA expression for eNOS was determined by quantitative real-time PCR method, using a two-step method. First, 2 μg total mRNA were converted into cDNA; then 10 ng of cDNA was used in the PCR reaction to quantitate the mRNA of the gene of interest. Sequences for probes and primers have been previously reported (46).

All samples were analyzed in triplicate. Samples from all animal groups in each study were analyzed for a single gene on the same plate, along with control wells with no template added for each primer/probe set. For all genes, the change in cycle threshold (ΔC$_T$) was calculated for each sample as the difference in mean C$_T$ of mRNA for eNOS relative to 18S RNA. Fold changes relative to the control group in each study were also calculated 2$^{-ΔΔC_T}$ with ΔΔC$_T$ = ΔC$_T$ of experimental sample-mean ΔC$_T$ of samples from control animals (21). In study I, the ADI group served as a control. In study II, ADX served as a control. Statistical analysis for mRNA data was done by using ΔΔC$_T$, as this variable should be linearly distributed.

Statistics

Data from study I were analyzed by two-way ANOVA to test for main effects of estradiol treatment and adrenalectomy, as well as for the interaction between adrenalectomy and estradiol on each variable. Differences between individual means were analyzed using Duncan’s post hoc test. Data from the two groups in study II were analyzed using Student’s t-test. All values are reported as the means ± SE.

RESULTS

Plasma Cortisol, Aldosterone and 17β-Estradiol Concentration

Plasma cortisol and aldosterone concentrations were significantly decreased in adrenalectomized ewes compared with adrenal intact groups in study I and decreased after steroid withdrawal in both groups of adrenalectomized ewes in study II (Tables 1 and 2). Plasma estradiol concentration was significantly increased in both ADI and ADX ewes infused with 17β-estradiol. Estradiol was significantly greater in ADX+E2 ewes after cortisol and aldosterone withdrawal on day 10 compared with ADI+E2 on day 10.

Plasma Electrolyte, Protein, and Hematocrit

In adrenalectomized ewes, plasma K$^+$ and protein concentrations were significantly higher, and plasma Na$^+$ concentrations were significantly lower, than those in the ADI group or before withdrawal of cortisol and aldosterone (Tables 1 and 2). In study I, there were no significant differences in any of these variables between ADI and ADI+E2 groups or between ADX and ADX+E2 groups. In study II, ewes in the ADX+E2 group had significantly higher plasma K$^+$ and lower plasma Na$^+$ than ewes in the ADX group (Table 2), and the changes in plasma electrolytes occurred at an earlier time point in the ADX ewes.

Statistics

Data from study I were analyzed by two-way ANOVA to test for main effects of estradiol treatment and adrenalectomy, as well as for the interaction between adrenalectomy and estradiol on each variable. Differences between individual means were analyzed using Duncan’s post hoc test. Data from the two groups in study II were analyzed using Student’s t-test. All values are reported as the means ± SE.
without estradiol treatment (Fig. 2). PCVs of ADX+E2 ewes were significantly higher than those in the adrenal intact groups in study I, but there was no significant difference in either PCV or plasma protein between the ADX and ADX+E2 groups in study I or study II.

Effect of 17β-Estradiol on MAP

After withdrawal of corticosteroid for 18–20 h, the MAP values among the four groups of ewes in study I were not significantly different (Fig. 3). However, the changes in MAP over time between days 9 and 10 were significantly greater in the ADX groups after withdrawal from corticosteroids compared with the ADI groups (ADX: −10.8 ± 3.2, ADX+E2: −15.4 ± 5.2, respectively) (Fig. 3).

In study II, which featured a longer study period after steroid withdrawal, the change in arterial pressure calculated as the mean value over 10 min also did not differ between the ADX and ADX+E2 groups (Fig. 4). However, the temporal pattern of arterial pressure was qualitatively different in the two groups of ewes. In the ADX+E2 ewes, MAP dropped to 70–80 mmHg and was sustained at this level until death. In the ADX ewes not treated with estradiol, changes in arterial pressure varied greatly between animals, and the experiment was terminated in 3 animals before 28 h due to periods of hypotension in the ADX group without estradiol treatment; four of six ewes exhibited profound rhythms in blood pressure occurring 22–26 h after corticosteroids withdrawal (Fig. 5). Although respiratory rate was not measured in these studies, changes in respiration occurring out of phase with the cardiac rhythm, but with the same periodicity, were also observed by the investigators. None of the adrenalectomized ewes treated with estrogen showed this pattern at the time of death.

Effect of l-NAME Administration on MAP

After l-NAME treatment, MAP was significantly increased in all groups. In study I, the increase in MAP after l-NAME

Table 1. Study I. plasma hormones, electrolytes, protein concentration, and packed cell volume at end of study

<table>
<thead>
<tr>
<th></th>
<th>ADI</th>
<th>ADI+E2</th>
<th>ADX</th>
<th>ADX+E2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol, ng/ml</td>
<td>6.4±3.6</td>
<td>7.5±4.4</td>
<td>1.6±1.3†</td>
<td>1.3±0.9†</td>
</tr>
<tr>
<td>Aldosterone, pg/ml</td>
<td>69±29</td>
<td>50±28</td>
<td>12±0.9†</td>
<td>14±2†</td>
</tr>
<tr>
<td>17β-E2, pg/ml (day 9)</td>
<td>(32±5) 38±9</td>
<td>(100±40) 64±27*</td>
<td>(22±6) 64±27</td>
<td>(86±9) 163±16†</td>
</tr>
<tr>
<td>Na⁺, meq/l</td>
<td>144.2±1.3</td>
<td>144.8±1.9</td>
<td>135.2±3.3†</td>
<td>138.6±2.5†</td>
</tr>
<tr>
<td>K⁺, meq/l</td>
<td>4.3±0.2</td>
<td>4.2±0.3</td>
<td>7.8±1.1†</td>
<td>7.0±1.8†</td>
</tr>
<tr>
<td>PCV, %</td>
<td>28±3</td>
<td>28±6</td>
<td>37±9</td>
<td>41±7†</td>
</tr>
<tr>
<td>Protein, mg%</td>
<td>7.7±0.4</td>
<td>7.6±0.4</td>
<td>9.4±0.1†</td>
<td>9.1±0.7†</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *Significantly different than ADI (P < 0.05). †Significantly different than ADI+E2 (P < 0.05). ADI, ovariectomized, adrenal-intact; ADI+E2, ovariectomized, adrenal-intact and infused with estradiol; ADX, ovariectomized and adrenalectomized; ADX+E2, ovariectomized, adrenalectomized and infused with E2. PCV, packed cell volume 17β-E2, 17β-estradiol.

Table 2. Study II. plasma hormones, electrolytes, protein concentration, and PCV before and after withdrawal of corticosteroids

<table>
<thead>
<tr>
<th></th>
<th>ADX Day 9</th>
<th>ADX+E2 Day 9</th>
<th>ADX Day 10</th>
<th>ADX+E2 Day 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol, ng/ml</td>
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<td>6.9±0.9</td>
<td>0.7±0.3*</td>
<td>0.5±0.2*</td>
</tr>
<tr>
<td>Aldosterone, pg/ml</td>
<td>38±6</td>
<td>37±4</td>
<td>&lt;12*</td>
<td>&lt;12*</td>
</tr>
<tr>
<td>17β-estradiol, pg/ml</td>
<td>31±5</td>
<td>74±11</td>
<td>57±8</td>
<td>137±28</td>
</tr>
<tr>
<td>Na⁺, meq/l</td>
<td>145.5±0.6</td>
<td>144.3±0.9</td>
<td>137.2±1.0*</td>
<td>138.2±1.4*</td>
</tr>
<tr>
<td>K⁺, meq/l</td>
<td>3.7±0.1</td>
<td>3.6±0.2</td>
<td>7.3±0.5*</td>
<td>6.4±0.7†</td>
</tr>
<tr>
<td>PCV, %</td>
<td>26±3</td>
<td>27±5</td>
<td>38±6*</td>
<td>36±10*</td>
</tr>
<tr>
<td>Protein, mg%</td>
<td>7.7±0.3</td>
<td>8.0±0.5</td>
<td>8.6±0.9</td>
<td>8.8±0.5*</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. *Significantly different than day 9 (before withdrawal of cortisol and aldosterone) (P < 0.05). †Significantly different than ADX (P < 0.05).
treatment was significantly reduced in all groups treated with estradiol relative to the respective groups without estradiol treatment (Fig. 3). In study II, however, there were no differences between the response to L-NAME in the ADX and ADX/E2 groups (Fig. 4).

Vascular Responsiveness

Injection of phenylephrine increased the MAP in all groups of animals (Fig. 6). In study I, there were no significant differences in the magnitude of the MAP response to phenylephrine among the groups of ewes, either before or after injection of L-NAME (Fig. 6). In study II, the MAP response to phenylephrine was significantly reduced in the ADX ewes, although the response in the ADX+E2 ewes was similar to that in study I. There was no effect of L-NAME injection on the responsiveness to phenylephrine.

Expression of eNOS mRNA and Protein by Endothelial Cells

In study I, adrenalectomy significantly reduced the expression of eNOS mRNA in aortic and uterine arterial endothelial cells. Estrogen treatment upregulated the abundance of eNOS mRNA in endothelial cells from uterine artery (Fig. 7). Expression of eNOS in uterine artery endothelium was significantly reduced in ADX compared with ADI ewes; estrogen treatment maintains expression in ADX ewes at levels not different than ADI ewes. In whole vessels, expression of eNOS mRNA in the aorta was significantly increased in intact estrogen-treated ewes compared with non-estrogen-treated ewes.

Expression of eNOS protein level tended to be decreased by ADX and increased with estradiol in the four types of endo-

Fig. 3. Top: mean arterial pressure (MAP) in study I at 0 h before (open bars) and at 20 h after (hatched bars) corticosteroid withdrawal. *P < 0.05, compared with before corticosteroid withdrawal. Bottom: MAP before (open bars) and 5 min after (hatched bars) injection of L-NAME. *P < 0.05, compared with before L-NAME. ADI (n = 6), ADI+E2 (n = 4), ADX (n = 6), and ADX+E2 (n = 6). (ADI: adrenal gland intact; ADX: adrenalectomized; E2: treated with 17β-estradiol.)

Fig. 4. Top: MAP from 0 to 28 h after withdrawal of corticosteroids (n = 6 at 0–22 h, n = 4 at 24 h, and n = 3 at 26–28 h) *Significantly different than time 0, P < 0.05. Bottom: MAP before (open bars) and 5 min after (hatched bars) injection of L-NAME. *P < 0.05, compared with before L-NAME ADX (n = 6), ADX+E2 (n = 6).
thelial cells. In uterine artery endothelial cells, estrogen treatment significantly increased eNOS protein expression, and ADX significantly decreased eNOS expression. In aortic endothelial cells, there was also a statistically significant overall stimulation of eNOS by estradiol and a tendency for suppression of eNOS with adrenalectomy, which was not significant; however, there were no significant differences in levels of eNOS protein between any two groups (Fig. 7). In mesenteric artery, only the overall inhibitory effect of adrenalectomy on eNOS was significant.

In study II, whole vessel eNOS mRNA shows a similar pattern; expression of eNOS mRNA was significantly increased by estradiol treatment in uterine artery (Fig. 8). Similarly, although eNOS protein expression tended to be increased in all vessels from ADX ewes treated with estradiol, this increase was only significant in the uterine artery.

DISCUSSION

In this study, we used an adrenalectomized sheep model to study the interaction between cortisol and estradiol on blood pressure and vascular reactivity. We had hypothesized that estradiol treatment would result in a more precipitous decline in blood pressure and vascular reactivity in hypoadrenal animals. We had further hypothesized that adrenalectomy would result in increased eNOS expression in endothelial cells, and that this increase would be augmented by estrogen treatment. However, the results of these experiments revealed that estradiol did not exacerbate hypotension or loss of vascular reactivity in adrenalectomized ewes. Although estradiol increased eNOS expression as expected in uterine artery, hypoadrenocorticism decreased eNOS expression in most of the vascular beds studied; this effect was most consistent in the uterine artery.

The reduction in eNOS expression in the adrenalectomized animals was an unexpected finding. Glucocorticoids increase the vasoconstrictive effects of catecholamines and ANG II (2), and conversely patients in Addisonian crisis have reduced vasoconstrictor responses to vasodilators (51). Previous studies in cultured endothelia and isolated vessels had suggested that glucocorticoids reduce eNOS expression in endothelia. In cultures of bovine coronary artery endothelia, cortisol has been shown to cause dose-dependent decreases in NOx release and eNOS protein expression (32). Cortisol has also been shown to cause dose-related increases in the vasoconstrictor response to norepinephrine in isolated uterine artery (48); this effect was associated with a decrease in eNOS protein expression and local NOx production. Other studies have found that cortisol blocked or reversed the stimulatory effect of estradiol on eNOS mRNA in human umbilical vein endothelial cells in culture (49). Our own previous studies in adrenalectomized ewes found that adrenalectomy resulted in reduced constriction in response to phenylephrine and that administration of L-NAME normalized this response (29). This suggested that NOS was upregulated in these adrenalectomized ewes. One difference between the experiments in the prior study and this present study is the design. In our previous study (29), we used a within-animal design, meaning that each animal had experienced more than one period of hypoadrenocorticism. Although animals were then treated with steroid and allowed to recover before the next day of study, it is possible that these preceding periods of steroid loss increased the expression of eNOS or slowed turnover or degradation of eNOS. In study I, we also
did not find any significant decrease in response to phenylephrine with corticosteroid withdrawal; in fact, the response to phenylephrine tended to be increased in the ADX group, suggesting that the decreased blood volume might increase the effective dose.

In study II, the reduction in eNOS expression may be related to the poor perfusion in the adrenalectomized ewes rather than to a direct effect of corticosteroids on endothelial cell expression of eNOS. The cyclic pattern of blood pressure in many of the adrenalectomized ewes in study II suggests there would be periods of cessation of blood flow in these ewes. The reduction in eNOS could therefore be secondary to episodes of reduced shear stress during these periods of low or no flow. Shear stress is an activator of eNOS expression, and several factors, includ-

Fig. 6. Responses to injection of phenylephrine in ewes in study I (top) or study II (bottom). pe1, injection of 3 μg/kg phenylephrine; pe2, injection of 3 μg/kg phenylephrine 5 min after injection of L-NAME. ADI (open bars), ADI+E2 (light gray bars), ADX (dark gray bars) and ADX+E2 (black bars); n = 6 per group.

Fig. 7. Effects of adrenalectomy and estradiol treatment on eNOS in study I. Expression of eNOS mRNA in endothelial cells from uterine artery and aorta (top) or whole blood vessels (middle), and of eNOS protein (bottom) in uterine artery, aorta, mesenteric, and renal arteries in study I. OD, optical density. aSignificantly different than ADI, P < 0.05. bSignificantly different than ADX, P < 0.05.
expression in study II. In study I, on the other hand, there were smaller and less dramatic changes in arterial pressure, yet expression of eNOS in the uterine vasculature was significantly decreased in aorta and uterine artery in the adrenalectomized ewes without estradiol treatment. However, it is likely that blood volume, and therefore blood flow, is reduced in the ADX ewes in study I, as is suggested by the increases in both plasma protein and PCV. However, studies by others have shown that in ewes in which uterine vessels were occluded to cause an approximate 40% reduction in uterine flow, the ipsilateral uterine venous NOx levels were decreased, but there was no reduction in eNOS protein (15). Although the presence of longer periods of intermittent flow or changes occurring in systemic circulation might counterbalance the effect in these studies, these results suggest that the decrease in eNOS in the uterine vasculature in study I may not be secondary to changes in shear stress. It is not known if in the in vivo situation withdrawal from corticosteroids activate other endogenous factors that result in inhibition of eNOS selectively in the uterine circulation.

The chronic administration of estradiol administration did not reduce MAP and vascular reactivity in adrenal intact ewes in our study. These results differ from studies showing consistent decreases in mean pressure in nonpregnant ovariectomized ewes treated with 17β-estradiol doses similar to those used in our study (24, 43). However, in the previous studies (24), the ewes were ovariectomized and given replacement doses of estradiol, then withdrawn from estradiol for several days before the start of the estradiol infusion. Estradiol infusions produced greater increases in plasma estradiol in that study than are reported here. It is possible that the previous estradiol treatment enhanced the vascular responsiveness to the subsequent infusion of estradiol or altered estradiol clearance over the longer period of estradiol infusion. In our study, estradiol treatment did increase eNOS expression in uterine artery, as expected based on the previous studies by others showing increases in eNOS protein in uterine artery (43). We also found significant increases in eNOS in aorta in the estradiol-treated ewes.

The greater increase in estradiol after withdrawal of cortisol and aldosterone (on day 10 compared with day 9 in the adrenalectomized groups) was also somewhat surprising. Estradiol is known to increase plasma volume (24, 42), whereas hypoadrenocorticism decreases plasma volume. The increase in PCV in our studies would predict that volume decreases by ~40% after corticosteroid withdrawal. However, it is unlikely that the changes in volume alone account for the doubling in plasma estradiol over the 18- to 28-h period, and we suspect that changes in clearance of estradiol may contribute to the measured increase in plasma estradiol concentration.

Estradiol also appeared to reduce the severity of the hypertensive crisis in the animals in study II and to slow the progression of hyponatremia and hyperkalemia. At the present time we do not know whether these effects are linked. It is likely that estradiol increased plasma volume, as has been shown by others (24, 42). Estradiol may exert effects on sodium and potassium via effects on renal transport mechanisms. Estradiol is known to increase sodium retention (5, 7, 14). Although the mechanism of this effect is not completely understood, estradiol has stimulatory effects on Na+ uptake by proximal and distal tubule (3). This effect may be mediated by an increase in the density of thiazide-sensitive NaCl cotransporter in the distal convoluted tubule (44) and/or by an increase in expression of the alpha subunit of the epithelial sodium channel, as shown in the rat kidney (9). Estradiol has also been found to reduce plasma K+ in ovariectomized rats (50); it was proposed that this action is mediated by enhancement of the renal responses to aldosterone because female rats have greater response to aldosterone than do male rats. We found that the development of hyperkalemia in the estradiol-treated, aldosterone-withdrawn sheep was slower. Therefore, we propose that estradiol may more directly alter K+ retention in the kidney.

The pronounced dysregulation of blood pressure, HR, and respiratory rate observed in many of the adrenalectomized ewes without estradiol treatment in study II was also somewhat unexpected. This marked rhythmicity was found in four of six ewes in this group. At the present time, we do not know whether this pattern in blood pressure reflects changes in cardiac function, secondary to changes in plasma potassium or glucocorticoid effects on myocytes, and/or the effects of glucocorticoids on CNS cardiovascular regulatory regions. The regularity of this rhythm, both within and between ewes, and its apparent association with the respiratory rhythm suggest that there is a central nervous system component. The frequency of this rhythm is ~0.05 Hz. This frequency is similar
to that of Mayer waves in humans. Waves with frequency similar to Mayer waves are predicted from mathematical models of the baroreflex when gain is increased (4, 16). Modeling of the human baroreflex loop demonstrated a sharp peak in frequency at 0.07 Hz when the hypovolemic state was modeled; this same frequency also appears in arterial pressure fluctuations in healthy standing adults (10). Although Mayer waves are generally lower in amplitude than the phasic changes we observed, it is possible that the more dramatic waves we observed are secondary to the more severe hypovolemia, which occurs with corticosteroid withdrawal and/or to a more dramatic change in baroreflex sensitivity. Glucocorticoids decrease baroreflex sensitivity (36, 37), and withdrawal of corticosteroid infusion might be expected to increase the gain of the reflex, producing oscillations in interbeat interval similar to those measured in the present experiments. The apparent effect of estradiol to slow the appearance of the dramatic changes in blood pressure suggests that this effect may also be secondary to the effects of estradiol on plasma volume, or alter central baroreflex sensitivity, rather than the effects of estradiol on the vasculature.

We conclude that there is a dramatic effect of corticosteroid availability and of estradiol treatment on endothelial eNOS expression. We also conclude that estradiol slows the progression of hypoadrenocorticism. These results suggest that estradiol, despite increasing eNOS expression in endothelial cells, does not contribute to the increased susceptibility to hypoadrenocorticism in pregnancy.

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REFERENCES


