Angiotensin II infusion in vivo does not modulate cortisol secretion in the late-gestation ovine fetus

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Poore, Kirsten R., I. Ross Young, Benedict J. Canny, and Geoffrey D. Thorburn. Angiotensin II infusion in vivo does not modulate cortisol secretion in the late-gestation ovine fetus. Am. J. Physiol. 275 (Regulatory Integrative Comp. Physiol. 44): R357–R362, 1998.—Maturation of the fetal adrenal gland is critical for the onset of ovine parturition. It has long been proposed that the fetal adrenal gland may be under inhibitory influences during late gestation. In vitro evidence has suggested that angiotensin II may be such an inhibitory factor and may help to prevent a premature increase in cortisol concentrations. The aim of this study was to test the effect of angiotensin II infusion in vivo on basal cortisol concentrations and fetal adrenal responsiveness to an ACTH-(1—24) challenge. Fetuses received a continuous infusion of either angiotensin II (100 ng·min⁻¹·kg⁻¹; n = 7) or saline (2 ml/h; n = 4), which commenced at 140 days of gestation (GA) and continued for a total of 50 h. Adrenal responsiveness to the administration of ACTH-(1—24) (5 µg/kg) was determined during angiotensin II or saline infusions at both 2 and 48 h after infusion onset. Angiotensin II had no significant effect on adrenal responsiveness after acute (2 h) or chronic (48 h) infusion. There was no effect of saline or angiotensin II infusion on basal immunoreactive ACTH or cortisol concentrations after 2 h, but there was a significant increase in basal cortisol concentrations in both treatment groups by 48 h, probably reflecting the normal rise in cortisol concentrations at this GA. Mean arterial blood pressure was significantly increased in angiotensin II-infused fetuses only. This study has therefore found no evidence to suggest that angiotensin II infusion in vivo modulates fetal basal cortisol concentrations or adrenal responsiveness in the last week of gestation, in contrast with previous in vitro studies. These results throw into question the proposed role of angiotensin II as a negative modulator of adrenal function in the ovine fetus.

hypothalamo-pituitary-adrenal axis; cortisol concentration; adrenal function

IT IS CLEAR THAT ACTIVATION of the fetal hypothalamo-pituitary-adrenal axis is a critical event for the onset of parturition in the sheep. The responsiveness of the fetal adrenal gland to ACTH increases in late gestation, and this event has been thought to contribute to the prepartum increase in cortisol concentrations and hence the timing of parturition (see Ref. 20). In addition to the positive influence of ACTH on adrenal function in the fetus, it has been proposed that there may be some factors that have an inhibitory action on the adrenal gland, because removal of the adrenal gland from in vivo influences has been shown to result in an increased responsiveness to ACTH (20). Jones and Roe-buck (8) first reported a discrepancy between adrenal responses to ACTH in vivo and in vitro. Similarly, Durand et al. (7) demonstrated that adrenal cells in vitro developed enhanced responsiveness to ACTH as the time in culture increased. These investigators suggested that this phenomenon was due to the removal of an inhibitory factor(s) normally present in vivo and Jones and colleagues (8, 17) suggested that these factors were the high molecular weight ACTH-containing peptides (8, 17). Further studies have added support to this proposal (18; unpublished observations from our laboratory). It is a corollary of this proposal that the effects of such inhibitory factors must be withdrawn or overridden before term.

More recently, Rainey and colleagues (2, 13, 15) have suggested that angiotensin II may also have an inhibitory role in the regulation of adrenal responsiveness in ovine fetuses, because angiotensin II inhibits ACTH-induced cortisol production and 17α-hydroxylase activity in the adult and fetal sheep adrenal gland in vitro (2, 13, 15). It was therefore suggested that angiotensin II may act to prevent a premature increase in basal cortisol concentrations in late gestation (15). The action of angiotensin II, however, varies considerably between species, and the effect of angiotensin II on the fetal adrenal gland has not been examined in vivo. The aim of this study, therefore, was to test fetal adrenal responsiveness, as determined by cortisol responses to exogenous ACTH-(1—24) administration, during angiotensin II infusion in vivo. The effect of an acute (2 h) and chronic (48 h) infusion of angiotensin II was examined in intact fetuses at 140 days of gestation (GA), at which time adrenal responsiveness is normally increasing (20).

The angiotensin II infusion rate used in this study has been shown previously to increase plasma angiotensin II concentrations in fetal sheep from ~50 to 400 pg/ml (16). This infusion results in an increase in mean arterial blood pressure (MAP), a significant inhibition of plasma renin activity, a 58% increase in circulating aldosterone concentrations, and an increase in the urinary excretion rate of Na⁺ and Cl⁻, but no change in plasma electrolyte concentrations (16).

METHODS

Animal preparation. All procedures involving the use of animals were approved by the Standing Committee on Ethics in Animal Experimentation of Monash University. A total of 10 ewes was used in this study. The ewes were housed in individual metabolism cages and fed between 0900 and 1200 daily. Water was available ad libitum. One ewe carried twins, so both fetuses were studied. All other ewes carried single fetuses. Seven ewes and fetuses had previously been used for other experimental protocols, all of which terminated at least 5 days before any further experiments were conducted. These

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experiments included an overnight infusion of cortisol (3.5 mg/24 h) followed by a corticotropin-releasing factor challenge (1 µg; 3 fetuses), an ACTH-(1—24) challenge (2.5 µg/kg; 2 fetuses), or a 30-min hypoxic challenge (2 fetuses). Basal cortisol concentrations in these fetuses were all within the normal physiological range at the start of the current experiment.

Surgery was performed on ewes between 113 and 121 (118 ± 1) days GA with the use of aseptic techniques. General anesthesia was induced by intravenous administration of 20 mg/kg thiopentone sodium in water (Pentothal; Bomac Laboratories, NSW, Australia) and maintained after endotracheal intubation with 0.5—2% halothane (Fluothane; IC1). All fetuses were fitted with a carotid artery catheter and a double-lumen jugular vein catheter. A jugular vein was catheterized in all ewes. Fetal well-being was routinely monitored by measuring fetal arterial blood gases using an ABL30 blood gas analyzer and OSM2 hemoximeter (Radiometer, Copenhagen, Denmark). At the completion of all experiments, ewes and fetuses were killed by barbiturate overdose (Lethabarb; Arnolds of Reading, Victoria, Australia) administered via the maternal jugular vein catheter. Some animals were killed immediately after the completion of the experiment, whereas the remaining animals were used for other purposes in our laboratory and were allowed to continue to term.

Experimental protocols. All experiments were carried out at 140—142 days GA. Saline or angiotensin II infusion (2 ml/h) commenced on 140 days GA (day 1) via the jugular vein (catheter 1). Angiotensin II (Aeuspe, West Melbourne, Australia) was infused at a dose of 100 ng·min⁻¹·kg⁻¹ estimated fetal body weight (4). ACTH-(1—24) (Synacthen, Ciba Geigy Australia, Pendle Hill, NSW, Australia; 5.0 µg/kg) was first administered by rapid intravenous injection (catheter 2) 2 h after infusion onset (acute experiment). Fetal arterial blood samples (3 ml) were collected at 30 min, 15 min, and immediately before saline or angiotensin II infusion onset; at 105, 90, 75, 60, 30 min, and immediately before ACTH-(1—24) injection (time 0); and at 10, 30, 60, 90, and 120 min after ACTH-(1—24) injection. Blood samples were collected into chilled sterile tubes containing EDTA (BDH Chemicals; 50 µl/3 ml blood; 11.2% solution in 0.9% saline) and were centrifuged for 5 min at 3,000 rpm at 4°C. All plasma aliquots were stored immediately at −20°C until assayed for immunoreactive (ir)-ACTH and cortisol concentrations (see below). ACTH degradation inhibitor mix [containing aprotinin (1,000 kallikrein inhibitor units/ml), N-ethyl-maleimide (25 mg/ml, Sigma, St. Louis, MO), and EDTA (18.6 mg/ml)] was added (20 µl/ml plasma) to all plasma aliquots for ir-ACTH assay. Blood cells were returned to the fetus during the experiment in a volume of Hartmann’s solution (compound sodium lactate, Baxter Healthcare, Old Toongabbie, NSW, Australia) equal to that of the blood cells. Sterility was maintained throughout this procedure.

At the completion of the first ACTH-(1—24) challenge experiment, the saline or angiotensin II infusion was allowed to continue for a total of 50 h. Three fetal arterial blood samples (3 ml) were collected in the 30 min preceding 12, 24, and 36 h from the start of the infusion. At each of these time points, results from the three samples were averaged. At 48 h from the start of the infusion, a second ACTH-(1—24) challenge experiment (see above) was performed (chronic experiment).

Fetal arterial blood pressure was recorded from the carotid artery catheter throughout both ACTH-(1—24) challenge experiments (recording was interrupted during blood samples). MAP was measured immediately before infusion onset and every 30 min during the first ACTH-(1—24) challenge experiment. Each value was the average of that measured at three time points, each 2 min apart. Amniotic catheters were not implanted in these animals, hence MAP could not be corrected for amniotic pressure. MAP during the infusion was therefore expressed as a percentage of the control (before infusion onset) value on 140 days GA. MAP was also recorded for −0.5 h at 24 h after the start of the infusion and for −0.5 h to the second ACTH-(1—24) challenge experiment.

Adrenal responsiveness for each ACTH-(1—24) challenge experiment was measured by integrating the plasma cortisol response to ACTH-(1—24) (0—120 min) above the mean preinjection (−30–0 min) cortisol concentration.

Radioimmunoassays. Fetal plasma cortisol concentrations were measured by RIA after extraction with dichloromethane, as previously described (4). The intra- and interassay coefficients of variation were 9 and 18% (n = 21), respectively, at values of 85.0 ± 3.3 and 12.8 ± 0.5 ng/ml. The sensitivity of the assay was 1.2 ± 0.3 ng/ml (n = 20). The average recovery for the cortisol extraction procedure was 95 ± 1% (n = 21). Ir-ACTH concentrations in unextracted fetal plasma were measured by RIA using an antiserum generously donated by Dr G. E. Rice (Royal Women’s Hospital, Melbourne, Australia), as previously described (11). The intra- and interassay coefficients of variation were 7 (n = 10) and 20% (n = 22), respectively, at values of 645.1 ± 15.0 and 1,518.8 ± 65.5 pg/ml. The sensitivity of the assay was 16.8 ± 4.1 pg/ml. The ACTH antiserum crossreacts with peptides containing the ACTH-(1—24) sequence, including the high molecular weight precursor peptides of ACTH.

Statistical analyses. All results are expressed as means ± SE. Data were first tested for homogeneity of variance using Bartlett-Box F and Cochran’s C tests. Data found heterogeneous were rendered homogeneous by square root or logarithmic transformation. The effects of treatment, experimental day, time, and individual animals were tested using multifactorial ANOVA for repeated measures. Where appropriate, least-significant difference (LSD) tests were applied to identify significant (P < 0.05) differences between means.

RESULTS

Effect of infusion onset on basal ir-ACTH and cortisol concentrations. The effect of saline or angiotensin II infusion onset at 140 days GA [for 2 h before ACTH-(1—24) administration] on basal ir-ACTH and cortisol concentrations is presented in Fig. 1. There were no significant effects of saline or angiotensin II treatment on basal ir-ACTH or cortisol concentrations. There was a significant (P < 0.05) variation in basal ir-ACTH concentrations in the 2.5 h before ACTH-(1—24) administration on 140 days GA in both saline- and angiotensin II-infused groups of fetuses; however, no time point after infusion onset was significantly different from the three values before infusion onset (as indicated by LSD test). Saline or angiotensin II infusion onset had no significant effect on basal cortisol concentrations.

Ir-ACTH and cortisol concentrations during chronic infusion. Ir-ACTH and cortisol concentrations during chronic saline and angiotensin II infusion, from 140 to 142 days GA, are presented in Fig. 2. Basal ir-ACTH or cortisol concentrations during the experimental period were not significantly different between saline- and angiotensin II-infused fetuses. There were no signifi-
cant changes in ir-ACTH concentrations in either saline- or angiotensin II-infused fetuses from 140 to 142 days GA; however, there was a significant ($P < 0.001$) change in cortisol concentrations between 140 and 142 days GA that occurred in both treatment groups to the same extent.

Increases in ir-ACTH and cortisol concentrations after ACTH-(1—24) administration. Increases in ir-ACTH and cortisol concentrations after ACTH-(1—24) administration during acute (140 days GA) and chronic (142 days GA) saline and angiotensin II infusion are presented in Fig. 3, A and B, respectively. There were no significant effects of saline or angiotensin II infusion on basal ir-ACTH or cortisol concentrations. There was a significant ($P < 0.001$) change in cortisol concentrations over the experimental period in both treatment groups; however, there was no significant change in ir-ACTH concentrations during saline or angiotensin II infusion. Different letters indicate values that are significantly different from each other as indicated by LSD test (across both treatment groups).

Effect of infusion on fetal blood pressure. Because MAP could not be corrected for amniotic pressure, MAP was expressed as a percentage of the control value on 140 days GA. The percentage change in MAP on 140 days GA and from 141 to 142 days GA in saline- and angiotensin II-infused fetuses is presented in Fig. 4. There was a significant ($P < 0.005$) interaction between treatment group and time during the infusion for fetal MAP. Subsequent analysis of each treatment group showed that there was no significant effect of saline infusion on fetal MAP; however, there was a significant ($P < 0.001$) change in fetal MAP during angiotensin II infusion. Mean absolute values for MAP (uncorrected for amniotic pressure) before infusion onset in saline-infused and angiotensin II-infused fetuses were 52 ± 2 and 49 ± 4 mmHg, respectively, and were not significantly different from each other.
Maturation of the fetal adrenal gland is a critical event for the onset of parturition in the sheep. It has long been proposed that, in addition to the stimulatory influence of ACTH on the fetal adrenal gland, a number of inhibitory influences may regulate adrenal corticosteroid output during late gestation (see Ref. 20). Several previous studies have proposed that angiotensin II is such an endogenous inhibitor of adrenal function (2, 14, 15). Fetal plasma angiotensin II concentrations are similar to those found in the maternal circulation (10), and it has been shown that angiotensin II treatment inhibits ACTH-induced cortisol secretion from ovine fetal adrenal cells maintained in vitro.

A number of studies have shown that, in addition to causing an inhibition of ACTH-induced cortisol secretion, angiotensin II inhibits ACTH-induced 17α-hydroxylase activity in vitro (2, 14, 15). It was not the aim of this study to specifically test this hypothesis, because this parameter could not be directly measured in the current study; however, these experiments were performed at a GA (140 days) when 17α-hydroxylase activity is normally increasing (6). The fact that cortisol responses to ACTH-(1—24) stimulation were not diminished after angiotensin II infusion suggests that this infusion had no effect on adrenal steroidogenic capacity in the current experiment. It is possible that, in vivo, the proposed effects of angiotensin II as an inhibitor of adrenal function are no longer discernible at 140 days.

**DISCUSSION**

Maturation of the fetal adrenal gland is a critical event for the onset of parturition in the sheep. It has long been proposed that, in addition to the stimulatory influence of ACTH on the fetal adrenal gland, a number of inhibitory influences may regulate adrenal corticosteroid output during late gestation (see Ref. 20). Several previous studies have proposed that angiotensin II is such an endogenous inhibitor of adrenal function (2, 14, 15). Fetal plasma angiotensin II concentrations are similar to those found in the maternal circulation (10), and it has been shown that angiotensin II treatment of both fetal and adult ovine adrenal cells in vitro inhibits ACTH-induced cortisol secretion (13, 15). Because these previous studies were performed in vitro, the aim of the present study was to examine the effect of in vivo angiotensin II treatment on adrenal responsiveness in fetal sheep.

Adrenal responsiveness, as given by the cortisol response to exogenous ACTH-(1—24) administration, was determined after acute (2 h) and chronic (48 h) intrafetal angiotensin II infusion. Fetal angiotensin II infusion, acute or chronic, was shown to have no effect on basal cortisol concentrations or adrenal responsiveness to ACTH-(1—24) compared with saline-infused control fetuses. This result is therefore in contrast to the study of Rainey et al. (15), which showed that angiotensin II treatment inhibited ACTH-induced cortisol secretion from ovine fetal adrenal cells maintained in vitro.

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**Fig. 3.** Increases in ir-ACTH (A) and cortisol (B) concentrations and adrenal responsiveness (area under the cortisol response curve; C) after ACTH-(1—24) administration (5.0 µg/kg) during acute (140 days GA) and chronic (142 days GA) saline (n = 4) and angiotensin II (n = 5, except on day 140, where n = 7) infusion. There were no significant effects of saline or angiotensin II treatment or experimental day on any of these parameters.

**Fig. 4.** Fetal mean arterial pressure (MAP) immediately before and during chronic saline (n = 4) and angiotensin II (n = 4, except on day 140, where n = 7) infusion that commenced at time 0 on 140 days GA. MAP during the infusion is expressed as a percentage of the control measurement at time 0. There was no significant effect of saline infusion on fetal MAP; however, there was a significant (P < 0.001) change in fetal MAP during angiotensin II infusion. Different letters indicate values that are significantly different from each other as indicated by LSD test.
GA, due to the overriding effects of increasing ACTH levels or other factors causing adrenal maturation in the last weeks of gestation. In the previous in vitro study of Rainey et al. (15), angiotensin II was shown to have an inhibitory effect on adrenal cells taken from fetuses at an earlier GA, 126–130 days. We chose to investigate the effect of angiotensin II infusion at 140 days GA because, as mentioned above, circulating cortisol concentrations and adrenal responsiveness to ACTH in the fetus are increasing rapidly at this time (20). We believed that any possible inhibitory effect of angiotensin II would be most easily detected at this stage of gestation. Further investigations of the effects of angiotensin II on adrenal function in vivo could, however, be performed at an earlier gestational age.

Although there was no effect of angiotensin II infusion on basal cortisol concentrations in the 2 h immediately after infusion onset, there was a significant increase in basal cortisol concentrations during chronic infusion. Rainey and co-workers (3) have shown that angiotensin II treatment of bovine fetal adrenal cells in vitro stimulates 17α-hydroxylase expression and basal cortisol secretion and suggested that, in the absence of ACTH, angiotensin II could maintain a low level of activity of steroidogenic enzymes (3). This effect was not observed, however, in adrenal cells from ovine fetuses (2, 15). Because, in the present study, the increase in basal cortisol concentrations during the experimental period was also observed in saline-treated fetuses, it seems likely that, rather than a positive effect of angiotensin II, this effect was simply that which normally occurs in late gestation (20). Clearly again, chronic angiotensin II infusion at the dose regimen chosen had no inhibitory effect on fetal adrenal function, in terms of basal corticosteroid output, at this gestational age.

The dose of angiotensin II infusion chosen for use in this study was based on previous reports that have characterized the effects of angiotensin II on aldosterone secretion, blood pressure, and renal function in fetal sheep (16, 19). In the current study, only one index of the effect of angiotensin II was reported, and it was demonstrated that the relative increase in fetal MAP observed during angiotensin II infusion was similar in magnitude to that previously reported (16, 19). Robillard et al. (16) showed that acute angiotensin II infusion, at the same rate as that used in this study, had no effect on plasma electrolyte concentrations; however, an increase in urinary Na+ excretion rate was observed, an effect that was thought to reflect a pressure-natriuresis. It has been suggested that the role of angiotensin II in Na+ reabsorption in the fetus may be absent or even reversed compared with that in the adult (10). Although the effect of angiotensin II on fetal renal function could not be determined in this study, we believe that the measurement of fetal MAP during our experiment was sufficient to substantiate the bioactivity of the infused peptide and to show that its effects were similar to those previously reported (16, 19). It remains possible that angiotensin II infusion at a different dose than that used in this study may affect fetal adrenal responsiveness.

A number of complex interactions between angiotensin II and ACTH may confound the interpretation of this experiment. Angiotensin II treatment of human fetal adrenal cells causes an increase in ACTH receptor mRNA levels and a potentiation of adrenal responsiveness to ACTH (9). In bovine adrenal cells, however, angiotensin II treatment causes a reduction in ACTH receptors (12). In addition, ACTH treatment has been shown to reduce angiotensin II receptor number in bovine adrenal cells (1). Whether chronic angiotensin II infusion or repeated administration of ACTH-(1–24) has such effects on ACTH and angiotensin II receptor populations in fetal sheep is unknown and remains speculative, given our findings that angiotensin II infusion had no effect on basal or stimulated cortisol concentrations in these experiments.

In conclusion, this study has examined for the first time the effect of angiotensin II infusion in vivo on the responsiveness of the ovine fetal adrenal gland. We have demonstrated that angiotensin II infusion, at a dose that has well-characterized biological effects, does not inhibit basal or ACTH-induced cortisol secretion in the fetal sheep in the last week of gestation. These results are in contrast with previous in vitro studies and, given the inconsistent in vitro effects of angiotensin II in different species, we therefore question the proposed role for angiotensin II as a negative modulator of fetal adrenal function in vivo in the sheep.

Perspectives

Activation of the fetal hypothalamo-pituitary-adrenal axis is a prerequisite for parturition in the sheep. In particular, maturation of the fetal adrenal gland has been thought to contribute to the rising cortisol concentrations that precede and trigger the maternal events of labor. The regulation of this adrenal maturation has been the subject of considerable research. It has been proposed that the fetal adrenal gland may in fact be under inhibitory influences in late gestation, such that an increase in basal cortisol concentrations occurs only at the appropriate time. It would be necessary, therefore, that these inhibitory factors be removed or overridden before term. Angiotensin II has been proposed in in vitro studies to have an inhibitory action on the fetal adrenal gland. The present study has examined this hypothesis by testing adrenal responsiveness during angiotensin II infusion to fetuses in vivo. We found no evidence to suggest that angiotensin II inhibited the ability of the fetal adrenal gland to secrete cortisol under basal or stimulated conditions. This result is in contrast to previous in vitro studies, and, because the effects of angiotensin II vary considerably between species, we have questioned the proposed inhibitory role of angiotensin II on the ovine fetal adrenal gland. The concept that the fetal adrenal gland is under a tonic inhibitory influence until close to term is appealing and we believe warrants further investigation.
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