Adenosine A₁ receptor mediated suppression of adrenal activity in near-term fetal sheep

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Adenosine A₁ receptor mediated suppression of adrenal activity in near-term fetal sheep. Am J Physiol Regul Integr Comp Physiol 298: R700–R706, 2010. First published January 13, 2010; doi:10.1152/ajpregu.00474.2009.—Activation of the hypothalamic-pituitary-adrenal (HPA) axis is a critical response to perinatal hypoxia. Recent data show that adenosine appears to inhibit baseline levels of fetal cortisol and to restrict the increase in ACTH and cortisol during moderate hypoxia. Because adenosine increases substantially during profound asphyxia, it is possible, but untested, that counterintuitively it might restrict the HPA response to more severe insults. It is unclear which receptors mediate the effects of adenosine on the HPA axis; however, adenosine A₁ receptor activation is important for adaptation to hypoxia. We therefore investigated whether adenosine A₁ receptor blockade modulates ACTH and cortisol levels in fetal sheep at 118 to 126 days gestation, randomly allocated to receive an intravenous infusion of either vehicle (vehicle-occlusion, n = 7) or 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, an A₁ receptor antagonist, DPCPX-occlusion, n = 7) infused 60 min before and during 10 min of umbilical cord occlusion, or infusion of DPCPX for 70 min without occlusion (DPCPX-sham, n = 6). Experiments were terminated after 72 h. Fetal ACTH levels increased significantly (P < 0.01) during occlusion, but not sham occlusion, and returned to baseline values by 60 min after occlusion. In the vehicle-occlusion group, fetal cortisol and cortisone plasma levels increased significantly (P < 0.05) 60 min after the occlusion and returned to baseline values by 24 h. In contrast, there was a marked increase in both fetal cortisol and cortisone during DPCPX infusion before occlusion to a level greater even than the maximum rise seen after occlusion alone. This increase was sustained after occlusion, with increased cortisol levels compared with occlusion alone up to 72 h. In conclusion, fetal cortisol concentrations are suppressed by adenosine A₁ receptor activity, largely through a direct adrenal mechanism. This suppression can be partially overcome by supraphysiological stimuli such as asphyxia.
fetal leads were exteriorized through the maternal flank, the fetus was returned to the uterus, and the uterus and abdominal incisions were closed. A maternal long saphenous vein was catheterized to provide access for postoperative care and death. Antibiotics (80 mg gentamicin; Roussel, Auckland, New Zealand) were administered in the amniotic sac before closure of the uterus. Postoperatively, all sheep were housed together in separate metabolic cages with free access to water and food. They were kept in a temperature-controlled room (16 ± 1°C, humidity 50 ± 10%) in a 12:12-h day-night cycle. During postoperative recovery, antibiotics were administered daily for 5 days intravenously to the ewe [600 mg benzylpencillin sodium (Crystapen) and 80 mg gentamicin]. Fetal catheters were maintained patent by continuous infusion of heparinized isotonic saline (20 U/ml at 0.2 ml/h), and the maternal catheter was maintained by daily flushing with heparinized saline. Experiments were started after 3 days of postoperative recovery, and fetal arterial blood was taken from the brachial artery for pH, blood gas, glucose, and lactate analysis to assess fetal health (26).

Experimental protocol and drug treatment. As previously reported, fetuses were randomized to two groups to receive vehicle (vehicle-occlusion, 5 males and 2 females, n = 7) or 8-cyclopentyl-1,3-dipropylxanthine (DPCPX-occlusion, 3 males and 4 females, n = 7) infused in the right axillary vein at a rate of 3.6 mg/min for 10 min, and then at 0.75 mg/min for 60 min (26). This regimen is known to block the bradycardia induced by cyclopentyl adenosine, a selective adenosine A1 agonist (31). After infusion for 60 min, profound asphyxia was induced by complete occlusion of the umbilical cord for 10 min, confirmed by characteristic fetal cardiovascular and pH and blood gas changes (26). The infusion of DPCPX was discontinued at the end of the occlusion period. A third group of fetuses was infused with DPCPX as above for 70 min without cord occlusion (DPCPX-sham, 3 males and 3 females, n = 6). Fetal arterial blood samples were taken at 90 and 30 min before occlusion, immediately before the start of occlusion (time 0), 8 min after the start of occlusion, and then at 1, 2, 4, 6, 24, 48, and 72 h after umbilical cord occlusion. Baseline was taken to be the ~90 min measurement for comparisons over time. After the hypoxic insult (3 days), the sheep were killed with pento-barbitone sodium (9 g iv to the ewe; Pentobarb 300, Chemstock International, Christchurch, New Zealand).

Hormone assays. Steroids were measured using mass spectrometry. The internal standards were cortisol-d2 for cortisol and corticosterone-d8 for cortisone. The internal standard (100 μl of 20 ng/ml in water) was added to 200 μl plasma. Steroids were extracted using 1 ml of ethyl acetate (Merck, Darmstadt, Germany). After removal of the organic supernatant, samples were dried, resuspended in 100 μl of mobile phase [80% methanol (Merck) and 20% water], and transferred to HPLC injector vials. Twenty-five microliters were injected onto an HPLC mass spectrometer system consisting of a Waters Alliance 2690 Separations Module (Waters, Milford, MA) followed by an Ion Max APCI source on a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer, all controlled by Finnigan Xcaliber software (Thermo Electron, San Jose, CA). The mobile phase was isocratic, flowing at 600 μl/min through a Luna 3 μm C18(2) 100A 250 × 4.6 mm column at 40°C (Phenomenex, Auckland, New Zealand). Retention times were as follows: 5.7 min cortisone and 6.1 min cortisol. Ionization was in positive mode and Q2 had 1.2 mTorr of argon. The mass transitions followed were as follows: cortisol-d2 365.3-121.2 at 24 V, cortisol 365.3-122.2 at 28 V, corticosterone-d8 355.3-125.2 at 24 V and cortisol 361.1-163.0 at 28 V. Mean inter- and intra-assay coefficient of variation values were 11.2 and 7.1% for cortisol and 20.4 and 10.3%, respectively, for cortisone. Cortisol and cortisone were measured using LC/MS/MS single reaction monitoring during an 11-min isocratic run following the addition of deuterated cortisol as an internal standard and extraction with ethyl acetate.

Plasma ACTH was measured by RIA, which was performed using a commercially available 125I RIA kit (24130; DiaSorin, Stillwater, MN) validated for use with both fetal and maternal ovine plasma. Analyses were performed in duplicate. The intra-assay and inter-assay coefficient of variation were 3.7 and 13.2%, respectively. ACTH concentrations were not measured in the DPCPX-sham group.

Data analysis. The effect of DPCPX on cortisol, cortisone, and ACTH levels, and mean arterial pressure (MAP) and fetal heart rate (FHR), was evaluated by using a nested, random effect, two-way repeated-measures ANOVA using JMP software version 5.1 (SAS Institute, Cary, NC). MAP and FHR values were calculated as 5-min averages at the same time that samples for hormone measurement were taken (except for 1-min averages at time 0 and 8 min during occlusion). When an effect of time or an interaction between time and treatment group was found, data were compared with baseline using the least-square mean test. Within-subjects regression analysis by the method of Bland and Altman (4) was used to examine the relationship between cortisol and MAP. Differences were considered significant when P < 0.05. Data are shown as means ± SE.

RESULTS

Hormones. There was a significant change over time in fetal cortisol levels in both occlusion groups (P < 0.05, Fig. 1). In the vehicle-occlusion group, fetal cortisol levels increased approximately fivefold (P < 0.05) following occlusion and recovered to baseline values by 24 h after occlusion. In the DPCPX-occlusion group, fetal cortisol concentrations were significantly increased (P < 0.01) from 30 min after the beginning infusion of DPCPX until 6 h following occlusion compared with baseline (~90 min) values. In contrast, the DPCPX-sham group showed a significant increase in fetal cortisol concentrations (P < 0.01) 30 min after the beginning of infusion, but levels then fell and were not significantly different from baseline by the end of the infusion. Fetal cortisol levels were significantly elevated in the DPCPX-occlusion group compared with the vehicle-occlusion group overall (P < 0.05), starting from halfway through the DPCPX infusion (~30 min) to 72 h (P < 0.05). Preocclusion fetal cortisol concentrations were not different between the DPCPX-occlusion and DPCPX-sham groups but were increased (P < 0.05) in the DPCPX-occlusion group compared with the DPCPX-sham group from the occlusion until 24 h.

Changes in fetal cortisone plasma concentrations showed a highly similar pattern to cortisol, with no significant differences in baseline values between groups and a significant change over time in all groups (P < 0.01, Fig. 1). In the vehicle-infusion group, fetal cortisone levels increased significantly (P < 0.05) by approximately two times baseline values following occlusion, and recovered to baseline values by 24 h after occlusion. In the DPCPX-occlusion group, fetal cortisone levels were significantly increased 30 min after the beginning of the infusion of DPCPX until 72 h after occlusion compared with baseline values. In the DPCPX-sham group, fetal cortisone concentrations were significantly increased (P < 0.05) 30 min after the beginning of infusion compared with baseline, with no significant differences at any other time point. Fetal cortisone levels were significantly elevated in the DPCPX-occlusion group compared with the vehicle-occlusion group from ~30 min to 6 h after occlusion and at 72 h (P < 0.01). The DPCPX-occlusion group also showed significantly higher cortisone levels (P < 0.05) after occlusion compared with the DPCPX-sham group up to 24 h.

There was a significant change in fetal ACTH levels over time in both occlusion groups (P < 0.001, Fig. 1). In vehicle-occlusion fetuses, fetal ACTH levels increased by ~30-fold.
fetuses at 8 min during occlusion and began to recover shortly after this time. There were no significant differences in fetal ACTH levels between the groups, except at the single time point at 8 min of occlusion when plasma ACTH levels were lower in fetuses receiving DPCPX (Fig. 1, top).

Blood gases and cardiovascular changes. Blood gas tensions and pH were not different between groups during the initial baseline period and did not alter significantly in response to DPCPX infusion (Table 1). Umbilical cord occlusion was associated with profound hypoxia, hypercapnia, and acidosis that were of similar magnitude in the DPCPX-occlusion and vehicle-occlusion groups.

There was a significant change over time in MAP in the two occlusion groups (P < 0.001, Fig. 2). In vehicle-occlusion fetuses, MAP was significantly lower at 8 min during occlusion compared with baseline values (P < 0.001). In contrast, in DPCPX-occlusion fetuses, there was no difference in MAP at 8 min during occlusion compared with baseline, but values were higher at 1 and 2 h after occlusion compared with baseline (P < 0.05). Furthermore, MAP was significantly higher in DPCPX-occlusion fetuses at 8 min of occlusion and 1 and 2 h after occlusion than in vehicle-occlusion fetuses (P < 0.05). There were no changes over time in MAP in the DPCPX-sham group, and MAP was not significantly different between the DPCPX-occlusion and -sham groups. MAP and cortisol levels following occlusion were positively correlated (r = 0.33, P < 0.05).

There were significant changes over time in FHR in both occlusion groups (P < 0.0001, Fig. 2). In vehicle-occlusion fetuses, FHR was significantly lower (P < 0.0001) at 8 min during occlusion and higher (P < 0.05) 1 h after occlusion compared with baseline values. In the DPCPX-occlusion group, FHR was significantly lower than baseline values (P < 0.01) at 8 min during occlusion, but was higher (P < 0.05) at all time points after occlusion except for 4 and 72 h. There were no changes over time in FHR in the DPCPX-sham group and no overall difference in FHR between the vehicle-occlusion and DPCPX-occlusion fetuses. FHR was significantly lower at 8 min during occlusion (P < 0.001) and 4 h after occlusion (P = 0.05 in DPCPX-occlusion fetuses compared with DPCPX-shams.

Discussion

The present study demonstrates that endogenous adenosine A<sub>1</sub> receptor activity markedly limits normoxic fetal release of cortisol and cortisone, but not ACTH, denoting a primarily direct adrenal effect through the A<sub>1</sub> receptor. A<sub>1</sub> blockade was not associated with any further rise in fetal cortisol levels after asphyxia, albeit levels remained significantly higher than in the vehicle-occlusion group. This suggests that the effect of adenosine to restrict glucocorticoid release is maximal at basal levels in the late gestation fetus and, despite the increase in adenosine activity during hypoxia (5, 13), can be partially overcome by supraphysiological stimuli such as asphyxia. The increase in glucocorticoid levels in the DPCPX-occlusion group was associated with an increase in fetal MAP during and for the first 2 h after occlusion, confirming that the HPA axis is a physiologically important modulator of adaptation to severe asphyxia.

Fig. 1. Effect of 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) or vehicle infusion 60 min before and during a 10-min umbilical cord occlusion or a 70-min DPCPX infusion only, on fetal plasma ACTH, cortisol, and cortisone plasma levels (n = 5–7). Vertical grey bar, duration of cord occlusion; horizontal hatched bar, duration of DPCPX or vehicle infusion. †††P < 0.001, ††P < 0.01, and †P < 0.05, compared with the −90 baseline sample within groups; ***P < 0.001, **P < 0.01, and *P < 0.05, DPCPX-occlusion vs. vehicle-occlusion groups; †††P < 0.001, ††P < 0.01, and †P < 0.05, DPCPX-occlusion vs. DPCPX-sham groups.

over baseline values (P < 0.001) at 8 min of umbilical cord occlusion, but slowly recovered after the occlusion and were not different from baseline values by 24 h. Similarly, fetal ACTH levels were increased (P < 0.01) in DPCPX-occlusion
Fetal cortisol and its metabolite cortisone levels were not increased during umbilical cord occlusion in the present study, but they increased by four- and fivefold after occlusion, and recovered to baseline values by 24 h. Although this is the first report of the HPA responses to complete umbilical cord occlusion in near-term fetal sheep, the magnitude of these increases is broadly consistent with responses to acute moderate isocapnic hypoxia (19, 27), reduced uterine blood flow (6), umbilical cord occlusion while ventilating the fetus (40), and repeated partial umbilical cord occlusion (43). In the present study, we saw an approximate 100-fold increase in fetal ACTH concentrations during occlusion, which is considerably higher than that previously reported in near-term animals (19, 21, 43). This difference most likely reflects the severity of the insult; our study used a model of severe asphyxia, which causes brain damage (16, 26, 33) compared with the relatively moderate or shorter intervals of hypoxia in other studies.

Consistent with this hypothesis, prolonged (25 min) umbilical cord occlusion in preterm fetal sheep was associated with much lower absolute cortisol concentrations than the present study but a greater relative increase, up to 30-fold, during occlusion and up to 48 h following occlusion (38). Furthermore, cortisol levels were increased after 20 min of occlusion, whereas there was no increase until after occlusion in the present study. It is difficult to compare responses between gestations, but the difference, a greater and more sustained relative HPA response in the preterm than the term fetus, most likely reflects the much more prolonged interval of asphyxia required to cause brain injury in the preterm than in the term fetus (22).

The present study shows for the first time that adenosine A1 receptor activity restricts cortisol, but not ACTH, release in the normoxic late-gestation sheep fetus. The increase in fetal cortisol concentrations during the baseline infusions of DPCPX was broadly consistent with the effect of nonspecific adenosine receptor blockade (11), but the relative increase was much larger. Critically, in the present study, cortisol concentration was higher after exposure to A1 receptor blockade than even the peak rise in the vehicle-occlusion group after umbilical cord occlusion. This surprising finding demonstrates that A1 receptor activity can partially restrict the response even to profound asphyxia. These data are in marked contrast with previous findings in vitro that adenosine enhances steroidogenesis (12, 44). Although this effect was largely mediated through the adenosine A2A and A3 receptors (12), nonspecific blockade also reduced steroid production. Speculatively, this may reflect maturational changes, differences in paracrine
control in cell culture compared with the intact adrenal gland (12), or species differences. In utero, there is also the further speculative possibility that adenosine could have altered placental transfer of glucocorticoids, as these hormones move bidirectionally across the ovine placenta (25), as well as altering their clearance rates from the fetal compartment. Thus the present experiments show that adenosine influences some combination of release, transfer, and clearance of glucocorticoids, independent of ACTH, in the basal state and during umbilical cord occlusion.

The overall pattern of changes in fetal ACTH levels in the present study, with an initial increase during umbilical cord occlusion in vehicle fetuses, followed by an increase in cortisol concentrations, is consistent with ACTH-driven adrenal activation, at least in the immediate peri-insult period. It is intriguing to note that vehicle-treated fetuses showed no significant increase in fetal cortisol levels during the period of umbilical cord occlusion although there was a delayed but sustained rise after asphyxia. The mechanism is unknown. The simplest explanation is that this delay reflects the time required for de novo synthesis and release of cortisol. In view of the dramatic increase in cortisol concentrations seen in the present study during adenosine A1 receptor blockade, it is possible that the rapid increase in adenosine levels during severe asphyxia may also have contributed to this delay (5).

In contrast to previous data (11), we did not find an increase in fetal ACTH levels during adenosine A1-receptor blockade above control values in the baseline period or during asphyxia (see Fig. 1). The mechanism of this difference is unclear but might in part reflect the use of highly selective A1 blockade in the present study, since the anterior pituitary cells express a range of adenosine and purinergic receptors. However, there are some data that adenosine A2A receptor activity interacts with corticotrophin-releasing hormone to enhance expression of pro-opiomelanocortin and thus should facilitate and not inhibit ACTH production (47). Alternatively, it might in part reflect the relatively severe insult in the present study compared with the noninjurious 30-min interval of moderate hypoxia induced by the ewe breathing an hypoxic gas mixture, studied by Chau and colleagues (11). Regardless, given that fetal ACTH concentrations before and following the occlusion were not increased by infusion of DPCPX, the increase in fetal cortisol and cortisone levels supports a direct effect of adenosine on fetal adrenal activity.

Indeed, as shown in Fig. 2, adenosine A1 receptor blockade was associated with reduced, not increased, ACTH levels near the end of the 10-min period of asphyxia. Although we cannot rule out a direct effect of adenosine to facilitate ACTH release, given that there was no difference at any other time point and that A1 receptor blockade was associated with rapid elevation of cortisol levels, we suggest that it is most likely an indirect effect mediated through feedback inhibition of ACTH by cortisol. Of course, these results do not exclude other regulatory factors operating individually or in combination with the exaggerated cortisol response that could contribute to ACTH suppression. For example, arginine vasopressin, a major mediator of ACTH release in the fetus (23), is released in response to hypoxia and to increased adenosine levels (32). Furthermore, prostaglandin production, mediated by prostaglandin endoperoxide synthase-2 activity in the fetal brain, is essential for fetal ACTH secretion in response to cerebral ischemia and parturition (18, 46). Although acidosis stimulates ACTH release (45), the severity of acidosis was comparable with and without A1 receptor blockade. The redundancy in regulatory controls at the hypothalamic-pituitary site is consistent with the importance of stress responses to hypoxia and ischemia and indicates diverse means by which adenosine could exert both stimulatory and depressive effects.

Glucocorticoids remained elevated for some time in both occlusion groups during recovery from asphyxia, independently of changes in plasma ACTH. As recently reviewed by Bornstein and colleagues (8), dissociation of ACTH and corticosteroid occurs in various circumstances, including stress, showing that glucocorticoid secretion is not exclusively linked to pituitary ACTH release. The ACTH-independent mechanisms involved appear to include altered adrenal sensitivity to ACTH and altered adrenal receptor activity caused by cytokines and vasoactive substances such as the catecholamines (8). In the newborn rat, for example, dissociation between ACTH and corticosterone occurs during exposure to hypoxia from birth (37).

In the present study, adenosine A1 receptor blockade was associated with a markedly sustained increase in cortisol levels after occlusion compared with vehicle controls. Hypothetically, this could reflect a direct effect of adenosine blockade, whether due to prolonged clearance of DPCPX or to altered responses to hypoxia (35). However, our finding that cortisol and cortisone concentrations had returned to baseline values by the end of the DPCPX infusion in the DPCPX-sham group strongly indicates that the prolonged increase in these hormones after DPCPX exposure plus umbilical cord occlusion was likely a secondary consequence of loss of protective effects of adenosine A1 activity during occlusion (26). We have previously shown that A1 receptor blockade leads to greater neural injury and more frequent seizures after occlusion (20, 26), accompanied by consequent physiological stressors, including reduced fetal oxygen tensions (20), and likely other stimuli of cortisol release such sympathetic activation (36).

The “desuppression” of fetal cortisol levels during A1 receptor blockade was associated with a significant improvement in fetal arterial blood pressure, but no change in heart rate, near the end (8 min) of the period of umbilical cord occlusion and for up to 2 h after occlusion compared with controls. This is similar to our previous findings from the same model (29), and is consistent with the finding of a small increase in MAP after an infusion of DPCPX (31). Conversely, a selective adenosine A1 receptor agonist was associated with reduced MAP in fetal sheep, but, since this was associated with marked bradycardia, this finding mostly likely reflects a direct cardiac effect of adenosine (7). Potentially, the increased blood pressure during and after DPCPX infusion observed in this study could be because of reduced peripheral vasodilatation associated with A1 receptor blockade (9) or nonselective blockade of A2A receptors (2). Against this possibility, we have previously reported that an A1 receptor agonist had no effect on femoral blood flow in the late-gestation fetus (7). Thus it is more likely related to the increased glucocorticoid levels. Supporting this hypothesis, fetal cortisol concentrations were positively correlated with MAP in the DPCPX group following cord occlusion.

These data are strongly consistent with a previous report in preterm fetal sheep that fetal cortisol concentrations were correlated with MAP following cord occlusion (38) and with...
increased fetal arterial blood pressure during exogenous infusions of cortisol (14, 15, 24, 42). The mechanisms by which glucocorticoids support blood pressure are not fully understood but include direct cardiac effects mediated by increased coupling of the β-adrenergic receptors to cellular postreceptor signal transduction (3) and by augmented cardiac and vascular responses to sympathetic stimulation (10, 39). More generally, the present finding that cortisol did not rise during asphyxia strongly infers that the major role of the HPA axis during an isolated episode of asphyxia is not in adaptation to the immediate event, but rather for support of fetal homeostasis during recovery from such severe insults.

In conclusion, the present study has demonstrated that adenosine A1 receptor activity suppresses fetal cortisol concentrations, largely through a direct adrenal mechanism, and that this suppression can be partially overcome by supraphysiological stimuli such as asphyxia.

Perspectives and Significance

Adenosine and its receptors are involved in the regulation of many aspects of physiology and pathophysiology. The finding that adenosine partially restrains the rise in cortisol even during immediate recovery from severe asphyxia may reflect the adverse effects of prolonged exposure to cortisol for the fetus (28, 30). Furthermore, because adenosine levels fall rapidly after birth, in parallel with the increase in arterial oxygen tensions (34), we may reasonably speculate that this fall may be an important contributor to the well-known increase in cortisol levels after birth (41). In this context, the subsequent stabilization of the HPA axis over the first week may represent resetting of the set point of adrenal regulation or involvement of other paracrine factors.

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DISCLOSURES

No conflicts of interest are declared by the authors.

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