Adrenocortical Sensitivity to ACTH in Neonatal Rats:

Correlation of Corticosterone Responses and Adrenal cAMP Content

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Short Title: Adrenal sensitivity to ACTH

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Abstract

A coordinated hypothalamic-pituitary-adrenal axis response is important for the survival of newborns during stress. We have previously shown that prior to post-natal day (PD) 5, neonatal rats exposed to hypoxia (one of the most common stressors effecting premature neonates) exhibit a large corticosterone response with a minimal increase in immunoassayable plasma ACTH and without a detectable increase in adrenal cAMP content (the critical second messenger). To explore the phenomenon of ACTH-stimulated steroidogenesis in the neonate, we investigated the adrenal response to exogenous ACTH in the normoxic neonatal rat. Rat pups at PD2 and PD8 were injected (IP) with porcine ACTH at low, moderate, or high doses (1, 4, or 20 µg/kg body weight). Trunk blood and whole adrenal glands were collected at baseline (before injection) and 15, 30, or 60 minutes after the injection. ACTH stimulated corticosterone release in PD2 and PD8 pups. In PD2 pups, plasma corticosterone at baseline and during the response to ACTH injection was greater than values measured in PD8 pups, despite lower adrenal cAMP content in PD2 pups. Specifically, the low and moderate physiological ACTH doses produced a large corticosterone response in PD2 pups without a change in adrenal cAMP content. At extremely high, pharmacological levels of plasma ACTH in PD2 pups (exceeding 3000 pg/mL), an increase in adrenal cAMP was measured. We conclude that physiological increases in plasma ACTH may stimulate adrenal steroidogenesis in PD2 pups through a non-cAMP mediated pathway.

Keywords: Corticosterone, adrenal gland, synthetic ACTH(1-39), second messenger, newborn
Introduction

The adrenocortical response to stress in newborns is essential for survival (19). For example, acute episodes of hypoxia are common in neonates born prematurely (16, 27, 28, 30). Furthermore, these stressors can affect the developing neonatal brain via the disruption of metabolic and neurologic function, and may have long-term endocrine and metabolic consequences (8, 10). A coordinated development of the hypothalamic-pituitary-adrenal (HPA) axis in neonates is important for promoting maturation of the immature lung, facilitating closing of the ductus arteriosis, and improving vasoconstrictor response to catecholamines (12, 31, 51). Since the rate of premature births in the US is increasing, it is important that we evaluate the mechanisms of steroidogenesis in the premature and full-term neonate (29).

The neonatal rat pup is a useful model of prematurity in humans and has been used to investigate the development of the HPA axis (41, 45). Corticosterone (the primary glucocorticoid in rats) is produced in the zona fasciculata of the adrenal cortex. The production of corticosterone is stimulated by adrenocorticotropic hormone (ACTH) which binds to, and activates the melanocortin 2 receptor (MC2R) leading to an increase in intracellular cyclic adenosine monophosphate (cAMP), together termed the ACTH-MC2R-cAMP cascade (17, 36, 40). Although it has been shown that there are other minor modulators of corticosterone production such as calcium, cAMP remains the accepted obligatory second messenger of ACTH action (17). Increased cAMP leads to the activation of protein kinase A and to a subsequent increase in the transport of free cholesterol from the cytosol across the mitochondrial membrane, in a process mediated by steroidogenic acute regulatory protein (StAR), the rate-limiting step in steroidogenesis (46).
The ability of the adrenal gland to secrete corticosterone is present in most mammals at birth, but decreases during the first one to two weeks of post-natal life. The adrenal gland in the developing rat displays an ACTH-hyporesponsive period between post-natal day (PD) 4 and PD14 (11, 25, 48, 49). We have previously performed ACTH stimulation testing in neonatal rats exposed to hypoxia from birth to 5-7 days. We found that there is an augmentation of the corticosterone, but not the aldosterone, response to ACTH, and that this increase is associated with an increase in StAR protein (37). Additionally, we have previously shown that up to PD5, pups exposed to acute hypoxia are capable of generating a large corticosterone response with a minimal increase in immunoassayable plasma ACTH and without a detectable increase in adrenal cAMP content (the critical second messenger) (23).

The prior studies with hypoxia described above were the motivation for the current studies examining neonatal adrenal responses to exogenous ACTH in *normoxic* pups. Prior studies have not carefully investigated the adrenal response to ACTH in the very young (PD1-4) neonatal rat; it has been assumed it is mediated in vivo by large increases in cytosolic cAMP since adrenal cells from rats of this age respond to cAMP and ACTH *in vitro* (2). However, this does not establish that the adrenocortical response to ACTH or stress *in vivo* in the first few days of post-natal life is dependent on increases in adrenocortical cAMP as the second messenger.

The primary goal of the present study was to determine if exogenous ACTH stimulates corticosterone production from the adrenal gland in the PD2 rat, and to investigate if this process is mediated by the classic ACTH-MC2R-cAMP cascade, as in the PD8 and adult rat adrenal gland (23). Specifically, we compared the corticosterone and adrenal cAMP responses of PD2 and PD8 rats using different doses of exogenous ACTH. We hypothesized that exogenous ACTH
would stimulate corticosterone production in the PD2 rat, but that the increase may not be associated with an increase in adrenal cAMP content.

Methods

Animal treatment and experimental protocol. The animal protocol was approved by the Institutional Animal Care and Use Committee of Aurora Health Care. Timed-pregnant Sprague-Dawley rats at gestational day 15-17 (N=48) were obtained from Harlan Sprague Dawley (Indianapolis, IN), maintained on a standard diet and water ad libitum in a controlled environment (0600-1800 lights on), and allowed to deliver and care for their pups without interference until experimentation. Pups (both sexes; N=574) were studied at postnatal days (PD) 2 and 8 (PD8). These ages were selected because we have previously shown that the dynamics of the HPA axis response to stress in PD8 rats are similar to a full-term human newborn, while the PD2 HPA axis response to stress models that of a premature human newborn (5, 6, 7, 18).

On PD2 or PD8, at approximately 0800 h, rat pups were removed from the dams and placed in a small chamber with adequate bedding, and were allowed free range of motion and room to huddle. A variable control heating pad (Moore Medical, Farmington, CT) was placed beneath the bedding and kept at the lowest setting required to maintain body temperature in normoxic rats at these ages (18). After 10 minutes in the chamber, at approximately 0830 h, rat pups were removed from the chamber and quickly weighed. Immediately after weighing, pups were sacrificed (baseline) or were injected intraperitoneally as described previously (37). Rats were injected with either vehicle (10 µL/kg body weight of isotonic saline) or porcine ACTH (1-39) (Sigma Chemical, St. Louis, MO) in isotonic saline at low, moderate, or high doses (1, 4, or 20 µg/kg body weight). After analyzing the plasma concentrations and corticosterone responses
achieved with the low, moderate, and high doses of ACTH (see Figures 1-3), we recognized that we needed a lower dose of ACTH to better evaluate the lower range of adrenocortical sensitivity. In order to obtain the most accurate dosing for this very low dose of exogenous ACTH, we conducted a pilot study and determined that an ACTH dose of 0.5 µg/kg body weight in PD2 pups and 0.2 µg/kg body weight in PD8 pups gave similar plasma ACTH concentrations. For vehicle and all doses of ACTH, pups were removed from the temporary holding chamber and decapitated at baseline (control), 15, 30, or 60 minutes post-injection.

In order to evaluate the early (5 minute) adrenal response to ACTH, an additional set of experiments were performed in which PD2 (N=81) and PD8 (N=55) pups were injected with low or moderate (1 or 4 µg/kg body weight) doses of ACTH, and blood and adrenals were obtained and pooled (as described below) at 5 minutes after injection (N=9 samples per age and dose).

**Blood and tissue collection.** Trunk blood was collected at baseline (before ACTH injection) and at 15, 30, or 60 minutes after the injection whereas adrenal glands were obtained at baseline and at 15 and 60 min after injection. Another set of experiments sampled at 5 minutes after ACTH injection. Trunk blood was pooled (2-3 pups/samples) in a tube with EDTA and treated as one sample for statistical purposes. Whole adrenals glands were pooled (4-6 adrenals/sample), flash frozen in liquid nitrogen, and stored along with the plasma samples at -70 ºC until later analysis.

**Plasma hormone and adrenal cAMP assays.** Plasma ACTH and corticosterone were measured by radioimmunoassay as described previously (MP Biomedicals, Orangeburg, NJ) (34). Frozen adrenal samples were weighed and then pulverized using a liquid nitrogen-cooled mini mortar (Bel-Art Products, Pequannock, NJ), reconstituted in cAMP assay buffer, and
frozen at -70°C until further analysis. Adrenal cAMP concentration was determined by ELISA according to the manufacturer’s protocol (Arbor Assays, Ann Arbor, MI) as described previously (23).

Statistical Analyses. The hormone and cAMP data were analyzed by two-way ANOVA (factor 1: time, factor 2: age). Post-hoc analysis was performed by Student-Newman-Keuls multiple range test (p < 0.05) (Sigmaplot 11.0).

Results

Plasma corticosterone response to exogenous ACTH in PD2 and PD8 pups at baseline, 15, 30 and 60 minutes post-injection are shown in Figure 1 (note the y-axis ranges are different for PD2 and PD8 results). Exogenous ACTH stimulated corticosterone release in both PD2 and PD8 pups at 15, 30, and 60 minutes. The PD2 pups had higher baseline plasma corticosterone concentrations compared to the PD8 pups. The corticosterone response of the PD2 pups to exogenous ACTH was greater than the corticosterone response of the PD8 pups. Note the dose-response relationship between ACTH injection and corticosterone response at 60 minutes in PD2 rats. However, in PD8 rats, the low and moderate doses resulted in statistically similar increases in corticosterone. Baseline plasma ACTH was 46 ± 3 pg/ml (N=15) in PD2 pups and 37 ± 2 pg/ml (N=18) in PD8 pups (P = 0.015). The peak plasma ACTH concentrations achieved 30 minutes after ACTH injection were 277 ± 42 pg/ml (N=17), 720 ± 150 pg/ml (N=16), and 6,198 ± 901 pg/ml (N=15) in the low, moderate, and high doses, respectively. The plasma ACTH concentrations achieved with low dose are comparable to those achieved in neonates injected with CRH (38). The concentrations achieved with the moderate dose are similar to those achieved in neonates exposed to intermittent hypoxia (7). The plasma ACTH concentrations
achieved with the high dose of ACTH were clearly pharmacological. The PD2 and PD8 pups injected with vehicle showed no change in plasma ACTH, plasma corticosterone, or adrenal cAMP content (data not shown).

The changes in adrenal cAMP content at 15 and 60 minutes after administration of ACTH are shown in Figure 2. Adrenal cAMP increased significantly and proportionately with low, moderate, and high doses of ACTH in PD8 pups. In PD2 pups, low and moderate ACTH doses produced a large corticosterone response in the absence of a change in adrenal cAMP content. At high (pharmacological) doses of ACTH in PD2 pups (leading to plasma ACTH levels exceeding 3000 pg/mL), increases in adrenal cAMP were detected. Baseline cAMP was 0.9 ± 0.2 pmol/mg (N=5) for PD2 adrenals and 1.1 ± 0.1 pmol/mg (N=5) for PD8 adrenals (not different).

Figure 3 summarizes the cAMP response at 15 minutes and the corticosterone response at 60 minutes after low, moderate, and high doses of ACTH in PD2 and PD8 pups. The plasma corticosterone responses to ACTH were much greater in at PD2 compared to PD8. As the dose of ACTH increased, there was a clear and proportional increase in adrenal cAMP content in PD8 pups. This increase in cAMP was accompanied by increases in plasma corticosterone. In PD2 pups, there was a large corticosterone response at all three doses; however, adrenal cAMP content did not increase significantly in the PD2 pups at 15 minutes post-injection in response to low and moderate doses of ACTH. The highest dose of ACTH did produce an adrenal cAMP response in both PD2 and PD8 pups. At this highest dose, the adrenal cAMP content in PD8 pups was more than double than that of PD2 pups, despite a substantially smaller corticosterone response.
After appreciating the data in Figures 1-3, we decided to use a much lower dose of ACTH to achieve a more modest corticosterone response. We achieved equivalent plasma ACTH concentrations by giving PD2 pups a higher dose of exogenous ACTH (Figure 4). The adrenal response to this very low dose ACTH followed a similar pattern to the higher doses, with the PD2 pups exhibiting a larger corticosterone response than PD8 pups (Figure 4). There was no significant difference between the baseline corticosterone values in PD2 and PD8 pups, although there was a tendency for baseline corticosterone to be higher in PD2 pups. We were unable to detect a change in adrenal cAMP at 15 minutes after ACTH injection in either age group.

Because of the cAMP data generated in Figure 2, we were concerned that we had missed a small spike in cAMP occurring during the time between the injection of ACTH and the 15 minute sampling time. Therefore, a new set of experiments was performed in which PD2 and PD8 pups were injected with the low or moderate dose of ACTH and blood and adrenal glands were obtained at 5 minutes after injection. At the 5 minute time point, plasma ACTH had increased to 142 ± 4 pg/ml for the low dose and 515 ± 69 pg/ml for the moderate dose of ACTH. Figure 5 shows adrenal cAMP and plasma corticosterone levels at baseline and 5 minutes after injection of ACTH. The low dose of ACTH did not result in a statistically significant increase in cAMP or corticosterone at 5 minutes post-injection. The moderate dose of ACTH resulted in a significant increase in plasma corticosterone in the PD2 pups without a significant increase in adrenal cAMP, whereas adrenal cAMP increased in the PD8 pups without a change in plasma corticosterone.

Discussion
The present study evaluated the plasma corticosterone and adrenal cAMP response to exogenous ACTH injections in PD2 and PD8 rats. We hypothesized that exogenous ACTH would produce a larger corticosterone response in PD2 pups, when compared to responses in PD8 pups. Furthermore, the response in the PD2 pup may not be accompanied by a sizeable increase in the obligate intracellular second messenger - cAMP. Our results confirmed this hypothesis by demonstrating: a) exogenous ACTH stimulated a larger increase in corticosterone in PD2 pups than in PD8 pups at each time point, b) adrenal cAMP content in PD2 pups did not change when injected with low and moderate ACTH doses, c) only high (pharmacological doses) of ACTH elicited a detectable increase in cAMP in PD2 pups, and d) adrenal cAMP levels in PD8 pups increased significantly and proportionately with increasing doses of ACTH.

The HPA axis of the neonatal rat undergoes a period of development that ultimately leads to a fully functional adrenal gland capable of producing corticosterone (33). In particular, it has been shown that the adrenal gland of the neonatal rat goes through a stress-hyporesponsive period (SHRP) (2). The neonatal rat SHRP is characterized by an attenuated corticosterone response to stress between PD4 and PD14 (11, 25, 48, 49). Previous studies have investigated the adrenal responsiveness in the neonatal rat during this hyporesponsive period (2). Additionally, we have focused on the adrenocortical response of neonatal rats during periods of hypoxia. The studies regarding the effects of acute and chronic hypoxia on the HPA axis of the developing neonatal rat are the motivation for the current study (5, 6, 37, 38, 39). Specifically, we continued the investigation of a phenomenon observed that, prior to PD5, when exposed to hypoxia, the neonatal rat exhibits a large corticosterone response without a drastic increase in immunoreactive plasma ACTH and without a detectable increase in adrenal cAMP content (the canonical second messenger) (23). These previous studies led us to question whether increases in ACTH in the
normoxic neonatal rat stimulate corticosterone production via a rise in adrenal cAMP content or via another second messenger pathway.

We have demonstrated that the PD2 adrenal has a much larger corticosterone response to ACTH compared to the PD8 pup confirming what has been demonstrated previously in dispersed adrenal cells in vitro (2) and in the adrenal response to hypoxia in vivo (23). This was not due to changes in plasma corticosterone binding capacity since we have previously demonstrated that PD8 pups have higher corticosterone binding globulin levels compared to PD2 pups (23).

The current study demonstrated a large corticosterone response to physiological doses of ACTH in PD2 pups without a measurable increase in adrenal cAMP. We understand that cAMP is the accepted intracellular second messenger for the adrenal response to ACTH in mature rats (17). Chronic ACTH stimulation can exert its steroidogenic effects through a non-cAMP mechanism (26). In adult rats, it has been shown that ACTH-stimulated adrenal corticosterone content increases within 3 minutes without a change in adrenal cAMP content particularly when the ACTH is injected in the morning (9). In addition, it has been suggested that adrenal cell responses to very low concentrations of ACTH are cAMP-independent (52) or that only very small amounts of cAMP are necessary to stimulate steroidogenesis (32).

What, then, is mediating this large increase in ACTH-stimulated corticosterone in the PD2 rat pup? There are other potential second messengers, such as calcium and cGMP; however, most studies do not support these as principal second messengers in this system (17, 23). There are other, less potent pathways that ACTH is capable of stimulating, including mitogenic activating protein kinase (MAPK), inositol phosphate, and diacylglycerol (17). These other pathways will be considered for further investigation. Non-ACTH mechanisms for controlling
steroidogenesis include sympathetic input via the splanchnic nerves (14, 47) and/or local factors such as vasoactive intestinal peptide and catecholamines (3, 4). However, these non-ACTH mediated pathways are unlikely to be the cause of increased corticosterone because we injected exogenous ACTH, and because the vehicle injection group did not show an increase in corticosterone.

It is thought that steroidogenic acute regulatory (StAR) protein is the principal mediator of cholesterol transport process essential for steroidogenesis (46). A previous study from the same group showed that exogenous ACTH and cAMP each resulted in a U-shaped curve of adrenal responsiveness in dispersed cells (2). In that study, the corticosterone responses to exogenous ACTH, cAMP, and a soluble (non-rate limited) form of cholesterol were higher in PD1 adrenal cells compared to PD10, and responses increased again in cells from adult rats. It was concluded that it was unlikely that cholesterol transport served as a major contributing factor in hyporesponsiveness in neonatal adrenal cells (2) and, therefore, is unlikely to explain the results of our study.

At first, we analyzed adrenal cAMP content at 15 minutes after injection of ACTH because previous studies have shown that the maximal effect of ACTH on cAMP production occurs after 15 minutes and returns to basal levels after approximately 40 minutes (17). Since, there is a possibility that very small increases in adrenal cAMP content in PD2 adrenals may have occurred earlier than 15 minutes (9), we repeated the low and moderate dose in PD2 and PD8 pups and sampled at 5 minutes. The 5-minute results with the moderate dose of ACTH essentially validated the 15 min data shown in Figures 1 and 2 as there was a corticosterone response in PD2 pups without a significant change in adrenal cAMP, whereas there was an adrenal cAMP response in PD8 pups without a change in plasma corticosterone. This confirms
that the PD2 rat adrenal appears to have a non-cAMP mediated increase in steroidogenesis whereas steroidogenesis the PD8 rat adrenal appears to be relatively insensitive to increases in cAMP stimulated by ACTH.

It is also possible that very small increases in cAMP were generated, but only in the appropriate compartment to stimulate steroidogenesis (42). Our method measured total cAMP content, so a small change in a specific intracellular compartment may not have been detected. The possibility of cAMP-independence is even more significant considering that the corticosterone response to a very low dose of ACTH did not correlate with an increase in adrenal cAMP content in PD8 pups (Figure 4).

Therefore, we conclude that either the PD2 adrenal does not require an increase in cAMP in the response to ACTH, or that it is ultrasensitive to very small, compartmental changes in adrenal cAMP content. Previous in vitro studies, however, do not suggest ultrasensitivity to cAMP in dispersed adrenal cells from PD1 compared to PD10 rat pups (2). Based on these findings, we still infer that another second messenger or intracellular mechanism may be involved in the large corticosterone responses to ACTH injection in the neonatal rat.

Perspectives: Our study has revealed a unique, but as yet, unidentified intracellular mechanism linking ACTH stimulation to an augmented increase in corticosterone production in the neonatal rat. This potentially novel phenomenon may have significant clinical implications. In particular, it is now established that transient, relative adrenal insufficiency is a significant clinical entity in the premature and low-birth weight human infant, and may be associated with hypotension, respiratory failure, and increased morbidity (15, 20, 21, 34, 35, 43, 50). Furthermore, an altered adrenocortical response to physiological and pharmacological doses of
exogenous ACTH is useful in the evaluation of adrenal function in the premature and low-birth
weight infant (1, 13, 20, 22, 24, 43, 44). ACTH stimulation is an important diagnostic test of
human adrenal function, the results of which can influence the implementation of glucocorticoid
therapy (35, 44). It is important to evaluate the mechanism of ACTH action on the adrenal cortex
using the rat model of human prematurity. An increased understanding of these mechanisms
could lead to improved diagnostic and treatment strategies. The current study has provided
evidence that ACTH may stimulate corticosterone production through an unidentified second
messenger system in the very young neonatal rat. Future studies investigating the identity of this
unknown intracellular mechanism will be helpful in gaining a full understanding of the
development of adrenal function in neonates.

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The authors have no conflicts of interest to disclose.
References:


Figure Legends

Figure 1. Plasma corticosterone response to ACTH injection in PD2 vs. PD8 rats. Postnatal day (PD) 2 and 8 pups were injected with porcine ACTH at low, moderate, and high doses (1, 4, or 20 µg/kg body weight, respectively). ¹High dose different from low dose (p<0.05); ²All doses different from each other (p<0.05); ³High dose different from low and moderate dose (p<0.05). Baseline (N=15-18). Post ACTH injection (N=7-10). Data are presented as mean ± SEM. Note the y-axis range is lower for PD8 pup results.

Figure 2. Changes in adrenal cAMP content in PD2 and PD8 pups injected with 3 different doses of ACTH. Postnatal day (PD) 2 and 8 pups were injected with porcine ACTH at low, moderate, and high doses (1, 4, or 20 µg/kg body weight, respectively). ¹Different from low dose (p<0.05); ²Different from low and moderate doses (p<0.05); ³Different from high dose by log transformation after the removal of one outlier (p<0.05). N = 5. Data are presented as mean ± SEM.

Figure 3: Changes in adrenal cAMP content at 15 min vs. plasma corticosterone at 60 min (post-ACTH injection). Postnatal day (PD) 2 and 8 pups were injected with porcine ACTH at low, moderate, and high doses (1, 4, or 20 µg/kg body weight, respectively). ¹PD2 different from PD8 (p<0.05); ²Different from 1 µg/kg body weight (low dose) (p<0.05); ³Different from 1 and 4 µg/kg body weight (low and moderate doses, respectively) (p<0.05). cAMP (N = 5); Corticosterone (N = 7-10). Data are presented as mean ± SEM.

Figure 4: Plasma ACTH and corticosterone after injection of a very low dose of ACTH in PD2 and PD8 pups. Postnatal day (PD) 2 and 8 pups were injected with porcine ACTH at very low doses (0.5 and 0.2 µg/kg body weight, respectively). ¹Different from baseline (0
min).\(^b\) Significant difference in plasma corticosterone between PD2 and PD8 pups (p<0.05). N=5-6. Data are presented as mean ± SEM.

**Figure 5: Adrenal cAMP and plasma corticosterone 5 minutes after injection of low or moderate doses of ACTH in PD2 and PD8 pups.** Postnatal day (PD) 2 and 8 pups were injected with porcine ACTH at low and moderate doses (1 or 4 \(\mu\)g/kg body weight, respectively). \(^a\) Different from baseline (0 min). \(^b\) Significant difference between PD2 and PD8 pups (p<0.05). N=9. Data are presented as mean ± SEM.
Adrenal cAMP (pmol/mg)

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ACTH injection

Plasma Corticosterone (ng/ml)

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