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E. D. Bruder, P. C. Lee and H. Raff

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[Abstract] [Full Text] [PDF]

Elevated corticosterone and inhibition of ACTH responses to CRH and ether in the neonatal rat: effect of hypoxia from birth

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Adrenocortical responses to ACTH in neonatal rats: effect of hypoxia from birth on corticosterone, StAR, and PBR

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Raff, Hershel, Julie J. Hong, Martin K. Oaks, and Eric P. Widmaier. Adrenocortical responses to ACTH in neonatal rats: effect of hypoxia from birth on corticosterone, StAR, and PBR. *Am J Physiol Regul Integr Comp Physiol* 284: R78–R85, 2003. First published September 12, 2002; 10.1152/ajpregu.00501.2002.—The adrenocortical response to hypoxia may be a critical component of the adaptation to this common neonatal stress. Little is known about adrenal function in vivo in hypoxic neonates. The purpose of this study was to evaluate adrenocortical responses to ACTH in suckling rat pups exposed to hypoxia from birth to 5–7 days of age compared with normoxic controls. We also evaluated potential cellular controllers of steroidogenic function in situ. In 7-day-old pups at 0800, hypoxia from birth resulted in increased basal (12.2 ± 1.4 ng/ml; $n = 12$) and ACTH-stimulated (94.0 ± 9.4 ng/ml; $n = 14$) corticosterone levels compared with normoxic controls (basal = 8.3 ± 0.5 ng/ml; $n = 11$; stimulated = 51.3 ± 3.8 ng/ml; $n = 8$). This augmentation occurred despite no significant difference in plasma ACTH levels in normoxic vs. hypoxic pups before (85 ± 4 vs. 78 ± 8 pg/ml) or after (481 ± 73 vs. 498 ± 52 pg/ml) porcine ACTH injection ($20 \mu\text{g/kg}$). This effect was similar in the afternoon at 6 days of age and even greater at 5 days of age at 0800. The aldosterone response to ACTH was not augmented by exposure to hypoxia from birth. Adrenocortical hypoxia-inducible factor (HIF)-1 α mRNA was undetectable by RT-PCR. Steroidogenic acute regulatory (StAR) protein in adrenal subcapsules (zona fasciculata/reticularis) was augmented by exposure to hypoxia; this effect was greatest at 5 days of age. Peripheral-type benzodiazepine receptor (PBR) protein was also increased at 6 and 7 days of age in pups exposed to hypoxia from birth. We conclude that hypoxia from birth results in an augmentation of the corticosterone but not aldosterone response to ACTH. This effect appears to be mediated at least in part by an increase in controllers of mitochondrial cholesterol transport (StAR and PBR) and to occur independently of measurable changes in endogenous plasma ACTH. The augmentation of the corticosterone response to acute increases in ACTH in hypoxic pups is likely to be an important component of the overall physiological adaptation to hypoxia in the neonate.

adrenocorticotropin; aldosterone; newborn; steroidogenic acute regulatory protein; peripheral-type benzodiazepine receptor; hypoxia-inducible factor

HYPOXIA is one of the most common causes of neonatal morbidity and mortality (10, 17). Considerable attention has been paid to the short- and long-term neurological, cardiopulmonary, and renal consequences of neonatal hypoxia (3, 8, 14, 34, 36). In comparison, the adrenal adaptation in vivo to prolonged neonatal hypoxia has not been extensively studied. One pertinent study in human infants with hypoxemia due to bronchiolitis demonstrated an augmented cortisol, but not aldosterone, response to ACTH (12). This suggests a zone-specific adrenal adaptation to hypoxia.

We have extensively examined dispersed adrenal cells in vitro removed from suckling rats exposed to hypoxia from birth (28). We have found that, as opposed to adult rats (29), steroidogenesis in vitro is augmented in adrenal cells from hypoxic neonatal rats despite no changes in steroidogenic enzyme expression, in steroidogenesis in isolated mitochondria, or in morphology as assessed by immunohistofluorescence or electron microscopy (28). This suggests that hypoxia-induced changes in intracellular factors may alter steroidogenic enzyme activity independently of steroidogenic enzyme expression.

The critical question, however, is whether chronic hypoxia in the neonate alters adrenal function in vivo. Therefore, the purpose of the present study was to examine the corticosterone and aldosterone responses in vivo to ACTH injection in neonatal rats exposed to hypoxia from birth. We also evaluated the expression of steroidogenic acute regulatory (StAR) and peripheral-type benzodiazepine receptor (PBR) proteins, two principal intracellular controllers of steroidogenesis (2, 23, 35), as well as hypoxia-inducible factor (HIF)-1 α , a transcription factor thought to be involved in the cellular and molecular response to low oxygen tension (11).

MATERIALS AND METHODS

Animal Treatment and Exposure to Hypoxia

All animal experimental procedures were approved by Medical College of Wisconsin and Aurora Health Care Insti-

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tutional Animal Care and Use Committees and conformed to the "Guiding Principles for Research Involving Animals and Human Beings" of the American Physiological Society. Timed pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN; $n = 79$) were obtained at 14 days gestation and maintained on a standard sodium diet and water ad libitum in a controlled environment (0600–1800 lights on). Parturition occurred spontaneously on the afternoon of gestational day 21 during which rats were kept under observation. As soon as a litter was completely delivered, the pups were weighed and cross-fostered (8–10 pups/dam), and the dam and its pups were moved to an environmental chamber and exposed to normobaric normoxia (21% O₂) or hypoxia (12% O₂) as described in detail previously (28, 36). We have previously shown that this exposure leads to arterial P_O₂ levels in adults of about 50–55 Torr with sustained respiratory alkalosis with metabolic compensation (27, 30, 31).

ACTH Injection and Blood and Tissue Sampling

Experiment 1: 7 days of age. At 0800 at 7 days of age (60 litters), three to four pups per litter were quickly removed from the chamber, weighed, and decapitated (basal samples). Trunk blood was collected in sodium EDTA (3–4 pups/tube), adrenal glands were removed, and some were weighed. A subset of blood samples was allowed to clot, and serum was frozen for the measurement of corticosteroid-binding capacity (see below). Adrenal glands that were weighed were not saved for subsequent analysis because of the time delay required for accurate assessment of weight. Capsules (zona glomerulosa; ZG) and subcapsules (zonae fasciculata/reticularis; ZF/R) were separated and immediately frozen for subsequent analysis (see below). The remaining pups were weighed and injected intraperitoneally with porcine ACTH (Sigma) diluted in normal saline as described in detail previously (40). Pups were injected with 20 µg/kg ACTH (10 µl/g body wt) and immediately returned to their home cages with their dams in the appropriate normoxic or hypoxic environment. The pups were then decapitated at 15, 30, 45, and 60 min after ACTH injection, with blood and adrenal glands collected as described above. To generate a more complete ACTH-corticosterone dose-response curve, additional pups were injected with 10 µg/kg ACTH and decapitated at 30 min.

Experiment 2: 5–6 days of age. We then determined if increases in endogenous ACTH earlier in the hypoxic exposure, or in the afternoon, might account for changes in adrenal function. Another group of pups and their dams was exposed to hypoxia from birth to 0800 at 5 days of age (8 litters) or to 1400 at 6 days of age (11 litters). Sampling was performed as described above except that adrenal glands were not weighed but were all immediately separated and frozen for analysis. ACTH (20 µg/kg) was injected as described above, with pups decapitated 30 min after injection.

Hormone assays. Plasma ACTH and corticosterone were analyzed in unextracted plasma by radioimmunoassay using reagents purchased from ICN Pharmaceuticals (Costa Mesa, CA) as described previously (28–31). Because of hyperlipidemia that occurs in suckling rats (26), plasma was centrifuged at 16,000 *g* for 2 min before assay to avoid interference of lipids in the ACTH assay. Plasma samples after ACTH injection were diluted 1:5 for analysis of plasma ACTH. Plasma aldosterone was analyzed by solid-phase radioimmunoassay (Diagnostic Systems Labs, Webster, TX) following the manufacturer's specifications, except that standards and samples were assayed with 50 µl (instead of 100 µl), which still provided sufficient sensitivity (25 pg/ml).

Serum corticosteroid-binding capacity. Serum corticosteroid-binding globulin (CBG) was estimated by two different methods. Both involved assessing the difference in binding of [³H]corticosterone to diluted serum in the absence (total binding) and presence (nonspecific binding) of excess corticosterone. One method involved stripping the serum with charcoal before assay (7), while the other did not (18). Because hypoxia induces hyperlipidemia in suckling rats (26) and hyperlipidemia alters the serum binding of corticosterone (4, 13), we repeated these measurements after delipidation of serum using Lipoclear (Statspin, Norwood, MA).

RT-PCR for Adrenal HIF-1 α and StAR mRNA

Total cellular RNA was extracted by the guanidine thiocyanate method using kit-supplied reagents (RNAagents, Promega Biotec, Madison, WI). Single-strand cDNA was generated from 1 µg total cellular RNA with the use of Superscript II preamplification reagents (Life Technologies, Bethesda, MD) according to the manufacturer's instructions. PCR was carried out in 25-µl volumes of 1 × PCR buffer [60 mM Tris·HCl (pH 9.0), 15 mM (NH₄)₂SO₄, 2.5 mM MgCl₂] containing 1/10 the contents of the reverse transcription reaction, 0.2 mM each dNTP, 0.5 µM each primer, and 0.05 U/µl Taq DNA polymerase (Promega, Madison, WI).

HIF-1 α . The reactions were subjected to 35 amplification cycles on a Perkin Elmer-Cetus thermal cycler. The amplification cycle profile was 95°C denaturation for 1 min, followed by primer annealing at 48°C for 1 min and extension for 2 min at 72°C. Primers were designed with the aid of commercially available software (Primer Designer, S&E Software, State Line, PA) from previously published sequences of mouse (39) and human (38) HIF-1 α genes. The primers for rat templates were chosen based on maximum areas of sequence similarity between the mouse and human genes and similarity in GC content and melting temperature. The following are the 5' → 3' sequences of the sense (S) and antisense (AS) primers used in these studies: HIF-1 α S, ATACTGATTGCATCTCCATCTTCTACC; HIF-1 α AS, TCAGTTAACTTGATCCAAAGCTCTGAG. The primers were synthesized by Operon Technologies (Alameda, CA). PCR products were separated by gel electrophoresis.

StAR. PCR for StAR mRNA was performed using previously published methods and primer sequences (33). Semi-quantitative analysis was performed by normalizing the StAR signal to the housekeeping gene L19 as described previously (22). Gels were digitized and scanned using an Alphaimager System (Alpha Innotech, San Leandro, CA).

StAR and PBR Protein Immunoblot Analysis

StAR and PBR protein immunoblot analysis was performed as described in detail previously (40) with antibodies kindly provided by V. Papadopoulos (Georgetown Univ. School of Medicine). Protein was extracted from adrenal ZG and ZF/R and fractionated by one-dimensional SDS-PAGE on a 15% acrylamide gel. Proteins were transferred onto 0.45-µm nitrocellulose membranes for 30 min using a Trans-Blot Cell (Ideia, Corvallis, OR). Membranes were blocked for nonspecific absorption using 3% (wt/vol) dry nonfat milk. The blots were treated for immunodetection of PBR, stripped, and reblotted for detection of StAR protein using anti-PBR and anti-StAR at 1:1,000 dilution prepared as previously described (40). Data were normalized to β -actin protein using anti-mouse actin antibody at 1:1,000 dilution (Sigma, St. Louis, MO). Goat anti-mouse IgG-horseradish peroxidase was used as secondary antibody at 1:6,000 followed by chemiluminescent detection with reagents from Perkin Elmer

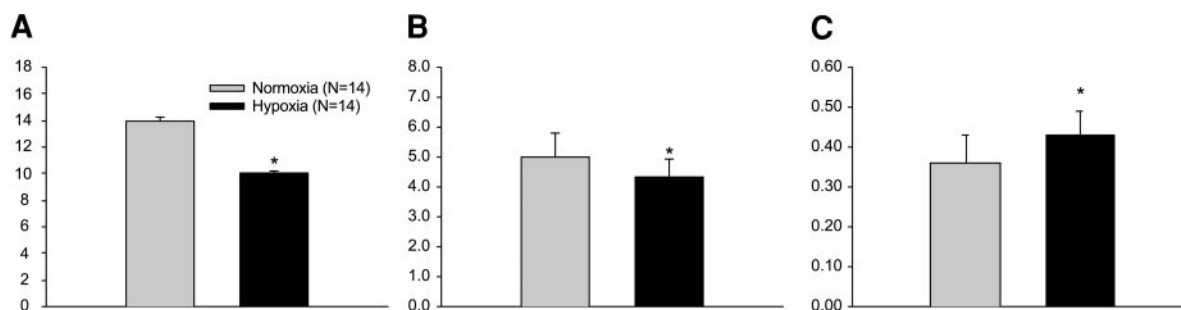


Fig. 1. Body weight (in g; A), adrenal weight (in mg; B), and the ratio of adrenal to body weight (in mg/g; C) in rat pups exposed to normoxia vs. hypoxia from birth to 7 days of age. *Hypoxia different from normoxia.

(Boston, MA). NIH Image J software was used to quantify blots.

Statistical Analysis

Data were analyzed by unpaired *t*-test and two- and three-factor ANOVA ($P < 0.05$). Data from gels were analyzed after logarithmic transformation. Post hoc analysis was performed by Duncan's multiple range test. Correlation analysis was performed by linear regression using Sigmastat software. Data are presented as means \pm SE.

RESULTS

Exposure to hypoxia from birth to 7 days of age resulted in $\sim 29\%$ lower body weight and $\sim 16\%$ lower adrenal weight compared with normoxic controls (Fig. 1). Because the difference in adrenal weight between normoxic and hypoxic rats was not as great as the difference in body weight, the ratio of adrenal weight to body weight was $\sim 16\%$ greater in pups exposed to hypoxia (Fig. 1).

Figure 2 shows plasma ACTH and corticosterone levels in rat pups exposed to normoxia or hypoxia from birth before (0 min) and after injection of ACTH (20 $\mu\text{g}/\text{kg}$). Hypoxia resulted in a small but significant increase in basal corticosterone (12.2 \pm 1.4 ng/ml) compared with normoxic controls (8.3 \pm 0.5 ng/ml) without any change in basal ACTH. Basal serum corticosteroid-binding capacity was so low using either method (7, 18) that the true binding could not be reliably distinguished from nonspecific binding even after delipidation (data not shown).

Injection of ACTH (20 $\mu\text{g}/\text{kg}$) resulted in a significant increase in plasma ACTH that was not different between normoxic and hypoxic pups (Fig. 2). There was a tendency for plasma ACTH to be lower in normoxic pups 60 min after injection, but there were no overall differences between hypoxic and normoxic data by ANOVA. Pups exposed to hypoxia demonstrated a marked augmentation of the corticosterone response to ACTH at all times assessed. The augmentation of the corticosterone response to ACTH in hypoxic pups was approximately 1.4-fold at 15 min, 1.7-fold at 30 min, 1.8-fold at 45 min, and 3.2-fold at 60 min.

Figure 3 shows plasma ACTH and corticosterone without and with 10 and 20 $\mu\text{g}/\text{kg}$ ACTH injection (30-min samples), with the 30-min results for 20 $\mu\text{g}/\text{kg}$ replotted from Fig. 2 for comparison. Although the

increase in plasma ACTH was related to the dose of ACTH, the corticosterone response on average was already maximal at 10 $\mu\text{g}/\text{kg}$. As in Fig. 2, basal and ACTH-stimulated corticosterone were augmented in hypoxic rats. These data were replotted (Fig. 4) individually (assuming linearity) to demonstrate the relationship between plasma ACTH and corticosterone (stimulus response). Hypoxia induced a significant shift upward in this relationship (i.e., *y*-intercept was significantly increased).

A prior increase in endogenous ACTH earlier in the hypoxic exposure or in the afternoon could have accounted for increased adrenal weight (relative to body

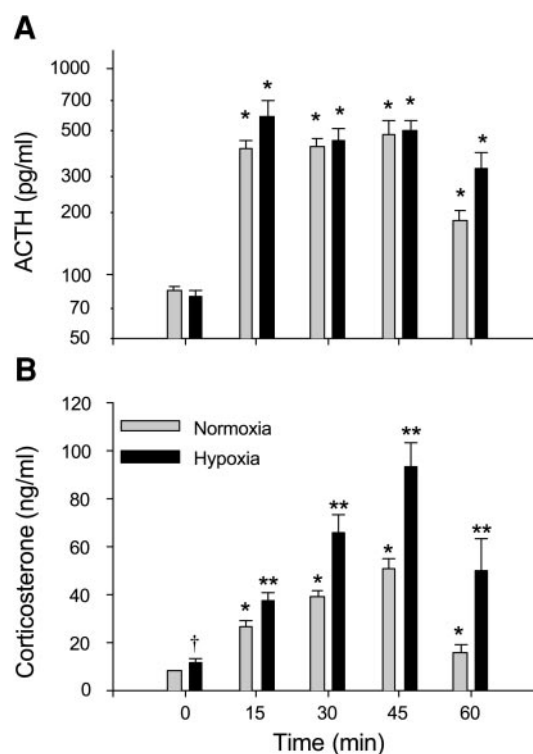


Fig. 2. Plasma ACTH (A) and corticosterone (B) before (0 min) and after injection of ACTH (20 $\mu\text{g}/\text{kg}$ ip) in pups exposed to hypoxia from birth to 7 days of age. Values are means \pm SE; $n = 6-8$. Note that ACTH data on this and subsequent figures are shown on a logarithmic scale so that basal values are visible. *Different from 0 min. **Different from 0 min and from normoxic value at same time point. †Basal hypoxia value different from normoxia.

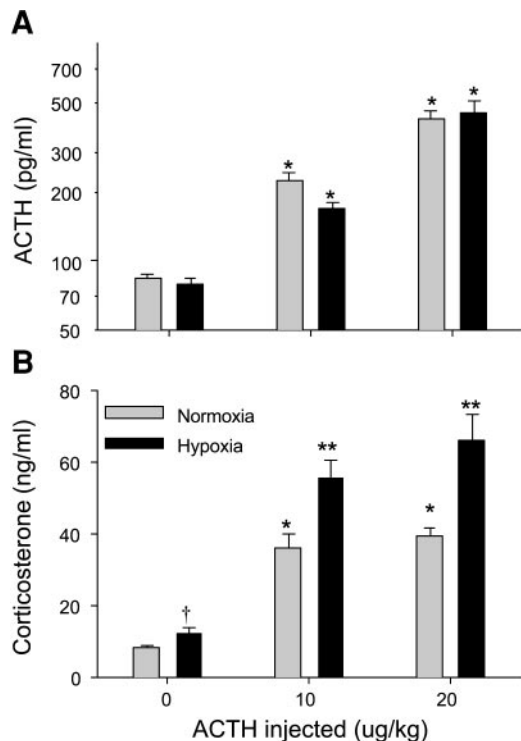


Fig. 3. Plasma ACTH (A) and corticosterone (B) responses to 0, 10, or 20 $\mu\text{g}/\text{kg}$ at 30 min. *Different from 0 min. **Different from 0 min and from normoxic value at same time point. † Basal hypoxia value different from normoxia. Values are mean \pm SE; $n = 6-14$.

weight) and augmented corticosterone responses to ACTH. To evaluate this possibility, we studied pups at 5 days of age in the morning and on the afternoon of *day 6* (the afternoon before experiments shown in Figs. 1–4). Figure 5 shows these results with data from 7-day-old rats replotted from Fig. 2 for comparison. Basal and ACTH-stimulated corticosterone was similar in normoxic pups regardless of age or time of day. Exposure to hypoxia from birth again resulted in an increase in basal corticosterone without a change in basal ACTH. This effect was more pronounced on *day 5* compared with *day 6* and on *day 6* compared with *day 7*. The hypoxia-induced augmentation of ACTH-stimulated corticosterone was more pronounced on *day 5* compared with *day 6* and on *day 6* compared with *day 7*. The qualitative response was not altered by the time of day. There was no significant overall effect of hypoxia from birth on basal or ACTH-stimulated plasma aldosterone (Fig. 6). However, at *day 5*, basal aldosterone was increased in hypoxic rats. Body weight in 5-day-old rats exposed to hypoxia (8.2 ± 0.2 g; $n = 26$) was less than normoxic controls (10.9 ± 0.01 g; $n = 26$). Body weight in 6-day-old rats exposed to hypoxia (9.5 ± 0.2 g; $n = 21$) was less than normoxic controls (12.9 ± 0.2 g; $n = 25$).

The ratio of StAR to L19 mRNA in adrenal subcapsules serves as a semiquantitative index of StAR expression as described previously (33). Exposure to hypoxia from birth to 7 days of age tended to increase ZF/R StAR/L19 mRNA (0.5 ± 0.1) compared with normoxic

controls (0.4 ± 0.1). Interestingly, StAR/L19 mRNA was higher in ZF/R from normoxic adult rats (0.6 ± 0.1) used as a positive control. There was no effect of hypoxia on ZG StAR/L19 mRNA (data not shown). We were unable to detect the expression of HIF-1 α mRNA by RT-PCR in neonatal adrenal glands from normoxic or hypoxic pups, although it was detectable in the kidney and liver (data not shown).

StAR and PBR proteins were analyzed by normalizing target protein band intensity to β -actin with the ratio in the adult adrenal arbitrarily set as unity. ZF/R StAR and PBR proteins were significantly greater in 5-day-old (AM) vs. 6-day-old (PM) or 7-day-old (AM) adrenal glands (Fig. 7). Exposure to hypoxia from birth resulted in a significant increase in ZF/R StAR protein at each age, with the effect being largest at 5 days of age. There was no effect of hypoxia from birth on ZF/R PBR at 5 days of age. However, at 6 days of age (PM) and 7 days of age (AM), there was a significant increase in ZF/R PBR in the rat pups exposed to hypoxia from birth. ACTH injection did not significantly alter StAR mRNA or StAR or PBR protein within the time frame (60 min) analyzed (data not shown). Finally, there was no effect of hypoxia and/or ACTH on StAR or PBR protein in the ZG (data not shown).

DISCUSSION

This study examined the control of corticosterone release *in vivo* in rat pups exposed to hypoxia from birth compared with normoxic controls. We found that 1) hypoxia resulted in a lower body weight and adrenal weight but an increased ratio of adrenal to body weight; 2) hypoxia from birth resulted in increased basal corticosterone despite no difference in plasma ACTH compared with normoxic controls; 3) hypoxia from birth resulted in an augmented corticosterone, but not aldosterone, response to exogenous ACTH; 4)

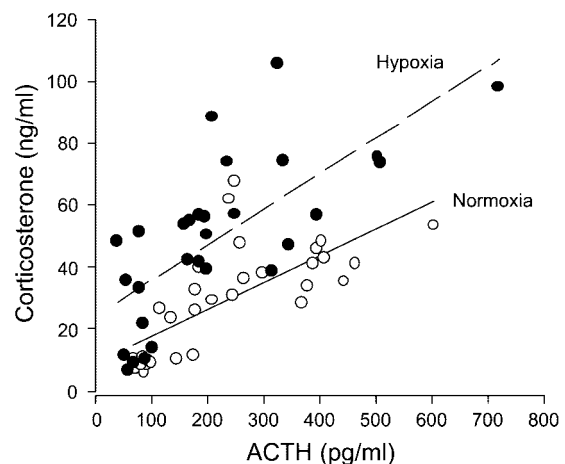


Fig. 4. Correlation of plasma corticosterone and plasma ACTH plotting the individual data points from Fig. 3 (0, 10, or 20 $\mu\text{g}/\text{kg}$ ACTH at 30 min after injection). For normoxia, slope = 0.09, y -intercept = 9.0 ng/ml, $r^2 = 0.51$, $n = 31$. For hypoxia, slope = 0.11, y -intercept = 24.1 ng/ml, $r^2 = 0.51$, $n = 29$. The hypoxia line was significantly elevated compared with the normoxia line (y -intercept: $P < 0.05$).

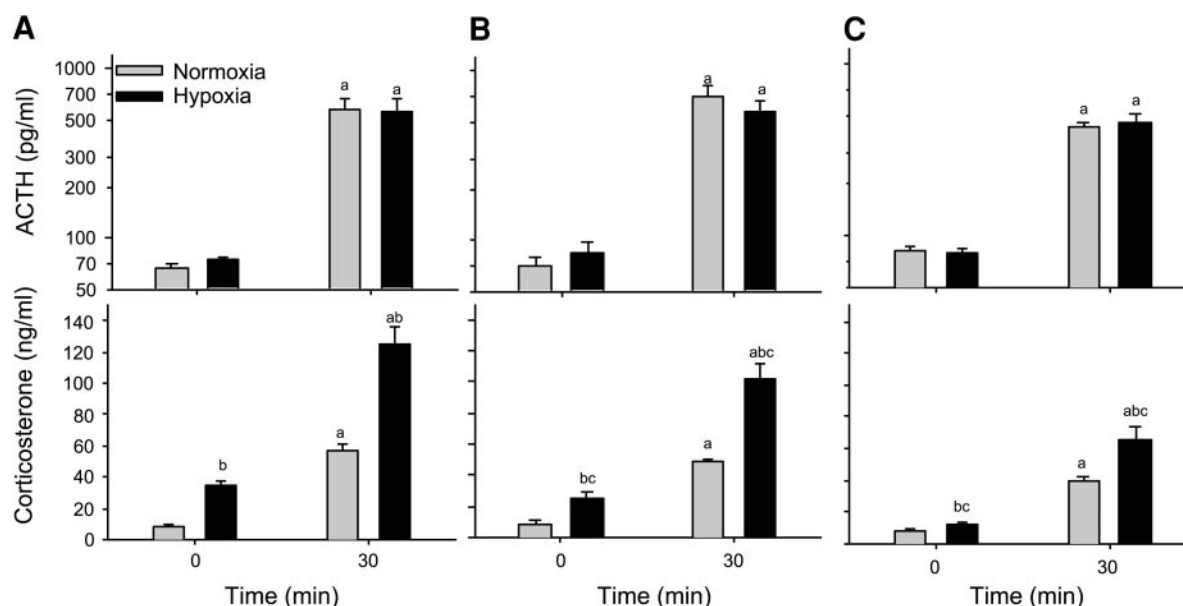


Fig. 5. Plasma ACTH and corticosterone before (0 min) and 30 min after injection of ACTH (20 $\mu\text{g}/\text{kg}$ ip) in pups exposed to hypoxia from birth to 5 days of age [0800–1000 (AM); A], 6 days of age [1400–1600 (PM); B], or 7 days of age [0800–1000 (AM); C; replotted from Fig. 2]. ^a30 min different from 0 min. ^bHypoxia different from normoxia at same time point. ^c6 and 7 day different from 5 day at same time point and treatment. Values are means \pm SE; $n = 6$ –14.

the augmentation in corticosterone response was greatest at 5 days of age compared with 6 and 7 days of age; 5) this effect could not be attributed to an increased ACTH in the AM or PM or earlier in the exposure; and 6) the increase in steroidogenesis was associated with an increase in StAR and, to a lesser extent, PBR protein, in the adrenal subcapsule (ZF/R).

We have previously demonstrated augmented early pathway (P450_{scc}) activity in intact dispersed adrenal cells from hypoxic rat pups but not in isolated mitochondria (28). This suggested that factors such as StAR or PBR proteins, which are known to mediate the rate-limiting mitochondrial cholesterol transport step, might be involved in the augmentation observed (2, 23, 35). If this were the case, we hypothesized that the adrenocortical response to exogenous ACTH *in vivo*

should be augmented in hypoxic pups compared with normoxic controls.

Because it is well known that body weight is lower in pups, juvenile, or adult rats exposed to hypoxia, it was important to ensure that the increase in ACTH was similar across groups. For that reason, ACTH was injected adjusting for body weight. We found that the increase in plasma ACTH achieved in normoxic and hypoxic rats was similar, leading to a controlled adrenal stimulus.

We also found an effect specific to corticosterone (subcapsule) because the aldosterone response to ACTH was not affected by hypoxia in neonatal pups. This is quite different from the response in adults, where aldosteronogenesis is decreased during hypoxia because of a decrease in expression of P450_{c11AS} (29).

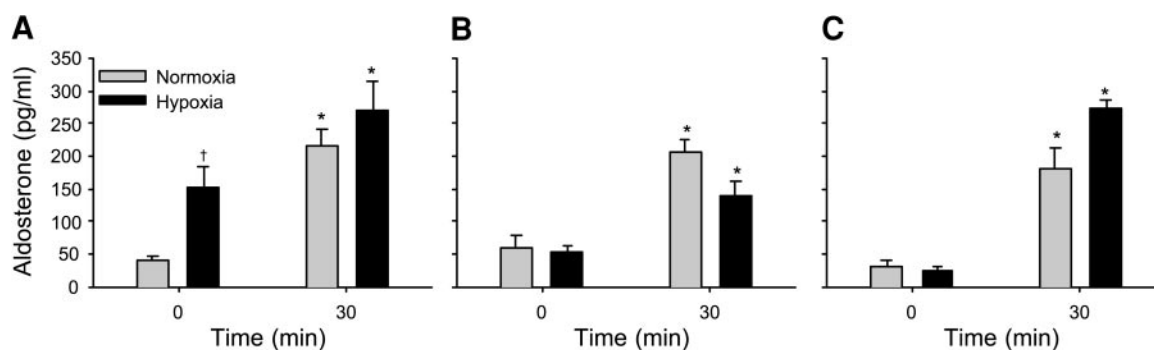


Fig. 6. Plasma aldosterone before (0 min) and 30 min after injection of ACTH (20 $\mu\text{g}/\text{kg}$ ip) in pups exposed to hypoxia from birth to 5 days of age [0800–1000 (AM); A], 6 days of age [1400–1600 (PM); B], or 7 days of age [0800–1000 (AM); C]. [†]0 min hypoxia different from normoxia. ^{*}30 min significantly greater than 0 min. Values are means \pm SE; $n = 6$ –14.

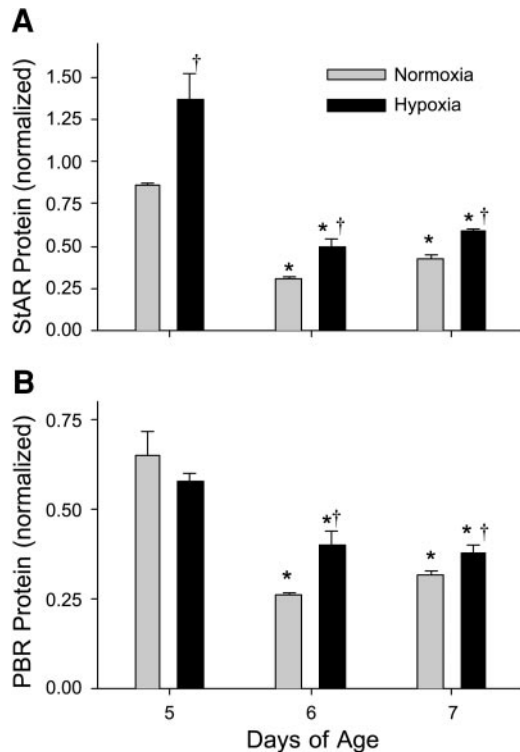


Fig. 7. Semiquantitative analysis of band intensities from protein immunoblots for steroidogenic acute regulatory (StAR) protein (A) and peripheral-type benzodiazepine receptor (PBR) protein (B) in pups exposed to hypoxia from birth to 5 days of age (0800–1000), 6 days of age (1400–1600), or 7 days of age (0800–1000). StAR and PBR bands were normalized to β -actin with the adult value arbitrarily set as unity. Values are means \pm SE; $n = 3$.

This confirms our previous proposition that the neonatal adrenal response to hypoxia is dramatically different from in the adult and is similar to a study in hypoxic human neonates (12).

What then could account for the augmented basal corticosterone response to ACTH in rat pups exposed to hypoxia from birth? Our initial hypothesis was that plasma ACTH was increased at some time during the exposure to hypoxia and that its trophic effects were responsible (6, 19). Consistent with that was the increase in relative adrenal weight we observed. We were unable, however, to demonstrate an increase in ACTH at 7 days of age. It remains possible that other post-translational products of proopiomelanocortin may be involved (6).

Even though neonatal rats have not been reported to have a significant circadian rhythm for corticosterone, we still thought that perhaps ACTH was increased in the PM and/or earlier in the exposure to hypoxia. This did not turn out to be the case. We have not completely eliminated the possibility that a small, essentially undetectable increase in ACTH, when integrated over time, might account for increased steroidogenesis and adrenal weight in vivo (1). We have also previously demonstrated that morphological changes in adrenal zonation and mitochondrial density are not responsible for changes in steroidogenesis in the neonatal rat ex-

posed to hypoxia from birth (28). The typical experimental approach using exogenous glucocorticoids to suppress ACTH release in adults is not useful in neonatal rats because even short-term dexamethasone significantly reduces the expression of P450c11 β (W. Engeland, personal communication). Our data do suggest that some factor(s) other than ACTH may be causing the increased corticosteronogenesis and adrenal weight in hypoxic pups. Because increased leptin has been shown to inhibit corticosterone but not aldosterone production in adult rats (20), it is possible that a decrease in leptin that we have demonstrated in hypoxic rat pups (24, 25) allowed an increased steroidogenic response in the ZF/R but not the ZG.

Another possible explanation for an increased corticosterone with no change in ACTH is that hypoxia resulted in an increase in CBG and/or albumin binding. We tried several different methods to assess serum corticosterone binding (7, 18). Because CBG levels were so low to start with, consistent with previous studies (7, 13), we were unable to reliably distinguish true binding from nonspecific binding. Adding to this difficulty was the dramatic hyperlipidemia that occurs in suckling rats exposed to hypoxia (26). Because of possible interference of increased serum lipids with the measurement of serum binding capacity (4, 13), we also measured CBG activity in serum after delipidation and still could not generate an acceptable signal-to-noise ratio. Therefore, we are not able to completely eliminate an increase in CBG as a possibility. Arguing against this is that serum binding of corticosterone is extremely low between 5 and 7 days of age and may not be able to account for dramatic changes in total steroid levels (7). Also, when corticosterone and ACTH were correlated (Fig. 4), there was a cluster of data points in which basal (pre-ACTH injection) corticosterone was not elevated.

Because mitochondria isolated from adrenals from hypoxic neonatal rats do not show augmentation in vitro but whole cells do (28), the final remaining probable mechanism is an intracellular factor or factors involved in steroidogenesis. We chose to study the two best described, StAR and PBR, both of which are involved in regulating the rate-limiting step of steroidogenesis (cholesterol transport from the outer to the inner membrane of the mitochondria) (2, 23, 35). Although the data are semiquantitative, the results are quite consistent with StAR and PBR involvement.

StAR protein was higher in adrenals from 5-day-old rats compared with 6- and 7-day-old rats, and hypoxia augmented this effect. This was associated with the large augmentation of the corticosterone response to ACTH in hypoxic pups at 5 days of age. PBR protein was not augmented at 5 days of age but was augmented at 6 and 7 days of age. These data suggest that a complex interaction of StAR and PBR proteins may account for some of the effect on corticosterone production observed. Adding that to the increase in relative adrenal weight, most of the effect of hypoxia could be accounted for. It has been previously reported by one of us (40) that the ontogenic expression of immunoreac-

tive PBR and PBR ligand-binding activity is correlated with the ontogenic pattern of ACTH-inducible steroidogenesis in neonatal rats during the so-called stress hyporesponsive period. The results suggested that PBR activity might be one of the factors determining the timing of the mature phenotype of the rat adrenal cortex. The present results are consistent with this hypothesis and extend those observations by implicating a possible role for both PBR and StAR protein in stimulus-induced enhancement of neonatal adrenocortical activity. This conclusion is also strengthened by the finding that hypoxia did not augment the aldosterone response to ACTH and that capsular (ZG) StAR and PBR were not significantly altered by hypoxia.

It is likely, however, that other factors are involved. We do not think that the transcription factor HIF-1 α is involved (11, 38), because its mRNA was not detectable in neonatal adrenal glands. We were concerned that the RT-PCR technique was not producing reliable results (even though it was able to detect HIF-1 α mRNA in the liver and kidney from these pups). We sent adrenal glands to G. Semenza at the Johns Hopkins Medical Institutions for analysis of mRNA by Northern blotting and protein by Western blotting (38), and Semenza, too, could not detect a signal for either (personal communication).

StAR protein was increased, but StAR mRNA was not. It is important to note that the RT-PCR for analysis of StAR mRNA is a semiquantitative method and that small changes may have not been detected. It is also important to realize that a change in StAR protein during posttranslational processing is the important functional endpoint and that it can occur without changes in mRNA levels (2). Of particular interest is that StAR and PBR proteins were highest at 5 days of age and that this correlated with the most robust steroidogenic response to ACTH whether in normoxic or hypoxic pups. This further suggests that StAR and PBR are important intracellular proteins mediating steroidogenesis in the newborn rat (40).

One interesting ACTH-independent controller that could be considered in future experiments is the possibility that factors from the adrenal medulla and/or innervation of the neonatal adrenal might be involved in adrenocortical function (9). In particular, it has been suggested that expression of tyrosine hydroxylase correlates with the stress-hyporesponsive period in the adrenal cortex of the rat pup at around the age we studied (21, 37). It may be that locally produced factors from the adrenal medulla (including ACTH) might be activated during hypoxia, which might alter adrenal sensitivity to exogenous ACTH or endogenous ACTH from the pituitary.

It seems likely, then, that some combination of a small, essentially undetectable change in ACTH (1) and/or some as yet unidentified systemic or local factor(s) results in an increase in adrenal weight and in StAR/PBR in rats exposed to hypoxia from birth. These factors lead to an increase in basal and ACTH-stimulated corticosterone in hypoxic rat pups.

Perspectives

We suggest that augmented corticosterone is an integral component of the metabolic adaptation to hypoxia in the neonate. Although increased circulating glucocorticoids may have detrimental effects on neurological development in the neonate (32), an increase in corticosterone in the hypoxic pups is likely to be instrumental in the cardiovascular and metabolic adaptation (5) and, ultimately, improved survival. Although these beneficial effects are numerous, several are worth special mention. One effect we have demonstrated involving increased corticosterone is a significant decrease in insulin sensitivity (24, 25). This is likely to maintain glucose delivery to the brain and heart under hypoxic conditions. Another phenomenon of particular interest is the effect of corticosterone on the development of the hepatic and exocrine pancreatic function, which we have shown is necessary to maintain neonatal hypoxic hyperlipidemia (15, 16). Therefore, in addition to the clear effects of corticosterone on maintaining cardiovascular reactivity during hypoxia, the effects on intermediate metabolism in the neonatal pup are vital components in the adaptation to hypoxia.

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REFERENCES

1. Akana SF, Shinsako J, and Dallman MF. Relationships among adrenal weight, corticosterone, and stimulated adrenocorticotropin levels in rats. *Endocrinology* 113: 2226–2231, 1983.
2. Artemenko IP, Zhao D, Hales DB, Hales KH, and Jefcoate CR. Mitochondrial processing of newly synthesized steroidogenic acute regulatory protein (StAR), but not total StAR, mediates cholesterol transfer to cytochrome P-450 side chain cleavage enzyme in adrenal cells. *J Biol Chem* 276: 46583–46596, 2001.
3. Boksa P, Wilson W, and Rochford J. Responses to stress and novelty in adult rats born vaginally, by cesarean section or by cesarean section with acute anoxia. *Biol Neonate* 74: 48–59, 1998.
4. Boonstra R and Tinnikov AA. Increased corticosteroid binding capacity of plasma albumin but not of corticosteroid-binding globulin caused by ACTH-induced changes in free fatty acid concentrations in snowshoe hares and rabbits. *J Endocrinol* 156: 205–212, 1998.
5. Chumas PD, Del Bigio MR, Drake JM, and Tuor UI. A comparison of the protective effect of dexamethasone to other potential prophylactic agents in neonatal rat model of cerebral hypoxia-ischemia. *J Neurosurg* 79: 414–420, 1993.
6. Coulter CL, Ross JT, Owens JA, Bennett HP, and McMillen IC. Role of pituitary POMC-peptides and insulin-like growth factor II in the developmental biology of the adrenal gland. *Arch Physiol Biochem* 110: 99–105, 2002.
7. D'Agostino J and Henning SJ. Hormonal control of postnatal development of corticosteroid-binding globulin. *Am J Physiol Endocrinol Metab* 240: E402–E406, 1981.
8. El-Khodori BG and Boksa P. Transient birth hypoxia increases behavioral responses to repeated stress in the adult rat. *Behav Brain Res* 107: 171–175, 2000.
9. Engeland WC, Wotus C, and Rose JC. Ontogeny of innervation of rat and ovine fetal adrenals. *Endocr Res* 24: 889–898, 1998.

10. **Frankel L and Stevenson DK.** Metabolic emergencies of the newborn: hypoxemia and hypoglycemia. *Compr Ther* 13: 14–19, 1987.
11. **Gassmann M and Wenger RH.** HIF-1, a mediator of the molecular response to hypoxia. *News Physiol Sci* 12: 214–218, 1997.
12. **Hanukoglu A, Fried D, Nakash I, and Hanukoglu I.** Selective increases in adrenal steroidogenic capacity during acute respiratory disease in infants. *Eur J Endocrinol* 133: 552–556, 1995.
13. **Haourigui M, Vallette G, Martin ME, Sumida C, Benasayag C, and Nunez EA.** In vivo effect of free fatty acids on the specific binding of glucocorticosteroids to corticosterone binding globulin and liver receptors in immature rats. *Steroids* 59: 46–54, 1994.
14. **Hoeger H, Engelmann M, Bernert G, Seidl R, Bubna-Lititz H, Mosgoeller W, Lubec B, and Lubec G.** Long term neurological and behavioral effects of graded perinatal asphyxia in the rat. *Life Sci* 66: 947–962, 2000.
15. **Lee PC, Jelinek B, Struve M, Bruder ED, and Raff H.** Effect of neonatal hypoxia on the development of hepatic lipase in the rat. *Am J Physiol Regul Integr Comp Physiol* 279: R1341–R1347, 2000.
16. **Lee PC, Struve M, Lewis SM, and Raff H.** Neonatal hypoxia in the rat: effects on exocrine pancreatic development. *J Pediatr Gastroenterol Nutr* 34: 542–547, 2002.
17. **Low JA, Froese AB, Galbraith RS, Smith JT, Sauerbrei EE, and Derrick EJ.** The association between preterm newborn hypotension and hypoxemia and outcome during the first year. *Acta Paediatr* 82: 433–437, 1993.
18. **Moraska A, Deak T, Spencer RL, Roth D, and Fleshner M.** Treadmill running produces both positive and negative physiological adaptations in Sprague-Dawley rats. *Am J Physiol Regul Integr Comp Physiol* 279: R1321–R1329, 2000.
19. **Nagaya M and Widmaier EP.** ACTH and stress accelerate maturation of adrenocortical function in neonatal rats. *Endocrine* 1: 247–252, 1993.
20. **Nowak KW, Pierzchala-Koziec K, Tortorella C, Nussdorfer GG, and Malendowicz LK.** Effects of prolonged leptin infusion on rat pituitary-adrenocortical function. *Int J Mol Med* 9: 61–64, 2002.
21. **Okimoto DK, Blaus A, Schmidt M, Gordon MK, Dent GW, and Levine S.** Differential expression of c-fos and tyrosine hydroxylase mRNA in the adrenal gland of the infant rat: evidence for an adrenal hypo-responsive period. *Endocrinology* 143: 1717–1725, 2002.
22. **Orly J, Rei Z, Greenberg NM, and Richards JS.** Tyrosine kinase inhibitor AG18 arrests follicle-stimulating hormone-induced granulosa cell differentiation: use of reverse transcriptase-polymerase chain reaction assay for multiple messenger ribonucleic acids. *Endocrinology* 134: 2336–2346, 1994.
23. **Papadopoulos V, Amri H, Boujrad N, Cascio C, Culty M, Garnier M, Hardwick M, Li H, Vidic B, Brown AS, Reversa JL, Bernassau JM, and Drieu K.** Peripheral benzodiazepine receptor in cholesterol transport and steroidogenesis. *Steroids* 62: 21–28, 1997.
24. **Raff H, Bruder ED, and Jankowski BM.** The effect of hypoxia on plasma leptin and insulin in newborn and juvenile rats. *Endocrine* 11: 37–39, 1999.
25. **Raff H, Bruder ED, Jankowski BM, and Colman RJ.** Effect of neonatal hypoxia on leptin, insulin, growth hormone and body composition in the rat. *Horm Metab Res* 33: 151–155, 2001.
26. **Raff H, Bruder ED, Jankowski BM, and Goodfriend TL.** Neonatal hypoxic hyperlipidemia in the rat: effects on aldosterone and corticosterone synthesis in vitro. *Am J Physiol Regul Integr Comp Physiol* 278: R663–R668, 2000.
27. **Raff H and Chadwick CJ.** Aldosterone responses to ACTH during hypoxia in conscious rats. *Clin Exp Pharmacol Physiol* 13: 827–830, 1986.
28. **Raff H, Jankowski BM, Bruder ED, Engeland WC, and Oaks MK.** The effect of hypoxia from birth on the regulation of aldosterone in the 7-day-old rat: plasma hormones, steroidogenesis in vitro, and steroidogenic enzyme mRNA. *Endocrinology* 140: 3147–3153, 1999.
29. **Raff H, Jankowski BM, Engeland WC, and Oaks MK.** Hypoxia in vivo inhibits aldosterone synthesis and aldosterone synthase mRNA in the rat. *J Appl Physiol* 81: 604–10, 1996.
30. **Raff H and Roarty TP.** Renin, ACTH, and aldosterone during acute hypercapnia and hypoxia in conscious rats. *Am J Physiol Regul Integr Comp Physiol* 254: R431–R435, 1988.
31. **Raff H, Sandri RB, and Segerson TP.** Renin, ACTH, and adrenocortical function during hypoxia and hemorrhage in conscious rats. *Am J Physiol Regul Integr Comp Physiol* 250: R240–R244, 1986.
32. **Rivest S.** Editorial: are glucocorticoids good or bad for brain development and plasticity. *Endocrinology* 143: 1157–1158, 2002.
33. **Ronen-Fuhrmann T, Timberg R, King SR, Hales KH, Hales DB, Stocco DM, and Orly J.** Spatio-temporal expression patterns of steroidogenic acute regulatory protein (StAR) during follicular development in the rat ovary. *Endocrinology* 139: 303–315, 1998.
34. **Soulier V, Dalmaz Y, Cottet-Emard JM, Lagercrantz H, and Pequignot JM.** Long-term influence of neonatal hypoxia on catecholamine activity in carotid bodies and brainstem cell groups of the rat. *J Physiol* 498: 523–530, 1997.
35. **Stocco DM and Clark RJ.** Regulation of the acute production of steroids in steroidogenic cells. *Endocr Rev* 17: 221–244, 1996.
36. **Thomas T and Marshall JM.** A study on rats of the effects of chronic hypoxia from birth on respiratory and cardiovascular responses evoked by acute hypoxia. *J Physiol* 487: 513–525, 1995.
37. **Walker CD.** Chemical sympathectomy and maternal separation affect neonatal stress responses and adrenal sensitivity to ACTH. *Am J Physiol Regul Integr Comp Physiol* 268: R1282–R1288, 1995.
38. **Wang GL, Jiang BH, Rue EA, and Semenza GL.** Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular oxygen tension. *Proc Natl Acad Sci USA* 92: 5510–5514, 1995.
39. **Wenger RH, Rolfs A, Marti HH, Guenet JL, and Gassman M.** Nucleotide sequence, chromosomal assignment and mRNA expression of mouse hypoxic-inducible factor-1 α . *Biochem Biophys Res Commun* 223: 54–59, 1996.
40. **Zilz A, Castello R, Papadopoulos V, and Widmaier EP.** Developmental expression of the peripheral-type benzodiazepine receptor and the advent of steroidogenesis in rat adrenal glands. *Endocrinology* 140: 859–864, 1999.