Development of the ACTH and Corticosterone Response

To Acute Hypoxia in the Neonatal Rat

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ABSTRACT

Acute episodes of severe hypoxia are among the most common stressors in neonates. An understanding of the development of the physiological response to acute hypoxia will help improve clinical interventions. The present study measured ACTH and corticosterone responses to acute, severe hypoxia (8% inspired O₂ for 4 h) in neonatal rats at post-natal days (PD) 2, 5, and 8. Expression of specific hypothalamic, anterior pituitary, and adrenocortical mRNAs was assessed by real-time PCR, and expression of specific proteins in isolated adrenal mitochondria from adrenal zona fascisulata/reticularis was assessed by immunoblot analyses. Oxygen saturation, heart rate, and body temperature were also measured. Exposure to 8% O₂ for as little as 1 h elicited an increase in plasma corticosterone in all age groups studied, with PD2 pups showing the greatest response (~3 times greater than PD8 pups). Interestingly, the ACTH response to hypoxia was absent in PD2 pups, while plasma ACTH nearly tripled in PD8 pups. Analysis of adrenal mRNA expression revealed a hypoxia-induced increase in Ldlr mRNA at PD2, while both Ldlr and Star mRNA were increased at PD8. Acute hypoxia decreased SpO₂ to ~80% and also decreased body temperature by 5-6 °C. The hypoxic thermal response may contribute to the ACTH and corticosterone response to decreases in oxygen. The present data describe a developmentally regulated, differential corticosterone response to acute hypoxia, shifting from ACTH-independence in early life (PD2) to ACTH-dependence less than one week later (PD8).

Key Words: adrenal cortex, pituitary, ontogeny, oxygen, body temperature
INTRODUCTION

Hypoxia may develop in the neonate due to the lack of lung development following preterm birth, or from a variety of cardiopulmonary disease states, such as patent ductus arteriosus (17,29). Paradoxically, hypoxia may also be precipitated by bronchopulmonary dysplasia caused by prior therapy with hyperoxia (9). Hypoxia in the premature neonate is a common and often devastating condition requiring mechanical ventilation, oxygen therapy, corticosteroids, and other supplementary therapies (39,51,58,59). This is of particular concern, as the number of preterm births in the United States has increased by 30% over the last two decades (12% of all live births in 2003) (30). Coordinated physiologic and metabolic responses to hypoxia, and a complete understanding of the development of these responses, are crucial for positive clinical outcomes (19,21,34,36).

Acute, severe hypoxia in newborns may occur as a result of apnea, respiratory infection, or other conditions (24,26,31). A coordinated hypothalamic-pituitary-adrenal (HPA) axis response may confer a variety of protective mechanisms aimed at coping with brief periods of severe hypoxia. For example, hypoxia-induced increases in plasma glucocorticoids may facilitate the closing of the ductus arteriosus, improve vasoconstrictor responses to circulating catecholamines, and promote maturation of the immature lung (11,33,67). Although acute hypoxia-induced increases in plasma corticosterone have been reported in neonatal rats (62,64), a complete understanding of the development, timing, and mechanisms of the HPA axis response to this stimulus has not been elucidated.
The primary goal of the present study was to evaluate plasma ACTH and corticosterone responses to acute hypoxia in rats at three critical ages in neonatal development (postnatal days [PD] 2, 5, and 8). Previous studies analyzed the pituitary-adrenal response to a shorter, more severe exposure to hypoxia (5% O2 for 20 min), reporting the absence of a plasma ACTH response until PD14, whereas plasma corticosterone displayed a U-shaped, age-dependent response to hypoxia (PD2-PD21) (62). Our laboratory has extensively examined HPA axis responses to chronic hypoxia in neonatal rats (5,7,44-46,48,50). Since episodes of acute, severe hypoxia are more likely to occur in the clinical setting, we have shifted our focus accordingly. We hypothesized that our acute hypoxia paradigm would elicit age-dependent differences in pituitary-adrenal responses. Furthermore, we hypothesized that these differences may be associated with changes in gene expression in the hypothalamus (e.g. Crh, Nr3c1, Nr3c2, Hsd11b2), anterior pituitary (e.g. Pomp, Crhr1, Hsd11b1), and the adrenal gland (e.g. Star, Ldlr, Cyp11a, Cyp21a1, Mc2r). Protein expression analyses were also performed on some of these gene products. Pulse oximetry, body temperature, and heart rate measurements were assessed in order to characterize cardiopulmonary responses to acute hypoxia.

METHODS

Animal Treatment

The Institutional Animal Care and Use Committee of Aurora Health Care approved the animal protocol. Timed-pregnant Sprague-Dawley rats (E15) or dams with their litters at PD 1 (Harlan Sprague Dawley, Indianapolis, IN; N=64 dams) were
received and maintained on a standard diet and water ad libitum in a controlled environment (0600-1800 lights on). The size of litters born in-house was normalized to 12 pups per litter (mixed sexes). In order to allow acclimatization to the animal facility, pups received at PD1 were studied at PD5 or PD8. On the morning of PD2, PD5, or PD8, litters (without dams) were placed in an environmental chamber. Litters were maintained together but were separated with plastic cage dividers, allowing the pups to nest and huddle on an adequate amount of bedding. During an initial 30 min period, room air (21% O₂) was supplied to the chamber at a rate of ~8 L/min. For baseline measurements, 2-3 pups from each litter were removed from the chamber and decapitated (control). Immediately following this, the chamber was closed and the input O₂ concentration was decreased to 8% (room air + N₂; ~8 L/min). Ambient O₂ concentration in the chamber reached the desired 8% within 10 min. At each subsequent time point (1, 2, 3, and 4 h), 2-6 pups/litter were removed from the chamber and sacrificed.

Blood and Tissue Collection

Trunk blood from 2-3 pups was pooled in an EDTA-plasma tube and treated as one sample. Whole adrenal glands for real-time PCR analyses, or adrenal zona fasciculata/reticularis (ZFR) for immunoblot analyses, were pooled and frozen on dry ice (4-12 adrenals/sample). Anterior pituitary glands were dissected, pooled (2-6 pituitaries/sample), and frozen on dry ice. Whole brains were frozen separately (1 brain/sample) by immersion in a container of 2-methylbutane (Thermo Fisher Scientific, Waltham, MA) chilled on dry ice. All plasma and tissue samples were stored
at –70 °C until analyses were performed. Real-time PCR and immunoblot analyses were performed on tissues from PD2 and PD8 pups. Intact hypothalami were obtained from whole brains, frozen on dry ice, and manually dissected with a scalpel in the coronal plane aided with a dissecting microscope. Landmarks were determined based on the atlas of Paxinos and Watson (42). Briefly, brains slices containing the hypothalamic paraventricular nucleus extended from a level immediately caudal to the crossing of the anterior commissure to the level of the dorsomedial hypothalamic nucleus. Sections were blocked dorsally at the dorsal edge of the third ventricle, ventrally at the border of the optic chiasm, and laterally at a position midway between the fornix and lateral border of the section (1 hypothalamus=1 sample). Dissected tissue was immediately processed for extraction of total RNA (see below).

Separate sets of PD8 litters were used to analyze possible gender differences in hormonal responses to hypoxia, as well as to provide a time control for the possible effects of maternal separation. Pups were separated by gender using anogenital distance as a visual marker (25), and exposed to hypoxia as described above. Visual gender determinations were confirmed by verifying the presence (or absence) of testes post-mortem. Time control pups were placed into the chamber as above, but were exposed to room air for the 4 h study duration. This experiment was performed to rule out effects of separation from the dam on plasma ACTH and corticosterone. Sampling methods were the same as above.
Hormone Assays, Pulse Oximetry, and Body Temperature Measurement

Plasma ACTH and corticosterone were measured by radioimmunoassay (MP Biomedicals, Orangeburg, NJ) as previously described (46). Arterial O₂ saturation (SpO₂; %) and heart rate (bpm) were measured using the MouseOx™ pulse oximetry unit (Starr Life Sciences, Allison Park, PA). This unit has been validated for use in rat pups per the vendor’s literature. One sentinel pup from each experiment (PD8 only; N=4) was instrumented by fastening sensor clips to each side of the head. The pup was kept near, but isolated, from the other pups in the chamber during the hypoxic exposure. Data from the MouseOx™ were gathered continuously (at each heartbeat) for the duration of the experiment. Over 50,000 data points were gathered over the 4 h experimental period. In order to analyze and summarize the data, 30 SpO₂ and heart rate measurements were selected at each time point, chosen at the point in the experiment immediately prior to pup removal from the chamber. The mean value of these 30 measurements was used as one datum, so each experiment yielded five data points (baseline, 1, 2, 3, and 4 h). In a separate group of rats (N=5 litters), body temperature was measured using a RET-3 rectal microprobe and a BAT-12 thermometer (Physitemp Instruments, Inc., Clifton, NJ) in PD2 and PD8 pups. Body temperature was recorded every 15 min during a 4 h exposure to 8% O₂ or 21% O₂ (normoxic time control). Instrumented pups were allowed to huddle with their littermates and ambient air temperatures remained constant.

RNA Isolation and Real-time PCR Assays

Total RNA for real-time PCR was isolated from whole adrenals, anterior pituitaries, and whole hypothalami using the RNeasy Mini Protocol (Qiagen). The
concentration of RNA was quantified using the absorbance value at 260 nm and the quality of the sample preparation was assessed using the A260/A280 ratio. All RNA preparations were diluted to a final concentration of 10-20 ng/µL, so that equal amounts were loaded across wells. Real-time PCR was performed using the Taqman One-Step RT-PCR Protocol (Applied Biosystems, Inc. (ABI); Foster City, CA). Pre-made primers and probes were purchased from ABI (see Table 1). The final reaction volume of 25 uL consisted of 1X AmpliTaq Gold® DNA Polymerase mix, 1X RT enzyme mix containing MultiScribe™ Reverse Transcriptase and RNase Inhibitor, 1X primer/probe mix, and 50-100 ng of total RNA. Amplification and detection were performed with the ABI Prism 7300 Sequence Detection System with the following thermal cycler conditions: 48 ºC for 30 min (RT), 95 ºC for 10 min, and 40 cycles at 95 ºC for 0.25 min and 60 ºC for 1 min. Each sample was assayed in triplicate. DNA digestion (ABI) was carried out on separate aliquots of RNA sample when the primer/probe mix for a specific gene could possibly detect genomic DNA (see Table 1).

Presentation of Gene Expression Data

Real-time PCR data are presented as the number of cycles needed to cross a pre-determined threshold (Ct), as previously published (6). The Ct value is set at a point where an increase in fluorescence (and therefore cDNA concentration) is exponential. Assuming 100% PCR reaction efficiency, the amount of cDNA doubles after each cycle. For example, a difference of 3.3 cycles between a control sample and an experimental sample translates to a 10-fold difference in mRNA expression. Note that the lower the Ct value, the greater the concentration of starting material (i.e. mRNA) in the sample of
interest. We chose to analyze 18S rRNA expression as an internal control (i.e. reference gene) in a subset of real-time PCR assays. We were then able to calculate normalized, relative changes in target mRNA expression (vs. baseline) using the $2^{-\Delta\Delta C_t}$ equation (28).

Isolation of Adrenal Mitochondria and the 100,000 g Membrane Fraction

Adrenal glands were dissected as mentioned above and quickly decapsulated to remove the outer zona glomerulosa/capsular layer. Subcapsules (adrenal ZFR and medulla) were then immediately frozen on dry ice and pooled (1 litter; 24 subcapsules/sample). Samples were homogenized on ice for 45 sec in ice-cold Tris (50 mM)/sucrose (0.25 M) buffer (“mito buffer”), pH 7.5, with an IKA® T10 Basic Ultra-Turrax Dispersing Tool (IKA Works, Inc., Wilmington, NC). Mitochondria were isolated by differential centrifugation as previously described (48). The supernatant from mitochondrial processing was further processed via differential centrifugation (100,000 g for 1 h). The resulting pellet, in addition to containing plasma membranes, also contained membranes from the following organelles: endoplasmic reticulum, microsomes, peroxisomes, Golgi apparatus, endosomes, and lysosomes (12). These samples will be referred to as “membrane” samples. Membrane and mitochondrial pellets were resuspended in mito buffer and the concentration of total protein was determined using a Qubit Fluorometer (Invitrogen Corporation, Carlsbad, CA). Following protein quantification, samples were centrifuged (10,000 rpm for 15 min) and pellets were resuspended in loading buffer. Final protein concentration was normalized across samples on any given gel. Samples were boiled for 10 min and frozen for subsequent immunoblot analyses.
**ImmunoBlot Analyses**

Mitochondrial and membrane protein samples from above were fractionated by one-dimensional SDS-PAGE on a 12% acrylamide gel (Invitrogen) (60 min @ 200 V). An equal amount of protein was loaded per well, and molecular weight markers (Precision Plus Protein WesternC Standards; Bio-Rad Laboratories, Inc., Hercules, CA) were run on each gel. Protein bands were transferred onto a 0.45 μm nitrocellulose membrane using the XCell II™ Blot Module (Invitrogen) (60 min @ 30 V). Membrane blots were blocked for non-specific absorption using Blocker™ Blotto in TBS (5% w/v nonfat powdered milk; Thermo Scientific, Rockford, IL). Mitochondrial blots were probed using anti-StAR (1:1000; kindly provided by Douglas Stocco at Texas Tech University, Lubbock, TX), anti-CYP11A (1:5000; Millipore Corporation, Temecula, CA), and anti-β-actin (1:200; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), while membrane blots were probed using anti-low-density lipoprotein receptor (LDLR) (1:300; Cayman Chemical Co., Ann Arbor, MI) and anti-β-actin. Donkey ECL anti-rabbit IgG-horseradish peroxidase (1:15,000; GE Healthcare, Buckinghamshire, UK) was used as a secondary antibody for detection of the proteins of interest. Precision Protein StrepTactin-HRP Conjugate (1:5000; Bio-Rad) was used as a secondary antibody for the detection of the molecular weight markers. Chemiluminescent detection was achieved using SuperSignal West Pico Luminescent Substrate (Thermo Scientific) and imaging with an Ultra-Lum camera (Ultra-Lum, Inc., Claremont, CA). Data were acquired by calculating area density of each protein band using the UltraQuant 6.0 software (Ultra-Lum).
**Statistical Analyses**

Data from pulse oximetry and heart rate measurements were analyzed by one-way ANOVA for repeated measures, with P<0.05 considered significant. Data from plasma ACTH and corticosterone assays, and body temperature measurements, were analyzed by two-way ANOVA for repeated measures, with P<0.05 considered significant. The ACTH and corticosterone response of each litter to 4 h of hypoxia was integrated as area under curve (AUC) above baseline by the trapezoidal rule and treated as one datum. Data from AUC calculations were log transformed and analyzed by one-way ANOVA. The Ct values obtained from real-time PCR, and densitometric values obtained from Western analyses, were analyzed by one-way ANOVA. All post hoc analyses were performed by Student-Newman-Keuls method for multiple comparisons (SigmaStat 2.03).

**RESULTS**

Pulse oximetry data for PD8 rats are shown in Figure 1. Thirty SpO2 or heart rate measurements were selected at each time point, and were chosen at the point in the experiment immediately prior to the chamber being opened. The mean value of these 30 measurements was used as one datum. Arterial O2 saturation (SpO2) was significantly decreased at all time points during exposure to 8% O2, when compared to SpO2 values at baseline (P<0.05). There were no differences between subsequent time points. Exposure to an 8% O2 environment consistently decreased SpO2 to around 80%, from a baseline value of nearly 100%. There was only a tendency for hypoxia to have an effect on heart rate, with rates decreasing from 305 ± 25 bpm at baseline to 229 ± 34 bpm at 2 h (P=0.254).
Figure 2 shows plasma ACTH (upper panel) and corticosterone (lower panel) measurements from PD2, 5, and 8 pups exposed to 8% O₂ for 0-4 h. Plasma ACTH concentrations were significantly increased, when compared to baseline, after 1 h in PD8 rats, and remained significantly elevated throughout the study (P<0.001). Acute hypoxia did not elicit significant changes in plasma ACTH concentrations at PD2 (P=0.977). Although there was only a tendency for ACTH to increase in PD5 pups by ANOVA, there was a significant increase after one hour of hypoxia when the data were log transformed and analyzed by t-test (P=0.04). Two of the five litters at PD5 showed a robust ACTH response to hypoxia, while the other three did not. This did not correlate with the magnitude of the corticosterone response. Plasma corticosterone was significantly increased at all time points compared to baseline in all three age groups (P<0.006). The most robust corticosterone response to hypoxia was in PD2 pups, where peak plasma concentrations of this hormone were nearly three times greater than in PD8 pups. In addition, baseline plasma corticosterone concentrations in PD2 pups were significantly greater than those in PD8 pups when the data were analyzed by t-test (P=0.008). Plasma ACTH was unchanged from baseline (49 ± 3 vs. 50 ± 3 pg/mL) in pups exposed to 21% O₂ as a time control for 4 h of maternal separation (P=0.383). Maternal separation also had no effect on plasma corticosterone (9 ± 1 vs. 10 ± 1 ng/mL; P=0.264). Female pups at PD8 exhibited a more robust plasma ACTH response at 4 h at 8% O₂ when compared to males of the same age (from 85 ± 2 to 151 ± 26 vs. 81 ± 2 to 115 ± 10 pg/mL; P=0.025). There were no gender differences in the corticosterone response to acute hypoxia (P=0.148; data not shown).
Figure 3 shows the integrated ACTH and corticosterone response to acute hypoxia (calculated from data shown in Figure 1). Note that the ACTH response to hypoxia increased with age, whereas the corticosterone response decreased with age. These results illustrate age-dependent decreases in adrenal sensitivity to ACTH and an age-dependent disinhibition from corticosterone negative feedback of the ACTH response to hypoxia.

The expression of genes with known or putative roles in adrenocortical function was analyzed by real-time PCR (Table 2). A decrease in the C_t value, as compared to baseline, corresponds to an increase in mRNA expression. Normalized, relative fold-changes in mRNA expression (vs. baseline), calculated using the 2^{-ΔΔCt} equation, are included for a subset of genes. Exposure of PD2 and PD8 pups to 4 h of hypoxia (8% O_2) significantly increased the expression of Ldlr mRNA in (P<0.002). Interestingly, hypoxia increased Star mRNA expression in adrenals from PD8 pups only. Hypoxia tended to increase adrenal Npy mRNA in PD2 rats, but the change was not significant (P=0.087). The expression of 18S rRNA (reference gene) was not affected by hypoxia (data not shown). In the anterior pituitary gland, hypoxia for 4 h caused a significant decrease in the expression of Crhr1 mRNA in PD8 pups only (P<0.001). There were no effects of hypoxia on pituitary Pmc or Hsd11b1 mRNA expression (P>0.05). Hypoxia decreased Crhr1 and Npy1r expression and increased Fos expression in PD2 hypothalami (P<0.04). In PD8 hypothalami, the only hypoxia-induced change was a decrease in Nr3c1 (GR) mRNA expression (P<0.02).

Data from adrenal ZFR immunoblot analyses are summarized in Table 3. Hypoxia did not affect mitochondrial StAR or P450scc protein expression, nor did it
affect LDLR protein expression in membrane samples (P>0.05). Interestingly, hypoxia significantly decreased β-actin protein expression in membrane samples from PD8 rats (P=0.008), and tended to decrease β-actin expression in mitochondrial samples from PD2 rats (P=0.089). This effect obviated the use of β-actin as a loading control. While the data suggest a possible effect of age per se on the expression of StAR, P450scc, LDLR, and β-actin, statistical analyses could not be performed for this comparison because of the changes in β-actin expression.

Figure 4 shows the effects of acute hypoxia on body temperature. The data show no effect of maternal separation (21% O₂ for 4 h) on body temperature. Interestingly, PD2 pups had significantly higher baseline (T=0) body temperatures than those of PD8 pups (32.8 ± 0.4 vs. 30.8 ± 0.6 °C; P<0.05). Hypoxia (8% O₂) decreased body temperature in PD2 and PD8 pups, with significant changes measured after 30-45 min of hypoxic exposure (P<0.05). A further significant decrease in body temperature only occurred at the 4 h time point (as compared to values at 30 min; P<0.05). There was no significant interaction between age and hypoxia on body temperature (P>0.05).

**DISCUSSION**

The present study characterized the early development of the HPA axis response to acute, severe hypoxia in neonatal rats. The results support the hypothesis that ACTH and corticosterone responses to acute hypoxia are age-dependent. Most remarkable was the shift in adrenocortical response to acute hypoxia from ACTH-independent in PD2 pups to ACTH-dependent in PD8 pups, with PD5 pups showing an intermediate, transitional ACTH response. We also demonstrated the development of adrenal
hyporesponsiveness to hypoxia-induced increases in ACTH at PD8. Analysis of mRNA revealed hypoxia-induced increases in Ldlr expression in PD2 and PD8 adrenals, while Star expression was increased only in PD8 adrenals.

We have previously described the effects of chronic hypoxia on the HPA axis of newborn rats. Moderate, continuous hypoxia (12% O2) from birth to PD7 caused an ACTH-independent increase in plasma corticosterone concentrations (44,49,50). This increase in steroidogenesis was at least partially attributed to increased expression of both Star mRNA and StAR protein, as well as increased expression of Ldlr mRNA (7,44). Interestingly, the corticosterone response to chronic hypoxia could be attenuated via blockade of post-ganglionic sympathetic neurotransmission using guanethidine (50). We also described hypoxia-induced changes in the adrenal lipid profile that may play a role in increased steroid production (8). Analysis of central mechanisms revealed that chronic hypoxia attenuated the plasma ACTH response to exogenous corticotropin-releasing hormone (CRH), indicating intact negative feedback due to increased adrenal steroidogenesis (46).

Life-threatening episodes of severe, acute hypoxia may be frequently encountered in both term and preterm newborn humans. The causes may range from respiratory distress brought on by diminished lung function to apneic episodes of known or unknown etiology (24,26,31,32). Increased adrenal glucocorticoid production in response to hypoxemia serves a number of adaptive roles, such as augmenting vasoconstrictor responses to circulating catecholamines and promoting lung maturation (11,33). A maximal adrenal response to hypoxemia is often lacking in these patients, necessitating frequent administration of steroids such as dexamethasone and hydrocortisone (66-68).
We used a model of severe, acute hypoxia to examine the ontogeny of the HPA axis response in neonatal rats. Pulse oximetry verified the severity of hypoxemia, as SpO2 values were consistently around 80%. Heart rate and body temperature were decreased during the hypoxic exposure; however, only the latter reached statistical significance. Previous studies by Mortola et al. have described a hypometabolic phenomenon (e.g. decreased body temperature and heart rate) in neonatal rats exposed to hypoxia (18,34,35). It was suggested that decreased body temperature was due to hypoxic inhibition of shivering and non-shivering thermogenesis, due directly to effects of low oxygen or indirectly to increased circulating levels of corticosterone (35,55,55,56). Therefore, a decrease in body temperature during hypoxia could have interacted with hypoxia itself to alter the function of the HPA axis in our studies. A decrease in body temperature may be a beneficial response to hypoxia to allow a decrease in metabolism when oxygen is scarce. This may be compromised by artificially maintaining the body temperature at “normal” (i.e. isothermic) values. Most importantly, the age of the pups did not alter the decrease in body temperature in response to hypoxia. We feel the different ACTH and corticosterone responses between PD2 and PD8 were not significantly confounded by hypothermia.

The pituitary-adrenocortical response to hypoxia was highly dependent on age. Plasma ACTH concentration was significantly increased in pups at PD8, while pups at PD2 showed no changes. Pups at PD5 exhibited smaller average increases in plasma ACTH at 1 h, which only reached significance following log transformation and analysis by t-test or integration as AUC. This was due to a variable ACTH response that did not correlate with corticosterone responses. This suggests that PD5 is the critical time during
which the adrenal switches from ACTH independence to dependence. Surprisingly, despite the lack of an ACTH response to hypoxia, PD2 pups exhibited the greatest increase in plasma corticosterone. Peak corticosterone concentrations in PD2 pups were over three times greater than those measured in PD8 pups. It is clearly evident that a developmental pattern in the HPA axis response to hypoxia exists. Previous studies have described a period of attenuated HPA axis responses to various physiological and psychological stressors in neonatal rats (10,27,61,62,64,65). During this stress hyporesponsive period (SHRP), basal plasma corticosterone concentrations are slightly elevated when compared to older rats, while ACTH and corticosterone responses to stress are attenuated (62). Clearly, the stress hyporesponsiveness in PD8 rats exposed to acute hypoxia, as we have found in this study, is due to decreased adrenal sensitivity to ACTH (71).

Hypoxia, and other stressors such as maternal deprivation, can overcome the physiologic restraint placed on the HPA axis during the SHRP (60,62,64). A key component of the response to hypoxia is adrenomedullary catecholamine production, due to systemic (e.g. cardiovascular) effects and to the local stimulatory effect on adrenocortical function (3,13,52,53). Splanchnic innervation of the adrenal medulla and cortex becomes complete by PD8 (16). Adrenomedullary chromaffin cells possess a cellular O₂-sensing mechanism that, when activated by hypoxia, increase catecholamine production and release (38). This attribute is not evident in rats beyond PD8 (57). Based on these observations, and our previous studies with chronic hypoxia and chemical sympathectomy (50), we hypothesize that augmented adrenomedullary activity during acute hypoxia may be at least partially responsible for increased plasma corticosterone in
PD2 pups. Further experimentation will be necessary in order to evaluate this mechanism.

Real-time PCR analyses were employed to explore the molecular mechanism(s) of hypoxia-induced increases in plasma corticosterone. We examined the expression of mRNA for major enzymes and proteins in the steroid synthetic pathway. The expression of Star mRNA was increased by hypoxia in PD8 adrenals. This increase was likely a result of increased plasma ACTH concentrations in this age group, as ACTH has been shown to increase the expression of Star mRNA (2). Hypoxia increased Ldlr mRNA expression in PD2 and PD8 adrenals. This finding suggests that adrenal LDL uptake may have been augmented by hypoxia, supporting increased flux of cholesterol through the steroidogenic pathway (4). The expression of mRNA for other major mediators of adrenal steroidogenesis (i.e. CYP11A, CYP21A1, and MC2R) was not affected by hypoxia; however, there was a tendency for an increase in Npy mRNA expression in the PD2 adrenal. Although NPY has been shown to have little affect on adrenocortical function (14), an increase in its expression (likely of adrenomedullary origin) could reflect increased activation of the adrenal medulla (15). While it is possible that the observed increases in mRNA expression were due to hypoxia-induced alterations in post-transcriptional processing or mRNA stability, it is likely that these increases are part of the physiological response to hypoxia. We chose to measure mRNA expression at the 4 h time point based on evidence suggesting that changes in mRNA expression can be detected as early as 1 h after the application of a stimulus (43).

Interestingly, immunoblot analyses could not detect hypoxia-induced changes in mitochondrial StAR or membrane LDLR protein expression. This lack of correlation
with mRNA expression may be due a lack of sensitivity of the immunoblot method.
Alternatively, the expression and retention of LDLR protein in membranes, as analyzed 
by immunoblot, may not accurately reflect LDLR protein concentration in the whole cell.
It has been suggested that active intracellular processing of LDL and its receptor may 
mask increases in LDLR protein expression expected upon increased \( Ldlr \) mRNA 
expression (37). Therefore, we hypothesize that hypoxia-induced corticosterone 
production in PD8 rats may be partially explained by increased expression of adrenal 
\( Ldlr \) and \( Star \) mRNA, likely a consequence of increased plasma ACTH concentrations in 
these rats. We also hypothesize that, in PD2 rats, hypoxia \textit{per se}, or some other factor 
induced by hypoxia (e.g. catecholamines), increases adrenal \( Ldlr \) mRNA expression, 
LDL cholesterol uptake, and corticosterone production.

Although not an intended primary outcome, we found a hypoxia-induced decrease 
in \( \beta \)-actin protein expression in adrenal membrane samples from PD8 rats. Actin proteins 
have been implicated as a mediator of stimuli-driven steroid production in cells from the 
adrenal cortex and gonads (22,23). Cytoskeletal actin is involved in the cellular rounding 
that occurs upon stimulation of adrenocortical cells with ACTH (1,23). Through these 
actions, organelles involved in the steroidogenic pathway (e.g. endoplasmic reticulum, 
lipid droplets, mitochondria) are brought within closer proximity of each other. This 
allows efficient shuttling of steroid precursor (i.e. cholesterol) and steroid metabolites, 
and also mediates stimuli-induced intracellular Ca\(^{2+} \) signaling (40,41). The hypoxia-
induced decrease in membrane \( \beta \)-actin protein expression observed in the present study 
could be one mechanism by which the PD8 adrenal exhibits a relative insensitivity to 
ACTH stimulation.
Molecular analyses at the hypothalamic-pituitary level measured limited changes in mRNA expression. In the anterior pituitary of PD8 rats, hypoxia caused a decrease in the expression of Crhr1 mRNA. This finding illustrates the fact that negative feedback at the level of the pituitary (via increased corticosterone production) is present in rats at this developmental age (63). It also supports previous studies that concluded that certain stressors (physiological or psychological) could override the restrictive effects of the pituitary component of the SHRP (62,64). Hypothalamic expression of Crhr1 and Npy1r mRNA was decreased by hypoxia in PD2 rats. These changes may reflect an adaptation to the ACTH-independent, hypoxia-induced increases in plasma corticosterone.

Hypothalamic expression of Fos mRNA was increased by hypoxia in PD2 rats, while this response was absent in PD8 rats. Previous studies measured increased hypothalamic Fos mRNA expression following a mild stressor in PD12 rats (54). This change occurred without increases in hypothalamic Crh mRNA expression or increases in plasma ACTH and corticosterone, suggesting HPA axis insensitivity to stress during the SHRP (54).

Our findings confirm that stress-induced hypothalamic Fos mRNA expression and HPA axis responses to stress may occur independently, and that these responses occur in a time and stressor-specific fashion. Real-time PCR results from whole hypothalami must be interpreted with caution, since isolation of specific nuclei was not performed. Therefore, we cannot specifically evaluate hypoxia-induced changes in mRNA expression in the paraventricular nucleus (69).
Perspectives and Significance

Acute episodes of severe hypoxia may occur at any age and may cause significant morbidity and mortality. In adult humans, this has become abundantly clear as the consequences of obstructive sleep apnea are now being realized (20). During the perinatal period, episodes of severe hypoxia not only have an impact on health in the short-term, but may also have lasting pathophysiological effects that persist into adulthood (47,70). A thorough understanding of the mechanism and timing of the HPA axis response to acute hypoxia in neonates has yet to be fully elucidated. Decreased body temperature in response to hypoxia and its possible influence on the HPA axis deserves additional scrutiny, since standard of care in a typical neonatal ICU is to prevent severe hypothermia. Further studies will uncover the details of these physiological mechanisms, as well as possibly provide new insight into therapeutic interventions for hypoxia-induced morbidity.

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FIGURE LEGENDS

Figure 1. Pulse oximetry measurements from PD8 pups exposed to 8% O₂ for 4 h.
Arterial O₂ saturation (SpO₂; %) and heart rate (bpm) were measured by fastening sensor clips to each side of the head. One sentinel pup from each experiment was instrumented. Data were gathered continuously (at each heartbeat) for the duration of the experiment. Thirty SpO₂ or heart rate measurements were selected at each time point, chosen at the point in the experiment immediately prior to the chamber being opened. The mean value of these 30 measurements was used as one datum, so each experiment yielded five data points (baseline, 1, 2, 3, and 4 h). N=4 at each time point. *Significant difference from baseline with P<0.05.

Figure 2. Plasma ACTH and corticosterone responses to acute hypoxia in neonatal rats at three different ages. Pups were placed in an environment chamber and exposed to a gas mixture consisting of 8% O₂. At each time point, 2-6 pups were removed and immediately sacrificed. Blood from 2-3 pups was pooled and treated as one sample. N=5-7 measurements per age group (at each time point) for each analyte measured. *Significant difference from baseline within the same age group with P<0.05. †Significant difference from PD8 within the same time point with P<0.05. #Significant difference from PD5 within the same time point with P<0.05.

Figure 3. Integrated plasma ACTH and corticosterone responses to acute hypoxia. Data were calculated as area under the curve for each age group using the trapezoidal rule, from individual litter responses shown in Figure 1. N=5-7 litters per age group for
FIGURE LEGENDS (cont.)

each analyte measured. *Significant difference from PD2 with P<0.05. †Significant
difference from PD5 with P<0.05.

Figure 4. Body temperature (°C) in PD2 and PD8 rats exposed to acute hypoxia.
One pup from each litter was instrumented with a microprobe rectal thermometer and
allowed to huddle with its littermates. Body temperature was monitored continuously
during the entire hypoxic exposure and data were recorded every 15 min. N=4-5
instrumented pups per age group per exposure (21% O2 or 8% O2 for 4 h). Body
temperature significantly decreased from baseline values after 30 min at 8% O2, and
values at 240 min were significantly lower than those at 30 min (PD2 and PD8; P<0.05).
Exposure to 21% O2 for 4 h had no effect on body temperature in pups at either age
(P>0.05).
Table 1. TaqMan Gene Expression Assays Used for Real-Time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Symbol</th>
<th>Assay ID#</th>
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<tbody>
<tr>
<td>Steroidogenic acute regulatory protein</td>
<td>Star</td>
<td>Rn00580695_m1</td>
</tr>
<tr>
<td>Cytochrome P450, subfamily 11A</td>
<td>Cyp11a</td>
<td>Rn00568733_m1</td>
</tr>
<tr>
<td>Cytochrome P450, subfamily 21A, polypeptide 1</td>
<td>Cyp21a1</td>
<td>Rn00588996_g1</td>
</tr>
<tr>
<td>Melanocortin 2 receptor (ACTH receptor)</td>
<td>Mc2r</td>
<td>Rn02082290_s1</td>
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<tr>
<td>Low density lipoprotein receptor</td>
<td>Ldlr</td>
<td>Rn00598438_m1</td>
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<tr>
<td>Hypoxia-inducible factor 1 alpha</td>
<td>Hif1a</td>
<td>Rn00577560_m1</td>
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<tr>
<td>Corticotropin releasing hormone receptor 1</td>
<td>Crhr1</td>
<td>Rn00578611_m1</td>
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<tr>
<td>Pro-opiomelanocortin</td>
<td>Pomp</td>
<td>Rn00595020_m1</td>
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<tr>
<td>FBJ murine osteosarcoma viral oncogene homolog</td>
<td>Fos</td>
<td>Rn02396759_m1</td>
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<tr>
<td>Corticotropin releasing hormone</td>
<td>Crh</td>
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<td>Glucocorticoid receptor (GR)</td>
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<td>Rn00561369_m1</td>
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<td>Mineralocorticoid receptor (MR)</td>
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<td>Rn00565562_m1</td>
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<td>Hydroxysteroid 11-beta dehydrogenase 1</td>
<td>Hsd11b1</td>
<td>Rn00567167_m1</td>
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<td>Hydroxysteroid 11-beta dehydrogenase 2</td>
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<td>Neuropeptide Y</td>
<td>Npy</td>
<td>Rn00561681_m1</td>
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<tr>
<td>Neuropeptide Y receptor 1</td>
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<tr>
<td>Actin, beta</td>
<td>Actb</td>
<td>Rn00667869_m1</td>
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</table>

Each gene expression assay consisted of a mix of sequence specific forward/reverse primers and a FAM-labeled probe. Note: Primer/probe sets that cross an exon-exon boundary are designated “m1” at the end of the Assay ID#. Primer/probe sets designated “g1” cross an exon-exon boundary but may still detect genomic DNA, while an “s1” designation describes an assay target found within a single exon (i.e. detects genomic DNA). RNA samples used for assays with “g1” or “s1” designations were treated with DNase prior to PCR.
TABLE 2.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Gene Symbol</th>
<th>Cycles to Threshold (Ct)</th>
<th>Cycles to Threshold (Ct)</th>
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<th>2^ΔΔCt</th>
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<td></td>
<td>Postnatal Day 2</td>
<td>Postnatal Day 8</td>
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<tr>
<td></td>
<td></td>
<td>Baseline 4 h P 2-∆∆Ct</td>
<td>Baseline 4 h P 2-∆∆Ct</td>
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<td>Adrenal</td>
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<td>23.98 ± 0.15 22.84 ± 0.14* 0.001 2.10</td>
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<td>Ldlr</td>
<td>31.40 ± 0.40 29.72 ± 0.41* 0.002 2.46</td>
<td>30.59 ± 0.10 28.07 ± 0.08* 0.001 5.46</td>
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<td>Cyp11a</td>
<td>23.20 ± 0.10 23.44 ± 0.08 0.097 0.76</td>
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<td>Cyp21a1</td>
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<td>19.78 ± 0.14 19.59 ± 0.18 0.433 1.26</td>
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<td>Mc2r</td>
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<td>Hif1a</td>
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<td>Npy</td>
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<td>Anterior</td>
<td>Pomc</td>
<td>25.38 ± 0.07 25.61 ± 0.19 0.159 ND</td>
<td>22.02 ± 0.22 22.01 ± 0.33 0.980 ND</td>
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<tr>
<td>Pituitary</td>
<td>Crhr1</td>
<td>34.29 ± 0.26 35.00 ± 0.13 0.255 ND</td>
<td>29.42 ± 0.05 30.50 ± 0.17* 0.001 ND</td>
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<td>Hsd11b1</td>
<td>32.65 ± 0.19 32.80 ± 0.24 0.822 ND</td>
<td>32.08 ± 0.25 32.63 ± 0.22 0.171 ND</td>
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<td>Hypothalamus</td>
<td>Crh</td>
<td>34.37 ± 0.19 34.26 ± 0.24 0.731 ND</td>
<td>33.44 ± 0.18 33.62 ± 0.16 0.461 ND</td>
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<td>Fos</td>
<td>26.96 ± 0.11 26.25 ± 0.17* 0.005 ND</td>
<td>26.76 ± 0.07 26.57 ± 0.17 0.317 ND</td>
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<td>Crhr1</td>
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<td>Nr3c1 (GR)</td>
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<td>23.41 ± 0.04 23.72 ± 0.09* 0.011 ND</td>
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<td>Nr3c2 (MR)</td>
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<td>Hsd11b2</td>
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<td>Npy</td>
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<td>Npy1r</td>
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<td>Actb</td>
<td>13.78 ± 0.08 13.81 ± 0.08 0.797 ND</td>
<td>16.57 ± 0.08 16.73 ± 0.11 0.282 ND</td>
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</table>

Real-time RT-PCR analysis of adrenal, anterior pituitary, and hypothalamic gene expression in PD2 and PD8 pups exposed to acute hypoxia. Whole adrenal glands (4-12 adrenals/sample) and anterior pituitary glands (2-6 pituitaries/sample) were pooled. Whole hypothalami were dissected and treated as one sample. N=3-6 per tissue type for each gene of interest (at each age). RNA concentrations were normalized to 10-20 ng/µL and each sample was assayed in
triplicate for PCR analysis. A decrease in the C\textsubscript{t} value at 4 h, as compared to baseline, translates into an increase in mRNA expression for that particular gene. Comparisons between age groups were not performed. * Indicates a significant difference in C\textsubscript{t} value. Where indicated, the 2\textsuperscript{ -\Delta\Delta C_{t}} equation was used to calculate the fold change in target mRNA expression (vs. baseline). The 2\textsuperscript{ -\Delta\Delta C_{t}} equation utilizes a single reference gene (18S rRNA in this case) for the normalization of relative changes in target mRNA expression. See Table 1 for description of gene symbol abbreviations. ND=Not Determined.
<table>
<thead>
<tr>
<th>Cellular Fraction</th>
<th>Protein</th>
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<th>Postnatal Day 8</th>
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<td></td>
<td></td>
<td>Subcapsules</td>
<td>Baseline</td>
<td>4 h</td>
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<tr>
<td>Mitochondria</td>
<td>StAR</td>
<td>4.47 ± 0.27</td>
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<td>CYP11A</td>
<td>4.28 ± 0.20</td>
<td>3.65 ± 0.43</td>
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<td>β-actin</td>
<td>2.40 ± 0.25</td>
<td>1.49 ± 0.37</td>
<td>0.089</td>
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<tr>
<td>Membranes</td>
<td>LDLR</td>
<td>0.99 ± 0.11</td>
<td>0.68 ± 0.22</td>
<td>0.307</td>
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<tr>
<td></td>
<td>β-actin</td>
<td>4.18 ± 1.11</td>
<td>4.80 ± 0.16</td>
<td>0.544</td>
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</table>

**Immunoblot analyses of adrenal subcapsule protein expression in PD2 and PD8 pups exposed to acute hypoxia (8% O₂).** Adrenal subcapsules were pooled (1 litter/sample) and immediately frozen. Comparisons between age groups were not performed due to a significant effect of hypoxia on the loading control (β-actin). N=4 per cellular fraction for each protein of interest (at each age). * Indicates a significant difference with P<0.05. See Table 1 for descriptions of abbreviations. Note that samples titled “Membranes” consist of plasma membranes and membranes from the following organelles: endoplasmic reticulum, microsomes, peroxisomes, Golgi apparatus, endosomes, and lysosomes.
FIGURE 1

Hours at 8% O₂

SpO₂ (%)  Heart Rate (bpm)
FIGURE 2

[Graph showing changes in Plasma ACTH and Plasma Corticosterone over hours at 8% O2]
FIGURE 3
FIGURE 4

![Graph showing body temperature over time for PD2 and PD8 in Normoxia and Hypoxia conditions.](image-url)