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

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Raff, Hershel, Lauren Jacobson, and William E. Cullinan. Elevated corticosterone and inhibition of ACTH responses to CRH and ether in the neonatal rat: effect of hypoxia from birth. Am J Physiol Regul Integr Comp Physiol 285: R1224–R1230, 2003—Hypoxia is a common cause of neonatal morbidity and mortality. We have previously demonstrated a dramatic ACTH-independent activation of adrenal steroidogenesis in hypoxic neonatal rats, leading to increases in circulating corticosterone levels. The purpose of the present study was to determine if this ACTH-independent increase in corticosterone inhibits the ACTH response to acute stimuli. Neonatal rats were exposed to normoxia (control) or hypoxia from birth to 5 or 7 days of age. At the end of the exposure, plasma ACTH and corticosterone were measured before and after either ether vapors were administered for 3 min or CRH (10 μg/kg) was given intraperitoneally. Thyroid function, pituitary pro-opiomelanocortin (POMC) mRNA and ACTH content, and hypothalamic corticotropin-releasing hormone (CRH), neuropeptide Y (NPY), and AVP mRNA were also assessed. Hypoxia led to a significant increase in corticosterone without a large increase in ACTH, confirming previous studies. The ACTH responses to ether or CRH administration were almost completely inhibited in hypoxic pups. Hypoxia did not affect the established regulators of the neonatal hypothalamic-pituitary-adrenal axis, including pituitary POMC or ACTH content, hypothalamic CRH, NPY, or AVP mRNA (parvo- or magnocellular), or thyroid function. We conclude that hypoxia from birth to 5 or 7 days of age leads to an attenuated ACTH response to acute stimuli, most likely due to glucocorticoid negative feedback. The neural and biochemical mechanism of this effect has yet to be elucidated.

adrenocorticotropic; corticotropin-releasing factor; anoxia; newborn; pro-opiomelanocortin, neuropeptide Y

HYPOXIA IN THE NEWBORN is one of the most common causes of neonatal morbidity and admission to an intensive care unit (8, 9, 19). Postnatal hypoxia occurs from birth for days to weeks after parturition and is caused by a wide variety of cardiovascular and pulmonary disorders (11, 19, 26, 31). There have been many studies evaluating the cardiopulmonary, renal, metabolic, and gastrointestinal adaptations to neonatal hypoxia (3, 10, 15–17, 30). Glucocorticoid therapy is used extensively in the treatment of neonatal respiratory distress (31). Because hypothalamic-pituitary-adrenocortical (HPA) axis activity is critical in stressed states (27), it is important to understand the potentially suppressive effects of elevated glucocorticoids on the HPA axis in the hypoxic newborn.

Because postnatal hypoxia is often chronic, we and others have exposed experimental animals to hypoxia from birth for days to weeks as a model of cardiovascular and pulmonary disease in the newborn (3, 22, 23, 30). We recently discovered that the 7-day-old rat pup exposed to hypoxia from birth demonstrates an ACTH-independent increase in corticosterone production (23). This exuberant, ACTH-independent corticosterone production is unique because the neonatal rat may exhibit an attenuated glucocorticoid response to other neonatal stressors, a phenomenon known as the stress hyporesponsive period (27). The exact anatomic locus of this so-called hyporesponsiveness in the normal neonate is controversial but has been identified at the hypothalamus, pituitary, and adrenal levels (2, 5, 6, 21, 32–40, 43). In light of the markedly elevated glucocorticoids in hypoxic neonates (23), we hypothesized that regulation of higher levels of the HPA axis differed fundamentally between hypoxic and normoxic neonates. Specifically, we hypothesized that the primary increase in corticosterone in the chronically hypoxic neonate should suppress acute ACTH responses to acute stress or CRH administration. We also hypothesized that an attenuated ACTH response could be correlated with an inhibition of the hypothalamic controllers of ACTH release: corticotropin-releasing hormone (CRH), AVP, and neuropeptide Y (NPY) (6, 7, 18, 28, 39, 40). Furthermore, because thyroid axis function influences CRH levels in the neonate, we hypothesized that HPA changes in hypoxic neonates could also be correlated with altered thyroid hormone levels (4, 5).

Therefore, this study evaluated the ACTH response to ether stress and to CRH administration in 5- and 7-day-old rat pups exposed to hypoxia from birth.
These critical time points were identified based on our previous study of adrenal function in hypoxic neonates (23). We also analyzed the expression of hypothalamic CRH, AVP, and NPY mRNA, pituitary pro-opiomelanocortin (POMC) mRNA and ACTH content, and thyroid-stimulating hormone (TSH) and thyroid hormones. We hypothesized that acute ACTH responses would be attenuated by hypoxia-induced elevation in corticosterone and that the indexes of neuroendocrine control of the HPA axis would account for this attenuation.

METHODS

Animal treatment and exposure to hypoxia. Timed pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN; n = 26) were obtained at 14 days gestation and maintained on a standard 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 environment chamber and exposed to normobaric normoxia (21% O_2) or hypoxia (12% O_2) as previously described in detail (22, 23, 30). We have previously shown that this exposure leads to arterial PO_2 levels in adults of about 50–55 Torr and sustained respiratory alkalosis with metabolic compensation (24, 25).

Ether stress, CRH injection, and blood and tissue sampling. At 0800 at 5 or 7 days of age, three to four pups per litter were quickly removed from the chamber, weighed, and decapitated (basal samples). Because neonatal rat pups do not exhibit a circadian rhythm in the HPA axis (23), this time of day was chosen to be consistent with previous studies (22, 23). Time from cage opening to death of the last pup in the litter was <5 min. We have previously found that neonatal HPA activity does not increase over this interval (23). Trunk blood was collected in sodium EDTA and pooled from three to four pups for each sample. Whole brains were quickly frozen in dry ice for in situ hybridization histochemistry. Pituitary glands were removed from the sella turcica, the neural lobe was removed and discarded, and the anterior pituitary was quickly frozen in liquid nitrogen (with 0.3 M sucrose), to avoid the neuropeptides in the posterior pituitary being washed out during the freezing process. A subset of brains was cooled and quickly dissected to isolate the hypothalamus, which was quickly frozen in dry ice for measurement of NPY mRNA. Some pups were not weighed and were exposed to ether vapor for 3 min as described previously (35). Briefly, pups were placed in an inhalation chamber for 1 min and exposed to light ether anesthesia; ether exposure was continued for an additional 2 min with a nose cone. After 20 min, pups were decapitated, and trunk blood was saved as described above. The remaining pups were weighed and were injected intraperitoneally with rat CRH (Peninsula, diluted in phosphate-buffered saline) as described in detail previously (35, 36). Pups were injected with 10 μg/kg CRH (10 μl/kg body wt), returned to their lactating dams in the chamber, and decapitated 30 min after CRH injection, with blood collected as described above. Based on responsiveness assessed previously (35) and on pilot studies in which pups were decapitated at 10, 20, and 30 min after stimulation, the 20-min post-ether and 30-min post-CRH time points were chosen for sampling as the peak ACTH and corticosterone responses. Only brains and pituitaries from pups before acute stimulation (at 0 min) were analyzed in detail because of pilot studies showing no effect of acute stimuli on CRH or AVP mRNA, or pituitary POMC mRNA.

Hormone assays. Plasma ACTH and corticosterone were analyzed by RIA as described previously (23). Because of hyperlipidemia that occurs in suckling rats (22), plasma was centrifuged at 16,000 g for 2 min before assay to avoid interference of lipids in the ACTH assay. Rat TSH was measured by RIA using reagents purchased from Alpco (Windham, NH). Plasma thyroxine (T_4) and triiodothyronine (T_3) were measured by RIA using reagents purchased from Diagnostic Systems Laboratories (Webster, TX). Plasma corticosterone was measured as described previously (39). Briefly, anterior pituitaries were dissected from the neurointermediate lobe and homogenized in ice-cold acetic acid-HCl with peptatin. After centrifugation, the supernatant was frozen and assayed at 1:1000 and 1:200 dilution in the ACTH RIA described above. ACTH content was normalized to protein content measured by direct UV spectrophotometry.

Northern analysis. Northern analysis of pituitary POMC and hypothalamic NPY gene expression was performed using previously published techniques (14, 29). Anterior pituitaries were dissected from the neurointermediate lobe at death and snap-frozen in liquid nitrogen. Hypothalamic blocks were dissected from the rest of the brain 30 min after CRH injection. Total RNA was isolated from anterior pituitary or hypothalamic tissue using the TRI Reagent procedure (Molecular Research Center, Cincinnati, OH), fractionated on 1.4% agarose gels containing 0.6 M formaldehyde, transferred to nylon membranes in 20× SSC, and immobilized by UV cross-linking. Antisense 32P-labeled cRNA probes were transcribed from appropriate linearized plasmids using T3 or T7 RNA polymerase (Stratagene, La Jolla, CA) from cDNA clones complementary to mouse POMC (1), rat NPY (13), or rat b-actin (14). Membranes were hybridized at 65°C in 50% formamide, 2% SDS, 0.8 M NaCl, and washed 3× in 0.1× SSC, 0.1% SDS (65°C). Hybridization signal intensity was quantified densitometrically after exposure to PhosphorImager cassettes (Molecular Dynamics, Sunnyvale, CA), and normalized for loading by comparison to the hybridization signal for b-actin mRNA.

Hypothalamic in situ hybridization histochemistry. In situ hybridization histochemistry (ISHH) for detection of CRH and AVP mRNAs was performed according to a previously established protocol, with riboprobes generated using standard in vitro transcription methodology (12) (CRH and AVP cDNAs courtesy of Dr. R. Thompson, Univ. of Michigan). For CRH and magnocellular AVP, semiquantitative analyses of hybridization films were performed on digitized images in the linear range of gray values obtained from an acquisition system (Fotodyne Variquest 100 lightbox, Pulnix CCD camera model TM-745 connected via a Pulnix CCD-84 converter to a Power Mac G4, captured with NIH Image v. 1.59 software). Signal pixels of the area of interest were defined as 3.5 SDs above the mean of a nearby cell-poor area; the number of pixels and average pixel values above the set background was then computed, yielding an integrated densitometric measure. For analysis of vasopressin mRNA expression within parvocellular neurons, manual grain counts were assessed from emulsion dipped, Nissl-counterstained material. Grain counts were performed from a minimum of five to six sections per animal over labeled cell bodies located within the medial parvocellular division of the paraventricular nucleus (mpPVN) at levels of the nucleus ranging from 1.7 to 1.9 mm caudal to the bregma (coordinates according to the atlas of Paxinos and Watson). Ectopic magnocellular neurons within the mpPVN were excluded from the analysis.

Statistical analysis. Data were analyzed by unpaired t-test or two- and three-factor ANOVA (P < 0.05). Post hoc analysis was performed by Duncan’s multiple-range test. Body weight
of each litter was averaged and analyzed as one datum. Data are presented as means ± SE.

RESULTS

Hypoxic 7-day-old pups weighed 9.8 ± 0.6 g per litter \((n = 10 \text{ litters})\) and were significantly lighter than their matched normoxic litters \((12.9 ± 0.6 \text{ g}; n = 10)\).

Ether stress. Hypoxia from birth resulted in a small increase in basal plasma ACTH (Fig. 1A, top) and a large increase in plasma corticosterone in 5-day-old rats (Fig. 1A, bottom). Hypoxia from birth in 7-day-old rats resulted in an increase in basal plasma corticosterone (Fig. 1B, bottom) without a statistical increase in plasma ACTH (Fig. 1B, top). In 5- and 7-day-old pups, hypoxia from birth eliminated the ACTH response to ether (Fig. 1, top), and there were no further increases in corticosterone levels (Fig. 1, bottom).

CRH stimulation. The pattern of the ACTH responses to CRH (Fig. 2) was similar to the response to ether (Fig. 1). Hypoxia from birth resulted in a small but significant increase in basal plasma ACTH and a large increase in basal corticosterone in 5-day-old rats (Fig. 2A). In 7-day-old rats, hypoxia from birth resulted in an increase in basal corticosterone without any
detectable change in basal plasma ACTH (Fig. 2B). In 5- and 7-day-old normoxic pups, there was a significant increase in plasma ACTH in response to CRH (Fig. 2, top), and a dramatic increase in plasma corticosterone (Fig. 2, bottom). In contrast, there was no further increase in ACTH or corticosterone in response to CRH in the hypoxic pups.

Because an increase in activity of the thyroid axis can alter the development of the HPA axis (4, 5), we measured plasma TSH, total T₄, and total T₃ in 7-day old pups. There was no effect of hypoxia from birth (data not shown).

Figure 3 shows representative in situ hybridization histochemistry for CRH and AVP mRNA in the hypothalamic paraventricular nucleus in brains from rat pups before acute stimulation (0 min). Densitometric analysis indicated that there was no effect of hypoxia from birth on the expression of CRH or magnocell and parvocellular AVP mRNA in 7-day-old rat pups. There was a tendency for NPY expression to be increased in the hypothalami from hypoxic rat pups, although this was not statistically significant (Fig. 4).

**DISCUSSION**

This study demonstrated that the large increase in basal corticosterone, which occurs in 5- to 7-day-old rat pups exposed to hypoxia from birth, was associated with inhibition of acute ACTH responses to CRH or ether but not with inhibition of pituitary POMC or hypothalamic CRH, AVP, or NPY gene expression.

We recently demonstrated a primary increase in plasma corticosterone in vivo and steroidogenesis in vitro in neonatal rats exposed to hypoxia from birth (23). It was interesting that the increase in corticosterone during hypoxia per se was more pronounced in 5-day-old compared with 7-day-old pups. This is likely due to a combination of the small increase in plasma ACTH observed in 5- but not 7-day-old pups and the well-described changes in adrenocortical steroidogenic capacity that occurs during early development in the neonatal rat (2, 43).

Because glucocorticoid negative feedback is expressed in the neonate (36), we hypothesized that the hypoxia-induced increases in corticosterone inhibited the ACTH response to a direct stimulus to the corticotroph (e.g., CRH) or a stimulus acting via hypothalamic factors (e.g., ether vapors). Indeed, hypoxia eliminated the response to either of these stimuli.

What was surprising was that hypoxia-induced elevations in corticosterone did not lead to a decrease in the expression or anatomic distribution of paraventricular CRH and AVP, the known hypothalamic controllers of ACTH release (12, 34, 35, 39, 40). This does not exclude a role for CRH because a previous study in neonatal rat pups found no change in PVN CRH mRNA...
during cold stress despite that fact that the ACTH response was blocked by CRH antagonist (41). However, Dent et al. (6, 7) found an increase in PVN CRH and AVP mRNA 15 min after restraint or injection stress in the neonatal rat, particularly in maternally deprived pups. This suggests that maternal deprivation enhanced the stress response in neonatal rat pups. In the current experiments, dams were present in the chambers to attend to the pups, which may have attenuated an increase in PVN CRH or AVP mRNA only 20 min after exposure to ether. Furthermore, there was no change in hypothalamic gene expression of NPY, which may also be involved in stimulating ACTH release (18, 28). Hypoxia had no effect on the expression of POMC or on ACTH content in the anterior pituitary. Although we only chose a single time point after ether or CRH administration, it is unlikely that we missed a transient change in these factors because we were unable to detect any changes at 10, 20, or 30 min poststimulus in pilot studies. Finally, the thyroid hormone axis, which may be involved in the maturation of the HPA axis in developing rats (4, 5), was not altered by hypoxia.

What, then, might be the explanation for the elimination of the ACTH response to CRH or ether in the hypoxic pups? It remains likely that the dramatically increased basal corticosterone levels in the hypoxic pups inhibited acute ACTH responses via glucocorticoid negative feedback. It may be that this inhibitory effect is opposed by activation of central pathways, which prevents the inhibition of basal ACTH and hypothalamic CRH, AVP, and NPY. We do not presently know whether ACTH levels can be suppressed below the basal levels we found in this study. The data suggest that there may be a differential control of ACTH release in the neonate such that basal ACTH release is constitutive and not sensitive to endogenous glucocorticoid feedback, while acutely stimulated ACTH release is.

The control of the HPA axis changes significantly during the first few weeks of life in the neonatal rat. The so-called stress hyporesponsive period (SHRP) is thought to minimize the corticosterone response to stress and thereby minimize the detrimental effects of exposure to glucocorticoids in the neonatal period. On the other hand, the lack of an adrenal response to hypoxia reduces the ability of the newborn to survive (42). Therefore, a direct activation of the adrenal cortex is a mechanism to increase corticosterone while bypassing the developmental inhibition of the hypothalamic-pituitary responses to a chronic stress such as hypoxia. The result, however, is an inhibition of the pituitary response to additional acute stimuli like ether vapors or CRH. This is likely due to classical glucocorticoid negative feedback, which is functional in the neonate (36).

**Perspectives**

Neonatal hypoxia/anoxia is among the most devastating and common causes of neonatal morbidity. It occurs for days to weeks after parturition and can be due to a wide variety of cardiovascular and pulmonary disorders (11, 19, 26, 31). It has been common practice to administer glucocorticoid therapy to neonates experiencing respiratory distress to attempt to attenuate morbidity (31). The direct activation of the adrenal cortex during neonatal hypoxia, therefore, increases endogenous glucocorticoid activity, which is beneficial, while bypassing the SHRP-associated inhibition of the HPA axis. It is important to note that the pituitary response to CRH normally exhibited in the normoxic neonate is eliminated in the hypoxic rat pup probably due to glucocorticoid negative feedback. The failure to facilitate an ACTH response to an acute stress during chronic hypoxia may protect the central nervous system of the neonate against the toxic central nervous system effects of further elevations in glucocorticoids (27).

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**DISCLOSURES**

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