Glucocorticoid feedback control of corticotropin in the hypoxic neonatal rat

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Abstract

The objective of this study was to determine the effects of manipulating glucocorticoid negative feedback on acute ACTH and corticosterone responses to corticotropin-releasing hormone (CRH) injection in 7-day-old rats exposed to normoxia or hypoxia from birth. Chemical adrenalectomy was achieved with aminoglutethimide, and glucocorticoids were replaced with a low dose of dexamethasone. Hypoxia *per se* increased basal plasma corticosterone and attenuated the plasma ACTH response to CRH. Aminoglutethimide *per se* decreased plasma corticosterone and strongly increased basal plasma ACTH and anterior pituitary *POMC* gene expression. Dexamethasone partially attenuated elevations in basal plasma

ACTH due to aminoglutethimide in both normoxic and hypoxic pups, but inhibited anterior pituitary *POMC* expression and CRH-induced plasma ACTH only in hypoxic pups. Despite this inhibition, hypoxic pups treated with both dexamethasone and aminoglutethimide still exhibited a significant CRH-induced increment in plasma ACTH, which was lacking in hypoxic pups not treated with either dexamethasone or aminoglutethimide. We conclude that ACTH responses to acute stimuli in hypoxic neonatal rats are prevented by ACTH-independent increases in corticosterone, rather than by intrinsic hypothalamic–pituitary hypoactivity.

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Introduction

Hypoxia is a common neonatal stress leading to significant short-term distress and long-term complications (Frankel & Stevenson 1987, Friedman & Fahey 1993, Low *et al.* 1993, Rubaltelli *et al.* 1998). Successful adaptation to neonatal hypoxia requires a coordinated physiological response, including an increase in the release of glucocorticoids from the adrenal cortex (Hanukoglu *et al.* 1995). Understanding the mechanisms by which the resulting increase in glucocorticoid secretion occurs, as well as the physiological impact of this increase in glucocorticoids, will aid in devising strategies to mitigate the short- and long-term effects of neonatal hypoxia. We have previously demonstrated that the neonatal rat exposed to hypoxia from birth has increased plasma corticosterone that is driven by sympathetic input to the adrenal cortex rather than by corticotropin (ACTH; Raff *et al.* 2003a, 2004). It is possible that this ACTH-independent increase in corticosterone in the neonate exposed to chronic hypoxia from birth is a mechanism to increase circulating glucocorticoids by bypassing the stress-hyporesponsive hypothalamus and/or pituitary.

We have demonstrated that the ACTH response to corticotropin-releasing hormone (CRH) or ether stress was significantly attenuated in the 7-day-old rat exposed to

hypoxia from birth (Raff *et al.* 2003b). We hypothesized that this attenuated pituitary corticotroph response was due to the negative feedback effects of the aforementioned ACTH-independent, sympathetically driven increase in corticosterone. This hypothesis is supported by the evidence that increased sensitivity to glucocorticoid negative feedback is one of the possible mechanisms contributing to the stress-hyporesponsive period in the neonatal rat (Walker *et al.* 1986b, Proulx *et al.* 2001, Schmidt *et al.* 2005).

The present study evaluated the hypothesis that the attenuated ACTH response to CRH in the 7-day-old neonatal rat pup exposed to hypoxia from birth, is due to the ACTH-independent increase in corticosterone. Because it is virtually impossible to adrenalectomize hypoxic neonatal rats with any expectation of survival, we induced a chemical adrenalectomy with aminoglutethimide (Lerner *et al.* 1984) and then provided different levels of glucocorticoids by means of a vehicle or low-dose dexamethasone injection (Proulx *et al.* 2001). We have used corticotroph responses to aminoglutethimide and CRH, in the presence or absence of dexamethasone to assess the sensitivity of hypothalamic–adrenal–pituitary axis (HPA) axis in normoxic vs hypoxic 7-day-old rat pups to the removal or imposition of glucocorticoid negative feedback.

Materials and Methods

The animal protocol was approved by the Institutional Animal Care and Use Committee of Aurora Health Care. Timed pregnant, Sprague–Dawley rats (Harlan, Indianapolis, IN, USA, $n=24$) at 14 days gestation were obtained and maintained on a standard diet and water available *ad libitum* (0600–1800 h lights on). Immediately after parturition (days 21–22), dams and their pups were continuously exposed to either normoxia (21% O₂) or hypoxia (12% O₂) in an environmental chamber, as described in detail previously (Thomas & Marshall 1995, Raff *et al.* 2000, 2003a). The experimental design is shown in Fig. 1. On postnatal day (PND) 6 at 1600 h, pups were separated into four pretreatment groups, with all pups from a given litter assigned to the same pretreatment. The four pretreatments, each consisting of two i.p. injections with a time gap of 14 h, were as follows: (1) Aminoglutethimide (400 mg/kg aminoglutethimide tartrate in 5 ml/kg saline at 1600 h of PND 6, followed by 5 ml/kg saline at 0600 h of PND 7); (2) Dexamethasone (5 ml/kg saline at 1600 h of PND 6, followed by 5 µg/kg dexamethasone phosphate in 5 ml/kg saline at 0600 h of PND 7); (3) Aminoglutethimide plus Dexamethasone (each given at the time and dose indicated above); or (4) Vehicle (5 ml/kg saline at both times). Pups were weighed before each pretreatment injection. Aminoglutethimide and Dexamethasone were obtained from Sigma.

At 0800 h (2 h after dexamethasone or saline (Vehicle) injection), some pups within each litter (Basal) were decapitated and trunk blood was pooled (three pups/sample). Each pool was considered $n=1$ for statistical analysis. Pituitary glands were quickly removed and the anterior lobe was dissected from the neurointermediate lobe. The anterior pituitary lobes of the three pups were pooled for each sample and frozen in liquid nitrogen. Each pool was considered $n=1$ for statistical analysis. The remaining pups (+CRH) within each litter were weighed and injected i.p. with 10 µg/kg of CRH (Bachem/Peninsula Labs, San Carlos, CA, USA diluted in phosphate-buffered saline, 10 µl/g body weight).

The CRH-injected pups were decapitated 30 min later. Each litter, therefore, provided four pooled plasma samples (two basal; two +CRH) and one to two pooled basal anterior pituitary samples (not every anterior pituitary was successfully retrieved). A vehicle control for CRH injection was omitted because injection stress does not activate the neonatal HPA axis (Walker *et al.* 1986a, Arai & Widmaier 1991).

Plasma ACTH and corticosterone were measured by RIA as described previously (Raff *et al.* 2003a,b). Pituitary pro-opiomelanocortin (POMC) mRNA was assessed by northern analysis as described previously (Jacobson *et al.* 1997, Raff *et al.* 2003b). ACTH data were log-transformed before analysis of variance to achieve a normal distribution. Data were analyzed by three-factor analysis of variance followed by Duncan's multiple range test.

Results

Figure 2 shows the ACTH and corticosterone levels achieved before (basal) or 30 min after CRH injection (+CRH) in 7-day-old rats exposed to normoxia vs hypoxia from birth and treated with vehicle, aminoglutethimide, and/or dexamethasone. ACTH and corticosterone responded significantly to CRH injection in normoxic vehicle-treated rat pups (first pair of bars, top and bottom panels of Fig. 2). As we have previously reported (Raff *et al.* 2003a, 2004), hypoxic vehicle-treated pups had elevated basal levels of corticosterone without an increase in basal plasma ACTH. Unlike normoxic pups, hypoxic pups did not exhibit a significant, CRH-induced increment in either hormone over basal levels (second pair of bars in both panels of Fig. 2). Aminoglutethimide treatment *per se* significantly reduced corticosterone and increased basal ACTH; levels of both hormones were comparable between normoxic and hypoxic pups. In contrast to normoxic pups, hypoxic pups demonstrated a significant increase in ACTH in response to CRH after aminoglutethimide treatment (third and fourth pairs of bars, Fig. 2). Dexamethasone *per se* lowered plasma corticosterone to similar levels and blocked the ACTH response

Figure 1 Experimental protocol. At birth, pups were exposed to normoxia or hypoxia for the entire experiment. On day 6 at 0600 h, pups were injected with vehicle or aminoglutethimide. On day 7 at 0600 h, pups were injected with vehicle or dexamethasone. Two hours later, some pups within a litter were sampled (basal). The remaining pups were injected with CRH and sampled 30 min later.

Figure 2 Plasma ACTH (top) and corticosterone (bottom) before (Basal – 0800 h) and 30 min after i.p. injection of 10 µg/kg CRH (+ CRH) in 7-day-old rat pups exposed to normoxia (N) or hypoxia (H) from birth. Four treatment groups of normoxic and hypoxic pups were studied: Vehicle (two saline injections (1600 h the day before and 0600 that day)), Aminoglutethimide injection (400 mg/kg at 1600 h the day before, followed by saline injection at 0600 h that day), Dexamethasone (saline injection at 1600 h the day before, followed by Dexamethasone at 0600 h that day), and aminoglutethimide + dexamethasone (aminoglutethimide injection at 1600 h the day before, followed by dexamethasone injection at 0600 h that day). Plasma from three pups was pooled and considered $n=1$. Each bar is the mean $\pm$ s.e.m. of $n=5-9$ pooled plasma samples. a, Significantly different from basal within the same treatment and the same normoxia or hypoxia group. b, Significantly different from the vehicle normoxia group within the same basal or CRH group. c, Significantly different from vehicle basal or vehicle CRH respectively within hypoxia. d, Significantly different from Aminoglutethimide Basal or Aminoglutethimide + CRH respectively within normoxia or hypoxia.

to CRH in both normoxic and hypoxic pups (fifth and sixth pairs of bars, Fig. 2). Administration of dexamethasone to aminoglutethimide-treated pups resulted in corticosterone levels that were not significantly different from those in pups treated with one drug alone. Aminoglutethimide plus dexamethasone significantly decreased, but did not completely normalize basal plasma ACTH relative to the levels in the corresponding Vehicle controls. CRH induced significant increases over basal levels of ACTH in normoxic and hypoxic

pups that had been treated with both dexamethasone and aminoglutethimide (seventh and eight pairs of bars, Fig. 2). The seemingly small difference in plasma ACTH between basal and + CRH in the hypoxic pups was indeed significant, most likely because the *post hoc* comparisons factor in rank order as well as differences among mean values. However, CRH-induced ACTH levels in aminoglutethimide-treated pups were significantly reduced by dexamethasone only in hypoxic pups (seventh and eight pairs of bars, Fig. 2).

Figure 3 shows anterior pituitary POMC mRNA levels in the Basal groups of normoxic and hypoxic pups that were given vehicle, aminoglutethimide, dexamethasone, or aminoglutethimide and dexamethasone. Despite differences in basal corticosterone levels (Fig. 2), POMC mRNA was similar between vehicle-treated normoxic and hypoxic pups, and increased to equivalent levels after aminoglutethimide administration (first and second pairs of bars, Fig. 3). Dexamethasone *per se* did not lower POMC mRNA below the levels in vehicle-treated pups (third pair of bars, Fig. 3). Administration of dexamethasone to aminoglutethimide-treated pups decreased POMC mRNA in hypoxic, but not normoxic pups (fourth pair of bars, Fig. 3).

Discussion

This study demonstrated in 7-day-old rat pups that (1) aminoglutethimide-induced reductions in corticosterone reveal elevated basal plasma ACTH and ACTH responses to CRH in hypoxic pups and (2) providing glucocorticoid feedback by

Figure 3 Anterior pituitary POMC mRNA normalized to 28S mRNA in 7-day-old rat pups exposed to normoxia (N) or hypoxia (H) from birth and treated with vehicle, aminoglutethimide, and/or dexamethasone, as described in Fig. 1 and Materials and Methods. Pituitary glands from three pups were pooled and considered $n=1$. Each bar is the mean $\pm$ s.e.m. of four anterior pituitary pooled samples collected from pups in the Basal group. a, Different from normoxic pups within same treatment group. b, Different from the corresponding Vehicle controls within normoxia or hypoxia. c, Different from aminoglutethimide treatment alone within normoxia or hypoxia.

dexamethasone administration to aminoglutethimide-treated pups resulted in equivalent basal and CRH-stimulated ACTH levels in normoxic vs hypoxic pups, despite differential inhibition of anterior pituitary POMC gene expression.

We have previously demonstrated that hypoxia from birth induced an ACTH-independent increase in corticosterone in 7-day-old rat pups (Raff *et al.* 2003a). This appeared to be mediated by sympathetic input to the adrenal cortex (Raff *et al.* 2004) and might be enhanced by the development of splanchnic innervation of the medulla at this age (Mikhail & Mahran 1965, Slotkin & Seidler 1988). We also previously demonstrated a significantly attenuated ACTH response to CRH and ether stress in 7-day-old rat pups exposed to hypoxia from birth (Raff *et al.* 2003b). These differences are not due to differences in corticosteroid-binding globulin or therefore to differences in free corticosterone levels between hypoxic and normoxic pups (Raff *et al.* 2003a). We confirmed the diminished ACTH response to CRH in hypoxic pups in the present study. We hypothesized that the ACTH-independent increases in corticosterone suppressed ACTH responses to acute stimuli via negative feedback inhibition. The present study supports that hypothesis.

First, the present study clearly showed that chemical adrenalectomy with aminoglutethimide resulted in large increases in basal ACTH in a manner similar to those observed in older rats (Jacobson *et al.* 1989). The effects of aminoglutethimide are consistent with the prior evidence that glucocorticoid negative feedback is operational in neonatal rats and may be a component of the etiology of the stress-hyporesponsive period (Walker *et al.* 1986b, Proulx *et al.* 2001, Schmidt *et al.* 2005). The functionality of glucocorticoid feedback in neonates was further confirmed by dexamethasone administration *per se*, which inhibited basal corticosterone and CRH-stimulated ACTH release. Moreover, hypoxic pups responded at least as well as normoxic pups to aminoglutethimide-induced decreases in corticosterone, exhibiting increases in basal plasma ACTH, anterior pituitary POMC gene expression, and CRH-induced ACTH secretion, which were as great or greater than those in normoxic pups. Since normal corticotroph responses to the removal of glucocorticoid feedback require hypothalamic input (Levin *et al.* 1988, Walker & Dallman 1993), these results indicate that the attenuated ACTH responses to the stimuli of CRH or ether stress that we have previously demonstrated in the hypoxic neonatal rat pups are not due to inherent hypothalamic-pituitary hypoactivity. It is also interesting to note that, despite the prior increases in plasma corticosterone, the plasma ACTH rapidly increased after overnight aminoglutethimide, suggesting a rapid recovery from inhibition by chronically elevated glucocorticoids in hypoxic pups.

A relatively low dose of dexamethasone was chosen (Proulx *et al.* 2001), so as to reduce but not eliminate aminoglutethimide-induced increases in basal ACTH. When pups were treated with both aminoglutethimide and dexamethasone, CRH administration resulted in equivalent ACTH levels in

normoxic vs hypoxic pups. This result suggests that in the absence of differences in circulating glucocorticoids, hypoxia does not alter the neonatal ACTH response to CRH. Our findings also indicate that hypoxia does not specifically decrease responsiveness of the corticotroph to CRH, which is consistent with previous microanatomical studies showing an increase in the number and size of the corticotroph population after hypoxic exposure (Gosney 1984, Kaur *et al.* 2002). In fact, with aminoglutethimide alone, the ACTH response to CRH was larger in hypoxic when compared with normoxic pups.

We also demonstrated that anterior pituitary POMC mRNA levels are increased by aminoglutethimide in the neonatal rat, and that these increases are comparable between hypoxic and normoxic pups. Interestingly, administration of dexamethasone to aminoglutethimide-treated pups, which decreased basal ACTH significantly to similar levels in both normoxic and hypoxic pups, decreased POMC mRNA only in hypoxic and not in normoxic pups. It may be that decreases in POMC mRNA would have been evident in normoxic pups if we had used sampling times later than 2 h. However, at the time points we used, our data clearly show more rapid inhibition of ACTH and POMC in hypoxic pups after aminoglutethimide and dexamethasone treatment. Consistent with the POMC mRNA data, the ACTH response to CRH in aminoglutethimide-treated pups was also only inhibited by dexamethasone in hypoxic pups. The differential suppression of POMC and CRH-induced ACTH secretion in hypoxic pups is unlikely to be due to differences in circulating levels or clearance of dexamethasone, since basal plasma ACTH showed similar inhibition by dexamethasone in both normoxic and hypoxic pups. The apparently greater sensitivity of POMC and ACTH responses to CRH to dexamethasone in aminoglutethimide-treated hypoxic pups is particularly intriguing, given the lack of inhibition of POMC expression by the elevated corticosterone levels in vehicle-treated hypoxic pups. We currently cannot distinguish whether this enhanced sensitivity occurs at the corticotroph, hypothalamus, or higher levels in the HPA axis of the hypoxic neonatal rat.

The use of chemical adrenalectomy does introduce potential confounds. The primary use of aminoglutethimide in this study was as an inhibitor of P450_{sc}, the first step in the steroidogenic pathway (Chabner *et al.* 1996). However, in addition to inhibiting adrenal steroidogenesis, aminoglutethimide also decreases gonadal steroidogenesis and inhibits aromatase (Chabner *et al.* 1996). Despite these confounds, aminoglutethimide has been used for experimental adrenalectomy in previous studies (Lerner *et al.* 1984, Jacobson *et al.* 1989). Its advantages are several. First, our model is an exposure of neonatal rat pups to hypoxia from birth. General anesthesia and adrenalectomy of neonatal rats under hypoxic conditions is not a viable experimental model. Secondly, aminoglutethimide allows the maintenance of the integrity of the adrenal medulla (Kent & Parker 1993), which is important in the neonatal adaptation to hypoxia (Hedner *et al.* 1980,

Slotkin & Seidler 1988). Therefore, the theoretical downsides to the use of aminoglutethimide are outweighed by its advantages in this particular experimental model.

In conclusion, we have demonstrated that the attenuation of the ACTH response to acute stimulation in 7-day-old rat pups exposed to hypoxia from birth is most likely due to glucocorticoid negative feedback. Although total corticosterone levels are low in 7-day-old rats when compared with adults, this is most likely due to low corticosteroid-binding globulin levels (Viau *et al.* 1996, Raff *et al.* 2003a). In fact, we propose that free (biologically active) corticosterone is actually normal or even increased, accounting for at least a component of the stress-hyporesponsiveness observed by others (Walker *et al.* 1986b, Proulx *et al.* 2001, Schmidt *et al.* 2005). The present findings demonstrate that even if low, the ACTH-independent increases in glucocorticoid levels in hypoxic neonates are capable of suppressing the ACTH response to acute stimuli such as CRH administration or ether stress (Raff *et al.* 2003b). Since glucocorticoid therapy is used to treat pulmonary disease and hypoxia in premature and term neonates (Tzukahara *et al.* 1999), inhibitory effects of exogenous glucocorticoids, in addition to enhanced feedback due to elevated endogenous glucocorticoid secretion, could impair the ability of the neonatal HPA axis to respond to other stresses in the postnatal period. Because glucocorticoid excess in the perinatal period can also permanently alter the regulation of the HPA axis and glucocorticoid-sensitive endpoints (Raff 2004), elucidating the mechanisms of glucocorticoid feedback in the normal and hypoxic neonate will help avoid adverse long-term sequelae of glucocorticoid therapy.

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