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Glucocorticoid Feedback Control of Corticotropin (ACTH) 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 corticotrophin-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 that 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.

Keywords

Adrenocorticotropin; corticosterone; negative feedback; adrenal

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 ACTH (Raff *et al.* 2003a; Raff *et al.* 2004). It is possible that this ACTH-independent increase in corticosterone in the neonate exposed to chronic hypoxia from birth

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is a mechanism to increase circulating glucocorticoids by bypassing the stress-hyporesponsive hypothalamus and/or pituitary.

We have demonstrated that the ACTH response to corticotrophin-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 evidence that increased sensitivity to glucocorticoid negative feedback is one of the possible mechanisms contributing to the stress-hyporesponsive period in the neonatal rat (Proulx *et al.* 2001; Schmidt *et al.* 2005; Walker *et al.* 1986b).

The current 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 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 HPA axis in normoxic vs. hypoxic 7-day old rat pups to the removal or imposition of glucocorticoid negative feedback.

Material 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, N=24) were obtained at 14 days gestation and maintained on a standard diet and water ad libitum (0600–1800 lights on). Immediately after parturition (day 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 (Raff *et al.* 2000; Raff *et al.* 2003a; Thomas & Marshall, 1995). The experimental design is diagrammed in Figure 1. On postnatal day (PND) 6 at 1600 hr, pups were separated into 4 pre-treatment groups, with all pups from a given litter assigned to the same pre-treatment. The 4 pre-treatments, each consisting of 2 ip injections 14h apart, 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 ug/kg dexamethasone phosphate in 5 ml/kg saline at 0600h 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 pre-treatment injection. Aminoglutethimide and dexamethasone were obtained from Sigma (St. Louis, MO).

At 0800 (2 hrs after dexamethasone or saline [vehicle] injection), some pups within each litter (Basal) were decapitated and trunk blood was pooled (3 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 3 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 ip with 10 ug/kg of CRH (Bachem; diluted in phosphate-buffered saline; 10 uL per gram body weight). The CRH-injected pups were decapitated 30 min later. Each litter, therefore, provided four pooled plasma samples (two basal; two +CRH) and 1–2 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 (Arai & Widmaier, 1991; Walker *et al.* 1986a).

Plasma ACTH and corticosterone were measured by RIA as described previously (Raff *et al.* 2003a; Raff *et al.* 2003b). Pituitary pro-opiomelanocortin (POMC) mRNA was assessed by Northern analysis as described previously (Raff *et al.* 2003b; Jacobson *et al.* 1997). ACTH data were log-transformed before analysis of variance to achieve a normal distribution. Data were analyzed by 3-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,Raff et al. 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 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 either drug alone. Aminoglutethimide plus dexamethasone significantly decreased but did not completely normalize basal plasma ACTH relative to levels in 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 means. 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 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 dexamethasone administration to aminoglutethimide-treated pups resulted in equivalent basal and CRH-stimulated ACTH levelsin 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 and 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 and, hence, 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 current 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 prior evidence that glucocorticoid negative feedback is operational in neonatal rats and may be a component of the etiology of the stress-hyporesponsive period (Proulx *et al.* 2001; Schmidt *et al.* 2005; Walker *et al.* 1986b). 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 that 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 pup 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 number and size of the corticotroph population (Gosney, 1984; Kaur *et al.* 2002). In fact, with aminoglutethimide alone, the ACTH response to CRH was larger in hypoxic compared to 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 hours. 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.

It is important to point out that the use of chemical adrenalectomy with aminoglutethimide does introduce potential confounds. The primary use of aminoglutethimide in this study was as an inhibitor of P450scc, 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 (Jacobson *et al.* 1989; Lerner *et al.* 1984). Its advantages are several. First, our model is 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. Second, aminoglutethimide allows the maintenance of the integrity of the adrenal medulla (Kent and 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 compared to adults, this is most likely due to low corticosteroid-binding globulin levels (Raff *et al.* 2003a; Viau *et al.* 1996). 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 (Proulx *et al.* 2001; Schmidt *et al.* 2005; Walker *et al.* 1986b). The current 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 post-natal 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 to avoid adverse long-term sequellae of glucocorticoid therapy.

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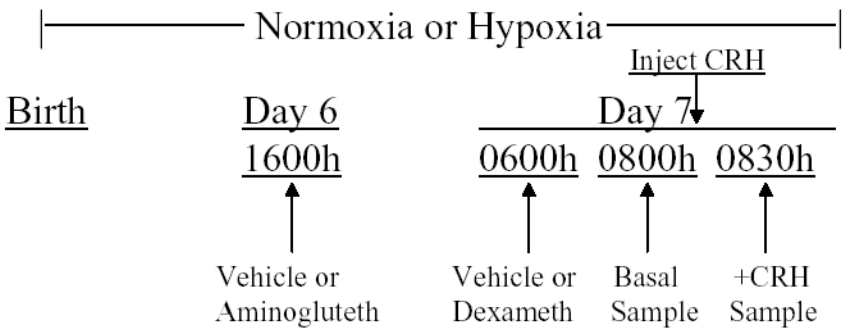

Figure 1.
Experimental protocol. At birth, pups were exposed to normoxia or hypoxia for the entire experiment. On Day 6 at 0600h, pups were injected with vehicle or aminoglutethimide. On Day 7 at 0600h, pups were injected with vehicle or dexamethasone. Two hours later, some pups within a litter were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

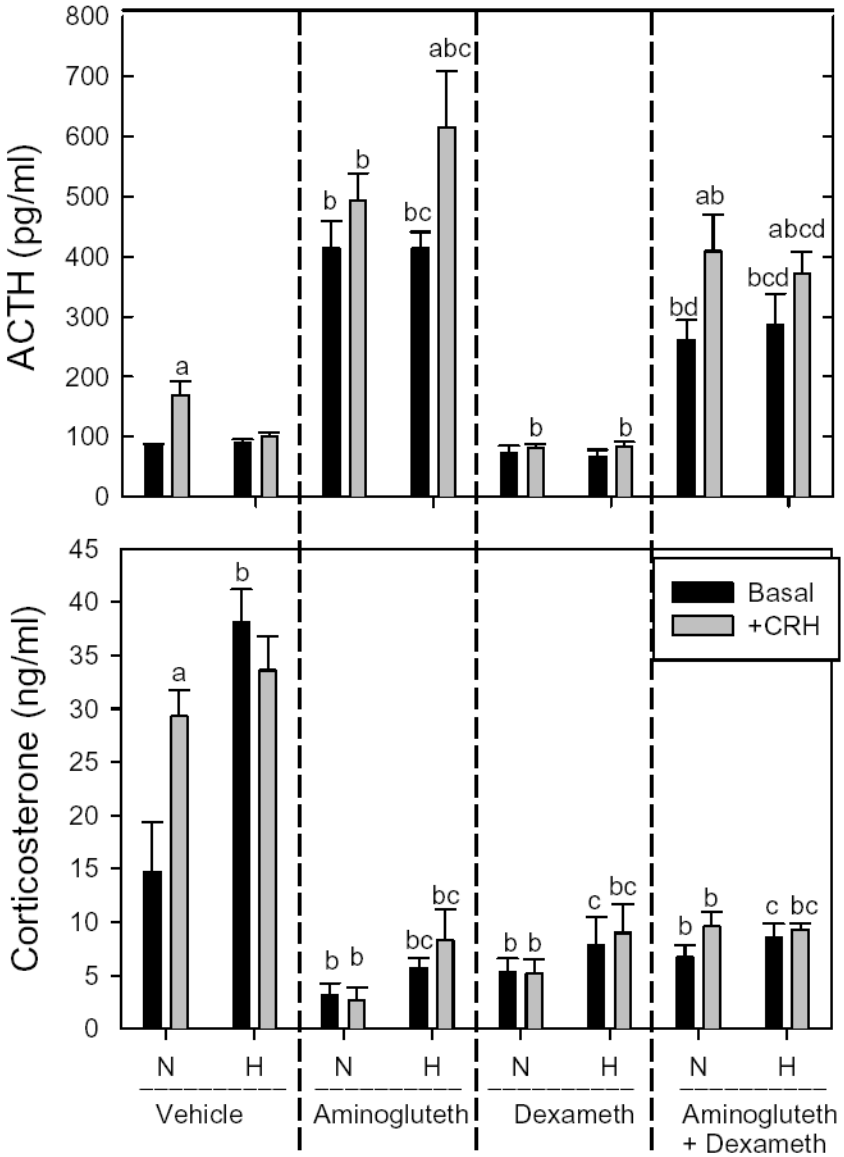

Figure 2.

Plasma ACTH (top) and corticosterone (bottom) before (Basal - 0800 hr) and 30 minutes after ip injection of 10 μg/kg CRH (+CRH) in 7-day old rat pups exposed to normoxia (N) or hypoxia (H) from birth. 4 treatment groups of normoxic and hypoxic pups were studied: Vehicle (2 saline injections [1600 h the day before and 0600 that day]), Aminoglutethimide injection [400 mg/kg at 1600 the day before] followed by saline injection at 0600 that day), Dexamethasone (saline injection at 1600 hr the day before followed by dexamethasone at 0600 that day), and Aminoglutethimide+Dexamethasone (aminoglutethimide injection at 1600 the day before followed by dexamethasone injection at 0600 that day). Plasma from 3 pups was pooled and considered N=1. Each bar is the mean±SEM 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

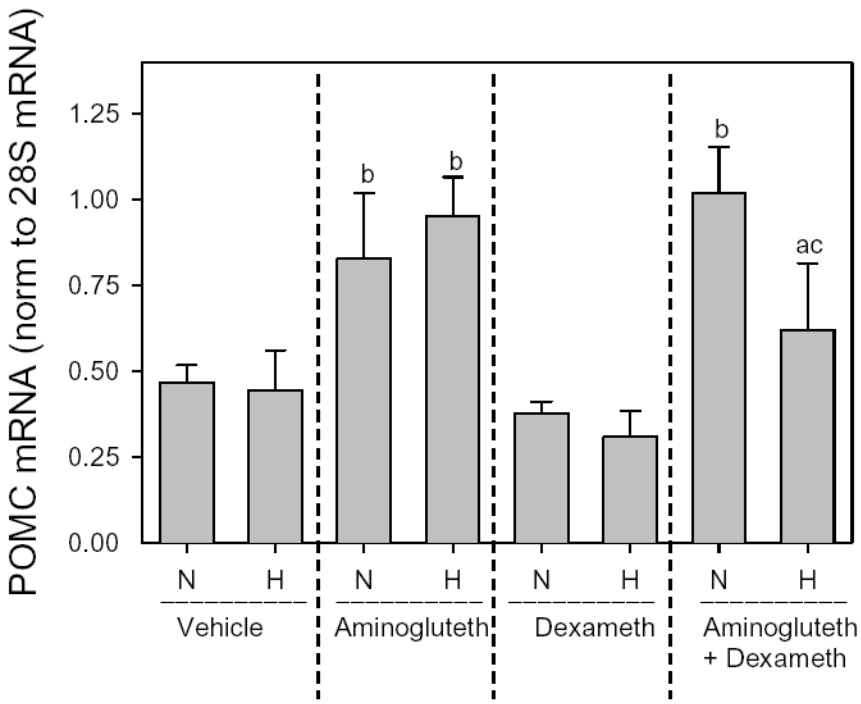

Figure 3.

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 Figure 1 legend and Methods. Pituitaries from 3 pups were pooled and considered N=1. Each bar is mean±SEM of 4 anterior pituitary pools collected from pups in the Basal group.

a, different from normoxic pups within same treatment group.

b, different from corresponding Vehicle controls within normoxia or hypoxia.

c, different from aminoglutethimide treatment alone within normoxia or hypoxia.