Published in final edited form as:
J Endocrinol. 2005 June ; 185(3): 477–484.

Plasma Leptin and Ghrelin in the Neonatal Rat: Interaction of Dexamethasone and Hypoxia

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Abstract

Ghrelin, leptin, and endogenous glucocorticoids play a role in appetite regulation, energy balance, and growth. The present study assessed the effects of dexamethasone (DEX) on these hormones, and on ACTH and pituitary POMC and CRHR1 mRNA expression, during a common metabolic stress – neonatal hypoxia. Newborn rats were raised in room air (21% O_2) or under normobaric hypoxia (12% O_2) from birth to postnatal day (PD) 7. DEX was administered on PD3 (0.5 mg/kg), PD4 (0.25 mg/kg), PD5 (0.125 mg/kg), and PD6 (0.05 mg/kg). Pups were studied on PD7 (24 h after last dose of DEX). DEX significantly increased plasma leptin and ghrelin in normoxic pups, but only increased ghrelin in hypoxic pups. Hypoxia alone resulted in a small increase in plasma leptin. Plasma corticosterone and pituitary POMC mRNA expression were decreased 24 h following the last dose of DEX, whereas plasma ACTH and pituitary CRHR1 mRNA expression had already increased (normoxia and hypoxia). Hypoxia alone increased corticosterone, but had no effect on ACTH or pituitary POMC and CRHR1 mRNA expression. Neonatal DEX treatment, hypoxia, and the combination of both affect hormones involved in energy homeostasis. Pituitary function in the neonate was quickly restored following dexamethasone-induced suppression of the HPA axis. The changes in ghrelin, leptin, and corticosterone may be beneficial to the hypoxic neonate through the maintenance of appetite and shifts in intermediary metabolism.

Keywords

dexamethasone; hypoxia; leptin; ghrelin; newborn

INTRODUCTION

An integrated endocrine response is a critical component of the physiological adaptation to metabolic disturbances in the neonate (Grongnet 1984, Frankel & Stevenson 1987, Friedman & Fahey 1993, Zayour *et al.* 2003). Critical hormones in the control of metabolism and appetite are leptin, produced by adipocytes (Neary *et al.* 2004), ghrelin, produced primarily by the stomach (Small & Bloom 2004), and adrenal corticosteroids (Dallman 2003). These hormones have complex interactions ultimately controlling food intake, growth, development, and energy balance in the neonate and adult (Meier & Gressner 2004). Of particular interest is the role that these hormones play in the neonatal adaptation to stress and disease (Zayour *et al.* 2003).

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This study was supported by NIH Grants DK54685 (HR) and DK62442 (LJ) and St. Luke's Medical Center (Aurora Health Care).

Neonatal bronchopulmonary dysplasia leads to hypoxia, and can occur in up to 23% preterm human births in the United States (American Academy of Pediatrics 2002). Glucocorticoid therapy is sometimes required in the treatment of neonatal respiratory distress and acts primarily to promote lung maturation (Sinkin *et al.* 2000). However, glucocorticoid therapy is associated with both short- and long-term negative consequences (Raff 2004, Yeh *et al.* 2004). We have examined many facets of the endocrine and metabolic adaptation to neonatal hypoxia (Raff 1999a, 1999b, 2001a, Raff 2003) and its interaction with dexamethasone therapy (Bruder *et al.* 2004a, 2004b). Of relevance to the current study is the dramatic decrease in growth that occurs, without a change in body composition, during hypoxia (Raff *et al.* 2001b). Glucocorticoid therapy also decreases growth rate (He *et al.* 2004), and interacts with hypoxia to lead to an almost complete cessation in neonatal growth (Bruder *et al.* 2004a). Finally, both hypoxia and glucocorticoid therapy lead to dramatic disturbances in lipid metabolism and GI function (Bruder *et al.* 2004a, 2004b, Lee *et al.* 2002, 2003).

The goal of the present study was to further evaluate the metabolic and developmental effects of neonatal hypoxia and its interaction with dexamethasone treatment. Hypoxia and dexamethasone may independently alter food intake and metabolism (Bruder *et al.* 2004a, Kayser 1992, Raff 2003, Raff *et al.* 1999a, Raff *et al.* 2001a). We hypothesized that hypoxia may attenuate dexamethasone-induced increases in leptin (Spinedi & Gaillard 1998), perhaps to encourage an increase in food intake. We also wanted to explore the effect of dexamethasone and hypoxia on ghrelin, another hormonal controller of appetite and metabolism in the neonate (Soriano-Guillen *et al.* 2004). Since leptin, ghrelin, and adrenal corticosteroids have reciprocal effects on one another (Ishida-Takahashi *et al.* 2004, Meier & Gressner 2004, Soriano-Guillen *et al.* 2004, Spinedi & Gaillard 1998), we assessed components of the hypothalamic-pituitary-adrenal (HPA) axis to determine if changes in leptin or ghrelin might be ascribed to altered HPA activity.

METHODS

Animal treatment

All experimentation was approved by the Institutional Animal Care and Use Committees of the Medical College of Wisconsin and St. Luke's/Aurora Sinai Medical Center. Timed pregnant Sprague Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, IN; N=16) were obtained at 14 days gestation and maintained on a standard sodium diet (Richmond Standard 5001, Brentwood, MO) and water *ad libitum* in a controlled environment (lights on, 0600–1800). Parturition usually occurred on the afternoon of gestational day 22, during which time rats were kept under observation. After litters were completely delivered, transferring no more than 1–2 pups from one dam to another equalized litter size. This is a standard technique to minimize the metabolic and hormonal effects of differences in numbers of pups in each litter (Routh *et al.* 1993, Young 2002). The dam and pups (~13 per litter) were then exposed to normobaric hypoxia (12% O_2) or kept in room air as control (21% O_2) as described previously (Raff & Chadwick 1986, Raff *et al.* 1999b). We have previously shown that this exposure leads to arterial PO_2 levels in adults of about 50–55 torr with sustained hypocapnia and alkalosis (Raff & Chadwick 1986, Raff *et al.* 1986).

Lactating dams were maintained with their litters for 7 days in a hypoxic or normoxic environment (Thomas & Marshall 1995). Dexamethasone phosphate (Sigma Chemical, St. Louis, MO) was administered subcutaneously in a tapering regimen to normoxic and hypoxic pups at 0800 as follows: post-natal day (PD) 3 (0.5 mg/kg), PD4 (0.25 mg/kg), PD5 (0.125 mg/kg), and PD6 (0.05 mg/kg) (Flagel *et al.* 2002). This tapering pattern of dexamethasone administration was designed to mimic glucocorticoid therapy used in the clinical setting. Control pups were injected with saline. Pups were weighed on each day of injection. At 0800 on PD7 (24 h after last dexamethasone injection), dams were removed from the chambers.

Pups were quickly decapitated and blood from each pup was pooled (3 pups per sample) and immediately placed on ice. Plasma was separated and frozen for subsequent analysis (N=4–10 per treatment). Pituitaries were removed and processed as described below. Samples were obtained from pups from 4 normoxic and 4 hypoxic litters.

Plasma measurements

All measurements were performed on pooled samples from each treatment group (3 pups/sample). Leptin was measured by enzyme-linked immunosorbent assay (Crystal Chem, Inc., Downers Grove, IL) with an inter- and intra-assay coefficient of variation (CV) of 7% and 5%, respectively. Leptin measurements were verified in some samples by radioimmunoassay (RIA) (Linco Research, Inc., St. Charles, MO) with an inter- and intra-assay CV of 6% and 5%, respectively. Ghrelin was measured by enzyme immunoassay (Phoenix Pharmaceuticals, Inc., Belmont, CA) with an inter- and intra-assay CV of <14% and <5%, respectively. Corticotropin (ACTH; inter- and intra-assay CV=11% and 7%) and corticosterone (inter- and intra-assay CV=7% and 6%) were measured by radioimmunoassay (MP Biomedicals, Inc., Orangeburg, NY).

Proopiomelanocortin (POMC) and corticotropin-releasing hormone receptor-1 (CRHR1) mRNA expression

Northern analysis of pituitary gene expression was performed using previously published techniques (Jacobson *et al.* 1997). Anterior pituitaries were dissected from the neurointermediate lobe at death and snap-frozen in liquid nitrogen (3 pituitaries per tube). Total RNA was isolated 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 20x SSC, and immobilized by UV cross-linking. Antisense ^{32}P-labeled cRNA probes were transcribed from appropriately linearized plasmids using T_3 or T_7 RNA polymerase (Stratagene, La Jolla, CA) from cDNA clones complementary to mouse POMC (Jacobson 2000, Raff *et al.* 2003), rat CRHR1 (Pozzoli *et al.* 1996), or rat 28S mRNA. CRHR1 probes were produced from a 461 bp cDNA clone based on a previously published sequence (Perrin *et al.* 1993), and generously provided by Neurocrine Biosciences (San Diego, CA). Membranes were hybridized at 65° C in 50% formamide, 2% SDS, 0.8 M NaCl, and washed three times in 0.1x SSC, 0.1% SDS (65° C). After washing, blots were exposed to phosphoimager screens (GE Healthcare, Sunnyvale, CA). The resulting autoradiographic images were analyzed using Imagequant 5.0 software (GE Healthcare, Sunnyvale, CA), with the CRHR1 and POMC signals normalized to 28S ribosomal RNA (N=7–11).

Statistical Analyses

Results are reported as mean ± SEM. Data were analyzed by two-way analysis of variance (ANOVA) and the Student-Newman-Keuls method for multiple comparisons (SigmaStat 2.03).

RESULTS

Average body weight at PD6 was 11.4 ± 0.2 g (N=99) in normoxic controls. Average body weight of normoxic pups at PD6 treated with dexamethasone was 23% lower than control (8.8 ± 0.2 g; N=95; P<0.05). Pups exposed to hypoxia had an average body weight that was 25% lower than normoxic controls at PD6 (8.6 ± 0.2 g; N=104; P<0.05). Pups exposed to hypoxia and treated with dexamethasone had an average body weight at PD6 that was 39% lower than normoxic controls (7.0 ± 0.1 g; N=112; P<0.05). The combination of dexamethasone and hypoxia had an additive negative effect on body weight.

The effects of dexamethasone on plasma concentrations of leptin (upper panel) and ghrelin (lower panel) in 7-day-old pups are shown in Figure 1. Daily dexamethasone administration on days 3–6 in a tapering dose regimen increased plasma leptin nearly seven-fold in 7-day-old normoxic pups ($P<0.001$). There was a small but significant increase in plasma leptin concentration during hypoxia alone ($P<0.02$). Hypoxia attenuated the leptin response to dexamethasone, although it was still increased compared to normoxic, vehicle-treated controls ($P<0.001$). There was a significant increase in the plasma concentration of ghrelin in normoxic pups after dexamethasone treatment ($P<0.001$). Hypoxia alone had no effect on plasma ghrelin and did not modify the dexamethasone-induced increase in ghrelin ($P<0.001$).

Figure 2 depicts plasma ACTH (upper-left panel), plasma corticosterone (upper-right panel), and pituitary POMC and CRHR1 mRNA expression (lower panels). Prior dexamethasone treatment decreased subsequent plasma corticosterone concentration in 7-day-old normoxic pups to levels nearly half that of vehicle-treated normoxic controls ($P<0.001$). Plasma corticosterone concentration was nearly doubled in hypoxic pups ($P<0.001$), but this effect was blocked by prior dexamethasone ($P<0.001$). Prior dexamethasone treatment also resulted in a significant increase in plasma ACTH on day 7 (24 h after last dexamethasone injection) in normoxic ($P<0.02$) and hypoxic ($P=0.007$) pups. Hypoxia alone had no effect on plasma ACTH concentration. Prior treatment with dexamethasone decreased pituitary POMC mRNA expression, measured 24 h after the last dexamethasone injection, over two-fold in normoxic ($P=0.002$) and hypoxic ($P=0.009$) pups. Hypoxia had no effect on pituitary POMC mRNA expression, and there were no differences in dexamethasone-induced decreases between normoxic and hypoxic pups ($P>0.05$). Prior treatment with dexamethasone increased pituitary CRHR1 mRNA expression ($P=0.013$), regardless of inspired O_2, when measured 24 h after the last dexamethasone injection (7 days of age).

DISCUSSION

The present study examined the interaction of glucocorticoid therapy and a common neonatal metabolic stress (hypoxia) on plasma leptin and ghrelin concentrations in the 7-day-old rat pup. Dexamethasone treatment *per se* significantly increased plasma leptin and ghrelin concentrations. Concomitant hypoxia attenuated the leptin, but not ghrelin, response to dexamethasone. These findings, to the best of our knowledge, are the first to describe dexamethasone-associated increases in plasma ghrelin in the normoxic or hypoxic neonatal rat.

Leptin

Dexamethasone treatment in preterm infants has been shown to increase serum leptin and insulin concentrations (Ng *et al.* 2002). We have previously observed significant hyperinsulinemia in rat pups treated with the same dexamethasone regimen as the current study (Bruder *et al.* 2004a). It has been suggested that there is no direct effect of dexamethasone on leptin, but rather an indirect effect of the dexamethasone-induced inhibition of ACTH (Spinedi & Gaillard 1998). We infer that ACTH was suppressed during dexamethasone therapy (PD3-6) since corticosterone levels were very low (see below for discussion of ACTH). It is possible, therefore, that dexamethasone-induced decreases in plasma ACTH may indirectly result in increased leptin. Dexamethasone may also increase the concentration of free leptin while having no affect on bound leptin or the soluble leptin receptor (Lewandowski *et al.* 2001). A recent study found that glucocorticoids antagonize leptin action through rapid inhibition of the signaling cascade associated with the leptin receptor (Ishida-Takahashi *et al.* 2004). The above findings confirm an intimate relationship between the HPA axis and adipocyte leptin production in the neonate.

We have previously shown that hypoxia from birth to seven days of age in unhandled rat pups resulted in a small but significant decrease in plasma leptin at 7 days of age (Raff *et al.* 2001a). This previous study used a leptin radioimmunoassay (RIA) while the present study utilized an enzyme-immunoassay. In order to verify the present results, we re-assayed some samples using the older RIA method and the two assay methods were in agreement. This can be attributed to the injection, handling, and associated periods of very brief separation from the dam, which influence endocrine responses in the pups (Salzmann *et al.* 2004, Walker *et al.* 1991). Catecholamine release from the sympathetic nervous system (SNS) may be a mediator of these responses (Young 2000). A previous study found that the transcription of the human leptin gene is activated by hypoxia, via the transcription factor hypoxia-inducible factor-1 (HIF-1) (Ambrosini *et al.* 2002). This lends support to the present study, but it does not provide a complete explanation of our findings.

To our knowledge, the present study is the first to report the effects of dexamethasone on plasma leptin in the hypoxic neonate. Interestingly, concomitant hypoxia attenuated the stimulatory effect of dexamethasone on leptin. A previous study suggested that catecholamines directly inhibit leptin production by binding to adipocyte adrenergic receptors (Scriba *et al.* 2000). It is possible that increased SNS activity in the hypoxic pup blunted the leptin response to dexamethasone.

Ghrelin

Information regarding the role of ghrelin in the control of appetite and growth in the adult is currently expanding, although less is known of its role in development (Bellone *et al.* 2004, Small & Bloom 2004). Ghrelin is most notably produced by endocrine cells of the gastric mucosa, but is also produced in the intestine, hypothalamus, and pancreas (Mozid *et al.* 2003, Wierup *et al.* 2004). We have previously shown that hypoxia does not affect total or active ghrelin in the plasma of neonatal rats, suggesting that the anorectic effect of hypoxia does not involve changes in ghrelin (Raff 2003). Ghrelin stimulates the HPA axis at the level of the hypothalamus, and glucocorticoids have been shown to be permissive for ghrelin-induced food intake and accumulation of fat mass (Tung *et al.* 2004). A study in humans found that endogenous and exogenous glucocorticoids decrease plasma ghrelin (Otto *et al.* 2004). The present results, to our knowledge, are the first to describe glucocorticoid-induced increases in ghrelin. This may be an important process in the developing animal. Dexamethasone-induced increases in plasma ghrelin in hypoxic neonates could be a mechanism by which appetite is stimulated to overcome the direct anorectic effects of hypoxia (Kayser 1992). We also speculate that the attenuation by hypoxia of dexamethasone-induced increases in leptin favors this orexigenic effect. These findings may be important in understanding the control of neonatal growth in health and disease.

ACTH/Corticosterone

We have previously shown that hypoxia from birth to seven days of age increases plasma corticosterone without affecting plasma ACTH (Raff *et al.* 2003). The present study confirmed these findings and also showed that pituitary POMC and CRHR1 mRNA expression are unaffected by hypoxia. The mediator of this sustained increase in corticosterone has yet to be elucidated, but our previous study indicated that it might be driven by increases in SNS activity (Raff *et al.* 2004).

Prior dexamethasone treatment (PD3-6) resulted in subsequent increases in plasma ACTH in 7-day-old pups with plasma corticosterone remaining low. It is likely that dexamethasone initially suppressed the HPA axis at the hypothalamus and pituitary, also decreasing adrenocortical function (Ford *et al.* 1997). After the discontinuation of dexamethasone, plasma corticosterone remained suppressed such that, in the absence of glucocorticoid negative

feedback, the pituitary rapidly increased ACTH production in order to reverse the suppression of adrenocortical function. The tapering dexamethasone regimen used in the current study likely facilitated this quick restoration of ACTH release, a finding in the neonate not found in the adult (Nicholson *et al.* 1984).

This temporal sequence of the recovery of the HPA axis is also observed clinically, albeit in a longer time domain (Chrousos 2001). That is, dexamethasone suppresses plasma ACTH, which results in a decrease in adrenocortical function. When exogenous corticosteroids are discontinued, ACTH recovers first and overshoots, which is necessary to reverse the decrease in adrenocortical function. The main difference between our results and this well-known clinical phenomenon is the time course of the recovery in that the increase in ACTH in the neonate occurred within 24 h of the last (and lowest) dexamethasone dose. It is likely that the short duration and tapering regimen of glucocorticoid therapy in the current study allowed the rapid recovery of ACTH secretion. We suspect that adrenocortical function, which normally lags behind ACTH, would have soon followed. Although this study was not designed to optimize dexamethasone dosing in the neonate, it appears that this regimen, which was designed to mimic dexamethasone therapy in human neonates (Flagel et al. 2002), allows a very rapid recovery of pituitary function.

Since pituitary POMC mRNA expression remained suppressed after the cessation of dexamethasone treatment, the increased ACTH secretion was likely the result of increased post-translational processing possibly driven by increased corticotropin-releasing hormone (CRH) during recovery from dexamethasone-induced inhibition (Lim *et al.* 2002). Previous studies have shown that dexamethasone treatment increases CRHR1 mRNA expression in the adult rat pituitary (Rabadan-Diehl *et al.* 1997). The present study measured increased CRHR1 expression in normoxic and hypoxic 7-day-old rat pituitaries, 24 hours after the final dose of a tapering regimen of dexamethasone. Increased CRHR1 expression may be CRH-driven and/or the result of an intracellular feedback mechanism in the pituitary (i.e. increased CRHR1 expression to overcome dexamethasone-induced suppression of POMC mRNA). Our findings illustrate that, following suppression with dexamethasone, the neonatal HPA axis regains responsiveness more quickly than that of the adult (Nicholson *et al.* 1984), and that this may occur predominantly at the pituitary level (Ford *et al.* 1997).

Summary

The present study demonstrated that a tapering dose regimen of dexamethasone in the neonatal rat modulates hormones involved in appetite and energy balance. Of great interest is the attenuation of the leptin response to dexamethasone in hypoxic pups. This may be a beneficial mechanism by which the developing animal attempts to maintain appetite in the face of the anorectic effect of hypoxia (Kayser 1992). Dexamethasone-induced ghrelin production, which was not inhibited by hypoxia, may produce a similar effect. The insulin-resistant state produced by hypoxia (Bruder *et al.* 2004a) serves to divert energy substrates away from peripheral tissues (i.e. adipose and muscle). Dexamethasone therapy is likely to augment this effect. This would allow critical tissues such as the brain to preserve function during the hypoxic insult, and would also explain growth failure (Bruder *et al.* 2004a). Hypoxia-induced increases in corticosterone (Raff *et al.* 1999b) are also likely to contribute to the insulin resistance and possibly play a role in maintaining appetite.

These findings have implications in short-term metabolic and endocrine control in the neonate. We also speculate that there may be long-term consequences of these short-term adaptations (Bruder *et al.* 2004a). Increases in leptin during development may permanently alter the neural mechanisms controlling food intake and energy balance (Bouret *et al.* 2004, Pinto *et al.* 2004). Likewise, neonatal hyperinsulinemia is also suspected of causing metabolic disturbances in the adult (e.g. insulin resistance and obesity) (Dorner & Plagemann 1994, Petry

et al. 2001). Growth failure, and associated periods of catch-up growth, may also lead to subsequent metabolic disease (De Souza & Moura 2000, Hales & Ozanne 2003). Increased concentrations of endogenous or exogenous glucocorticoids in the neonate have also been implicated in subsequent HPA axis dysfunction (Flagel *et al.* 2002), and may be detrimental to brain development (Lindahl *et al.* 1988, Yeh *et al.* 2004). Neonatal hypoxia also leads to long-term changes in sympathoadrenal function (Soulier *et al.* 1997). Therefore, it is important not only to understand the acute responses to neonatal hypoxia and dexamethasone treatment, but also to relate these responses to long-term maintenance of health.

Acknowledgements

The authors would like to thank Barbara Jankowski, Peter Homar, and Rebecca Kittell for their expert technical assistance. The authors would also like to thank Dr. Martin Oaks and Karen Hallett for the production of the CRHR1 clone.

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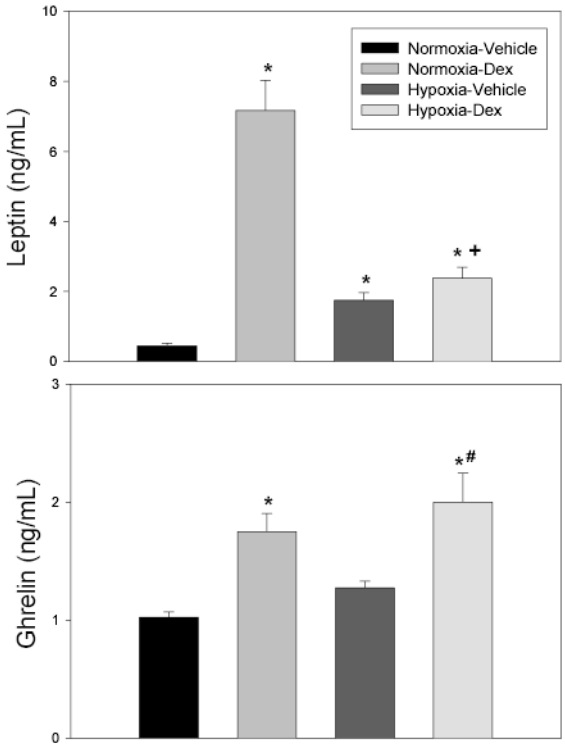

Figure 1. Effects of dexamethasone and/or hypoxia on plasma leptin and ghrelin concentrations in the neonatal rat.

Rats were exposed to hypoxia from birth to seven days of age and treated with a tapering dose regimen of dexamethasone (or vehicle) from postnatal day 3 to postnatal day 6. Plasma from 3 pups was pooled to create one sample. * Indicates a significant difference from Normoxia-Vehicle with $P<0.05$. # Indicates a significant difference from Hypoxia-Vehicle with $P<0.05$. + Indicates a significant difference from Normoxia-Dex with $P<0.05$. N values for leptin measurements were: Normoxia-Vehicle (11), Normoxia-Dex (7), Hypoxia-Vehicle (9), Hypoxia-Dex (7). N values for ghrelin measurements were: Normoxia-Vehicle (8), Normoxia-Dex (4), Hypoxia-Vehicle (8), Hypoxia-Dex (4).

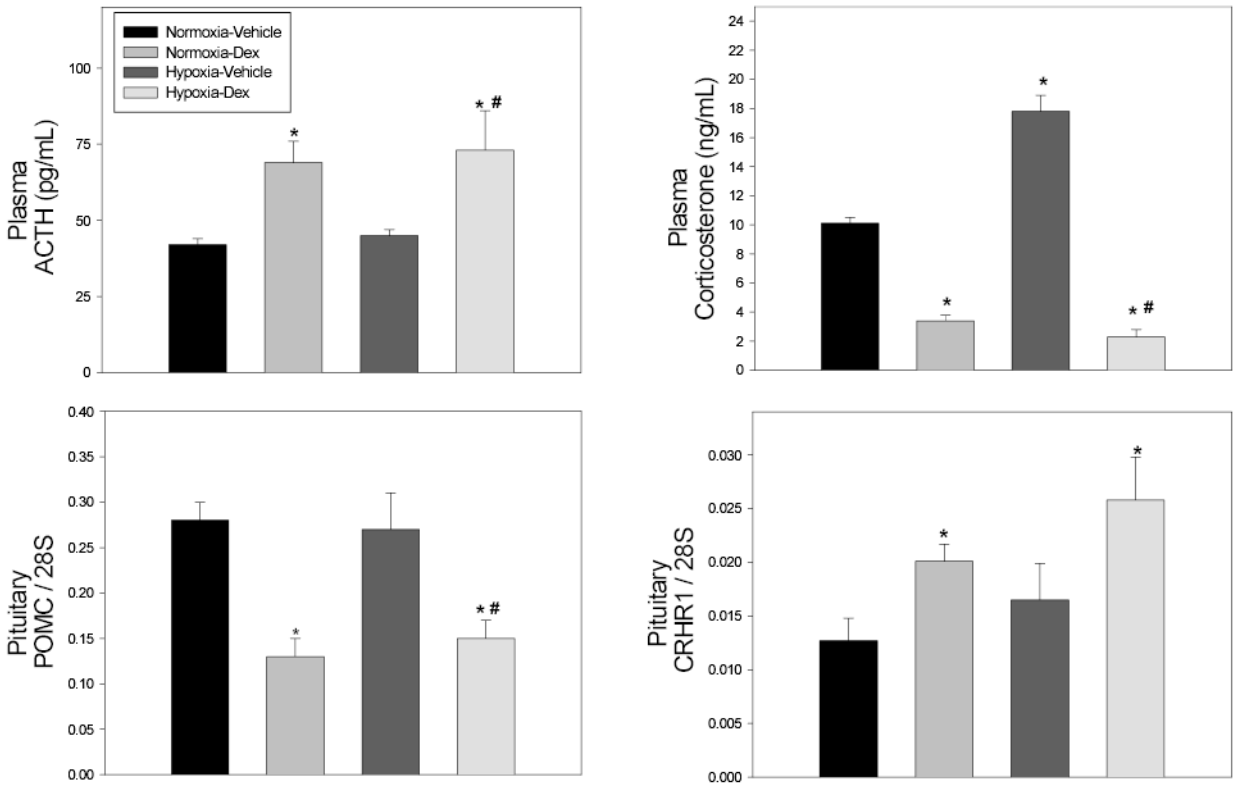

Figure 2. Effects of dexamethasone and/or hypoxia on plasma corticosterone, plasma ACTH, and pituitary POMC and CRHR1 mRNA expression in the neonatal rat.

Rats were exposed to hypoxia from birth to seven days of age and treated with a tapering dose regimen of dexamethasone (or vehicle) from postnatal day 3 to postnatal day 6. Plasma from 3 pups was pooled to create one sample. Pituitary POMC and CRHR1 expression was measured using a Northern blot technique. * Indicates a significant difference from Normoxia-Vehicle with $P<0.05$. # Indicates a significant difference from Hypoxia-Vehicle with $P<0.05$. N values for plasma corticosterone and ACTH measurements were: Normoxia-Vehicle (10), Normoxia-Dex (6), Hypoxia-Vehicle (10), Hypoxia-Dex (8). N values for POMC measurements were: Normoxia-Vehicle (11), Normoxia-Dex (7), Hypoxia-Vehicle (9), Hypoxia-Dex (7). N values for pituitary CRHR1 measurements were: Normoxia-Vehicle (5), Normoxia-Dex (6).