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NITRIC OXIDE INHIBITS ACTH-INDUCED CORTISOL PRODUCTION IN NEAR TERM, LONG TERM HYPOXIC OVINE FETAL ADRENOCORTICAL CELLS

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Abstract

We previously reported that in the sheep fetus, long term hypoxia (LTH) resulted in elevated basal plasma $ACTH_{1–39}$ while cortisol levels were not different from normoxic controls. We also showed that LTH enhances endothelial nitric oxide synthase (eNOS) expression in the fetal adrenal. This study was designed to determine the effect of nitric oxide on cortisol production in adrenocortical cells from LTH fetal sheep. Ewes were maintained at high altitude (3,820 m) from ~40 days' gestation (dG) to near term. Between 138–141 dG, fetal adrenal glands were collected from LTH and age-matched normoxic control fetuses. Adrenal cortical cells were pre-treated with sodium nitroprusside (SNP), L-NAME, L-arginine, or Diethyleneamine nitric oxide (DETA-NO) then challenged with 10nM ACTH. Cortisol responses were compared after 1 h. ACTH induced cortisol secretion was significantly higher in LTH vs. control ($p<0.01$). Enhancement of nitric oxide with L-arginine resulted in a significant reduction of ACTH-mediated cortisol production in the LTH group. DETA-NO also caused a significant decrease in ACTH-mediated cortisol production ($p<0.05$). Inhibition of NOS with L-NAME significantly increased cortisol production in the LTH group ($p<0.05$ compared to ACTH alone) while the effect on the control group was not significant. NOS activity was significantly higher in the LTH group compared to control but this difference was eliminated following ACTH treatment. These data indicate that LTH enhances adrenal cortical sensitivity to the inhibitory effects of NO on cortisol production. NO may therefore play an important role in regulating ACTH-induced cortisol production in the LTH fetal adrenal.

Keywords

nitric oxide synthase; L-NAME; sodium nitroprusside

Introduction

In the ovine fetus, there is an exponential increase in fetal plasma cortisol concentration during the final ~20 days of gestation in conjunction with the maturation of the fetal hypothalamo-pituitary-adrenal axis. 1 This increased cortisol production assures maturation of fetal organs and stimulates the parturition process. It is imperative that plasma cortisol is maintained at normal levels throughout gestation to allow for normal growth and development as evidenced

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by premature elevations in fetal plasma cortisol resulting in preterm delivery of a growth-restricted fetus.[2–5] Our laboratory has demonstrated that fetal sheep adapt to development under conditions of long-term hypoxia (LTH) by maintaining normal basal plasma cortisol concentrations despite elevated basal plasma adrenocorticotropic hormone (ACTH).[6–8] The mechanism(s) that govern this adaptation is not fully understood and a number of potential mechanisms may be involved in the regulation of fetal cortisol production under conditions of LTH. One potential regulatory effector is enhanced production of nitric oxide (NO).

Nitric oxide (NO) is a diatomic free-radical gas that has a wide range of physiological functions.[9, 10] NO is synthesized from L-arginine by a family of nitric oxide synthases (NOS);[11] the constitutively expressed neuronal NOS (nNOS/ NOS-I) and endothelial NOS (eNOS/NOS-III) and a third inducible isoform (iNOS/NOS-II). eNOS and nNOS are regulated by Ca^{2+} and calmodulin, while iNOS is Ca^{2+}/calmodulin independent.[12] NO produced by eNOS and nNOS regulates a range of physiologic functions while iNOS tends to be invoked in more pathological situations.

NO has a profound effect on steroidogenesis in endocrine tissues. NO inhibited steroidogenesis in ovarian tissue of women,[13] pigs,[14, 15] rabbits, [16, 17] and rats, [18] while inhibition of NOS increased testosterone production in Leydig cells.[19] Immobilization stress increased NO in adult rat testis and reduced production of testosterone.[20] Although less is known about the effects of NO on adrenal steroidogenesis, NO inhibits basal, ACTH and angiotensin II-induced aldosterone production in zona glomerulosa cells of adult rat adrenal cortex transfected with eNOS.[21] NO mediated inhibition of aldosterone was also shown to be *cGMP-independent*,[22] and reversed by NOS inhibitor thiocitrulline.[23] NOS inhibition also increased aldosterone production in humans.[24] NO donors decrease both un-stimulated and ACTH-stimulated corticosterone production in rat zona fasciculata cells while NOS inhibition enhances glucocorticoid output,[25] implicating NO-mediated inhibition of key rate-limiting steps in the steroidogenic pathway.[26] Adams and coworkers also found NO to act as a negative inhibitor of corticosterone synthesis.[27]

These data, together with evidence that NOS or the effects of NO may be regulated by hypoxia [28–30] and other stressors,[31] suggest NO may be involved in the adrenocortical adaptation to LTH in the ovine fetus by preventing premature maturation of the fetal adrenal cortex resulting in excess cortisol production in the face of the significantly elevated ACTH. We recently observed that LTH enhances expression of eNOS in sheep fetal adrenal cortex and that eNOS was located in the cortisol producing cells of the zona fasciculata.[32] This was the first study to demonstrate the presence of eNOS in the ovine fetal adrenal cortex and the pronounced upregulation of eNOS in response to LTH. However, it is not known whether differential NOS expression represents a functional mechanism of cortisol regulation in response to LTH. The present study was designed to determine the effect of nitric oxide on basal and ACTH-induced cortisol production in adrenocortical cells from LTH fetal sheep and to assess the effect of LTH on NOS activity.

Materials and Methods

Animals

Pregnant ewes were maintained at high altitude (3,820 m) from approximately day 40 of gestation to near term (term ~ 146 days). Following transportation to the laboratory, hypoxia was maintained by nitrogen infusion through a non-occlusive maternal tracheal catheter as previously described.[6, 33] Age matched, normoxic ewes served as controls. Between days 138–141 of gestation, the ewes were sedated with pentobarbital, intubated, and maintained under general anesthesia with 1.5–2% halothane in oxygen while the fetuses were delivered through a midline laparotomy. Procedures were preformed as previously described in detail.

8, 34, [35] Fetal adrenal glands were collected from the LTH and age-matched normoxic control animals for cell dispersion and subsequent study.

Cell Dispersion

Fetal adrenal glands were placed in phosphate buffered saline, divided in half along the longitudinal axis and the cortex was micro-dissected from the capsule and medulla.

The cortical tissue was minced then gently digested in a suspension of collagenase type IV and DNAse and filtered through a 100 μm cell strainer followed by treatment with red blood cell lysis buffer and centrifugation for cell collection. Cells were incubated in Ham's F10 medium (Mediatech, Manassas, VA) for a 2 hr recovery period, then aliquotted into individual tubes (2.5×10^5 cells per tube; in duplicate). Cell viability was confirmed by Trypan blue exclusion.

Effects of NO on Cortisol Production

Fetal adrenal cortical cells (FACs) were subjected to a 30 min pre-treatment with either media alone (control) the NO donor sodium nitroprusside (SNP, 1mM), NOS substrate (L-arginine (L-Arg), 2mM), or the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME, 1mM) at 37C. Concentrations were based on preliminary dose response experiments and other published reports. 22, 25, 36 After the pre-incubation, cells were stimulated with ACTH (10 nM) for one hour at 37° C. Following the 1 h incubation, the tubes were centrifuged, cells and media separated, and both stored at −80° C until analysis. Cortisol analysis was performed using a commercially available ELISA kit (Oxford Biomedical Research; #EA 65) as previously described and validated in our laboratory.37 Additional studies were performed to confirm the effects of the NO-donor SNP on cortisol production by using another NO donor, Diethyleneamine nitric oxide (DETA-NO, 0.5mM). This does was found to be effective in inhibition of steroidogenesis in adult rat adrenal fasciculata.25 We also treated FACs with a combination of L-NAME (1.0 mM) and L-Arg (2mM) to confirm that the effect of L-NAME was due to inhibition of NOS.

NOS Activity in Fetal Adrenocortical Cells

Additional FACs, incubated for 1 h at 37° C in the presence or absence of ACTH, were immediately snap frozen in liquid nitrogen for determination of NOS activity. At the time of assay, the cortical cells were homogenized in 200μl lysis buffer (20mM HEPES-KOH, 10mM KCl, 1.5mM $MgCl_2$, 1mM EDTA, 167mM dithiothreitol, 100mM PMSF, 5μg/ml Leupeptin, 0.8μg/ml aprotinin) for 30 seconds. The mixture was then centrifuged at 14000 × g for 15 minutes and the supernatant retained. Total protein was determined by Bradford method (Bio-Rad). NOS activity was determined using an enzyme linked immunoassay kit (Oxford Biomedical Research, NB 78) following the manufacture's instructions. NOS activity was measured as μM nitrite/μg protein.

Statistical analysis

Differences in cortisol responses and NOS activity were determined by two-way ANOVA and with Bonferonni post tests where appropriate (Prism 4.0 software) and $p<0.05$ was considered significant.

Results

Effects of NO Cortisol Production

Basal (un-stimulated) cortisol production and the effects of alterations in NO on adrenal cortical cells cortisol production from control and LTH adrenal cortical cells are illustrated in Figure 1A. Although there was a trend towards higher cortisol production from FACs obtained from

LTH fetuses under basal, SNP and L-Arg treatment, there were no differences in cortisol production between control and LTH FACs in any of the treatment groups examined in the absence of ACTH. Neither SNP, L-Arg or L-NAME had a significant effect on cortisol production from either control or LTH FACs.

In response to ACTH (Figure 1B), cortisol production was significantly greater in the LTH FACs compared with controls ($p<0.01$). Pre-treatment with SNP or L-Arg, abolished the enhanced cortisol production in response to ACTH observed in the LTH FACs. In contrast, the cortisol production was significantly greater in the LTH group compared to control in response to ACTH following L-NAME pre-treatment ($p<0.01$).

Since the LTH FACs produced significantly more cortisol in response to ACTH than the control group, we normalized the data to percent cortisol response to ACTH alone, and then compared the effects of alterations in NO synthesis for each group (Figure 1C). In the control fetal FACs, enhancement of NO synthesis with either SNP or L-Arg pre-treatment had no effect on cortisol production in response to ACTH. In marked contrast, in the LTH group compared to ACTH treatment alone, enhancement of NO synthesis with L-Arg pre-treatment significantly reduced cortisol production ($p<0.05$). A similar trend was observed with SNP but was not significant. In the LTH FACs, NOS inhibition with L-NAME resulted in a significant increase ($p<0.01$) in cortisol production compared to ACTH alone. A similar trend was noted in the control group but did not reach statistical significance.

In order to confirm the effect of NO on inhibition of cortisol synthesis, we also studied another NO donor, DETA-NO. Under basal conditions, there was no effect on cortisol production on either control or LTH FACs (Figure 2A). As observed above, LTH FACs had a significantly higher cortisol production in response to ACTH compared to controls (Figure 2B, $p<0.01$) while pre-treatment with DETA-NO eliminated this difference. Again, since LTH FACs produced significantly more cortisol in response to ACTH than the control group, we normalized the data to percent cortisol response to ACTH alone, and then compared the effects of alterations in NO synthesis within each group (Figure 2C). Pre-treatment with DETA-NO significantly reduced the cortisol response to ACTH in both control and LTH FACs ($p<0.01$). We also pretreated cells with both L-NAME and L-Arg to ensure that the previously observed effects (Figure 1) were indeed the result of specific effects on NOS. Although this treatment had no effect on basal cortisol production (Figure 3A), the combination of the NOS substrate and NOS inhibitor restored cortisol production to levels observed with ACTH alone (Figure 3B).

NOS Activity

Under basal conditions, NOS activity was significantly greater in the LTH FACs compared with control ($p<0.05$, Figure 3). Following ACTH treatment, NOS activity was unchanged in the control cells. In marked contrast, there was a significant reduction in NOS activity in LTH FACs ($p<0.05$), restoring levels to that observed in the control FACs.

Discussion

Previous studies from our laboratory demonstrated that in response to development under conditions of LTH, basal plasma cortisol concentrations in fetal sheep are in the normal range despite elevated basal ACTH. [6–8] Interestingly, in response to a secondary stressor, the cortisol response is enhanced in LTH fetuses compared to normoxic controls.[6, 38] Although the factors involved in these important adaptive responses remain undefined, recent studies from our laboratory together with other observations suggest that regulation of nitric oxide in response to LTH in the fetal adrenal cortex may play a critical role. In a recent study, we demonstrated that LTH significantly elevated eNOS expression in sheep fetal adrenal cortex[32]. However,

the mere presence of NOS does not necessarily indicate a functional role. In the present study, we demonstrate that stimulation of NO synthesis suppressed ACTH-stimulated cortisol synthesis in the LTH fetal adrenal while inhibition of NOS activity enhanced cortisol production. We further show for the first time that basal NOS activity was elevated in LTH fetal adrenocortical cells compared to normoxic control FACs and that ACTH treatment reduced NOS activity in adrenals cells from this group.

The effects of NO on vascular tissue are well established and largely depend on cGMP pathways (See 11 for review). In endocrine tissue however, the effects of NO on steroidogenesis appear to be cGMP-independent 21. NO interacts competitively with the oxygen binding site of key steroidogenic enzymes such as P450scc (CYP11A1) and P450c17 (CYP17)39. Peterson et al., 40 have suggested that since these enzymes use several rounds of attack of the heme-oxygen complex on the steroid substrate, such multi-step targets would be more sensitive to NO inhibition than other steroidogenic enzymes. These authors also suggested that since the lyase activity of CYP17 is a key branch point in differentiating glucocorticoid and androgen biosynthesis in the ovine fetal adrenal, inhibition by NO could play a role in maintaining the very low levels of DHEA that are associated with ovine adrenal activity while still allowing cortisol biosynthesis through 17-OH progesterone.40 Overall, this competitive interaction makes NO an effective inhibitor of steroidogenesis. Clearly, NO has been shown to inhibit key rate-limiting steps in the steroidogenic pathway and can inhibit steroidogenesis in a range of endocrine tissue including ovary 17, testis [41, 42] and adrenal glands [22, 25, 36]. All of these studies however used adult tissue or cells. To our knowledge, there have been no previous studies utilizing fetal tissue or cells and examining the response to LTH.

The present studied examined the effect LTH and NO on cortisol synthesis in FACs. Under basal conditions (absence of ACTH), cortisol output is relatively low and no differences were noted between control and LTH fetal adrenal cells. However, with ACTH stimulation, a number of important changes occurred. In response to ACTH alone, there was a significant increase in cortisol output compared to un-stimulated conditions in both control and LTH adrenal cortical cells with a greater increase observed in LTH adrenal cortical cells. This finding confirms previous *in vitro* data [43] as well as in vivo studies [6, 38] showing enhanced cortisol responsiveness to stress levels of ACTH stimulation in LTH fetuses. Following pre-treatment with either an NO donor or NOS substrate, the difference in cortisol production from the fetal adrenal cells was eliminated between control and LTH groups (Figure 1B,). When data were normalized to cortisol output in response to ACTH alone, there was a significant inhibition of cortisol synthesis in fetal adrenal cells in the LTH group that was not present in the control. The LTH-enhanced increase in eNOS message and protein expression [32] coupled with the increased NOS activity under basal conditions (Figure 3) may be responsible for the differential effects of NO on cortisol production.

Data from the LTH fetal adrenal cells are similar to the observations of Cymeryng et al 25, [36] in adult rat adrenal cells and Y1 adrenal cell line in that that NO suppresses glucocorticoid production. NOS inhibition with L-NAME resulted in increased cortisol production in fetal adrenal cells in the LTH group ($p<0.05$) compared to ACTH alone. A similar trend was noted in adrenal cells from the control fetuses but was not significant. The effects of L-NAME on steroidogenesis in the present study are in agreement with the effects of NOS inhibition on aldosterone production in the adrenal [24, 44] and androgen in the testis 20. It has become clear that the extent of the effect of NO is probably dependent on species, tissue type, and physiological condition.

Higher basal levels of NOS activity in the FACs from the LTH group were expected based on our previous study showing enhanced expression of adrenal cortical eNOS [32]. What was unexpected however, was the effect of ACTH on NOS activity. After ACTH treatment, NOS

activity in the LTH FACs was reduced to levels comparable to control cells under both basal and ACTH stimulation (Figure 3). A number of mechanisms are involved in the regulation of NOS activity that include post-translational modification via phosphorylation, availability of substrates/cofactors and protein–protein interactions including binding to calcium-dependent calmodulin, caveolin-1 and heat shock protein 90 (Hsp90)[45]. Relevant to the potential effects of ACTH, one of the key factors that may link ACTH stimulation and a decrease in NOS activity is ERK1/2. Although the role of ERK 1/2 on NOS expression/activity is controversial, it is apparent that it plays a role in regulating NOS activity. ERK1/2 inhibition upregulated ATP stimulated eNOS activity in COS-7 cells 46. MEK/ERK 1/2 inhibition also enhanced eNOS activity in porcine pulmonary arteries 47. These studies suggest therefore, that stimulation of ERK 1/2 could therefore inhibit NOS activation. Further, ERK 1/2 stimulation plays an important role in adrenal steroidogenesis.48 We have recently shown using a MEK inhibitor that the ERK1/2 signaling pathway plays a key role in ACTH and cAMP induced cortisol synthesis in the ovine fetal adrenal cortisol cells.[49] inhibiting ERK activity also had a more pronounced effect in the LTH cells suggesting that this pathway is upregulated in response to LTH. [43]

Hypoxia has also been shown to have significant effects on NOS activity. Chen and Meyrick[28] found that acute hypoxia stimulated Hsp90 binding to eNOS and activation of the PI3–Akt pathway resulting in increased eNOS phosphorylation. They suggested that this may be a mechanism whereby eNOS activity and subsequent NO production is upregulated in hypoxic coronary arteries. Other studies using hypoxic conditions for a longer duration confirmed that hypoxia increases eNOS generation of NO by enhancing Hsp-90 binding in myocardial tissue [50]. The same group also found the hypoxia decreased caveolin-3 [51], another mechanism that can contribute to enhanced NOS activity. Clearly future studies are warranted to address these key mechanisms regulating NOS function in the LTH fetal adrenal cells.

In the present study, the locus of the ACTH effect on NOS activity in the LTH cannot be established. However, together with the data showing significant inhibition of cortisol output with NO stimulation and enhanced cortisol output in response to NOS inhibition, it appears that this may be a mechanism of regulating cortisol responses under conditions of LTH. In vivo under basal conditions, despite higher basal levels of ACTH, LTH fetuses maintain plasma cortisol concentrations at a level similar to normoxic controls. It is possible however, that although the elevated basal ACTH concentrations may not significantly affect basal cortisol biosynthesis, they may play a role in regulating adrenal growth under hypoxic conditions. The previously reported upregulation of adrenal eNOS in the LTH group [32] may be responsible for enhanced basal NOS activity observed in the present study, which would exert an inhibitory effect on basal cortisol production overcoming the low level of stimulation by elevated basal ACTH. Stimulation with stress levels of ACTH in vitro as in the present study or those observed in vivo in response to a secondary stressor[6, 38] decrease NO activity, thus enhancing cortisol output.

In conclusion, the present study demonstrated for the first time in fetal FACs, that NO plays a role in the regulation of cortisol biosynthesis following LTH exposure. Further, we have demonstrated that LTH enhances adrenocortical sensitivity to NO and that ACTH treatment reduces NOS activity. Taken together these data strengthen the concept that NO may be a modulator of cortisol production and ACTH stimulation increases cellular sensitivity to NO following LTH. ACTH stimulation also reduces NOS activity in LTH adrenocortical cells, making NO a potential driving force in LTH fetal responsiveness to a secondary stressor. Future studies will focus on the precise mechanisms involved in LTH induced ACTH reduction of NOS enzyme activity.

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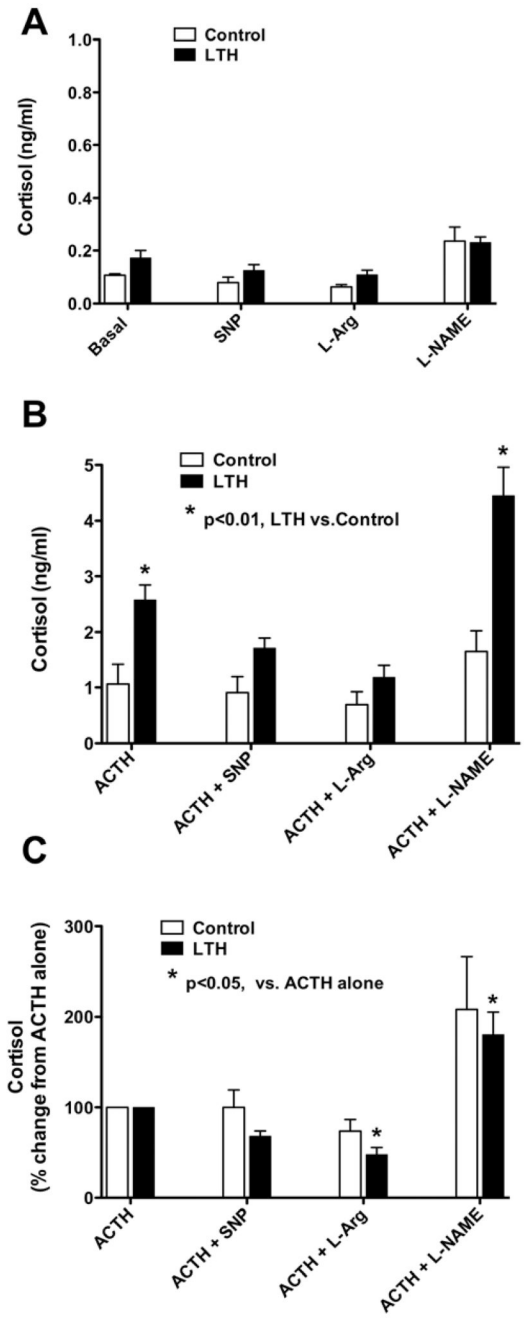

Figure 1.

Cortisol production in fetal adrenal cortical cells (FACs) from control and LTH ovine fetuses as described in the Methods section. A) untreated basal, or treated with sodium nitroprusside (SNP, 1mM), NOS substrate (L-arginine (L-Arg), 2mM) or the NOS inhibitor L-NAME (1mM) B) treated with ACTH (10nM) or pre-treated with sodium nitroprusside (SNP, 1mM), NOS substrate (L-arginine, 2mM) or the NOS inhibitor L-NAME (1mM) followed by ACTH treatment as described in the Methods section. C) Cortisol data normalized to % change from ACTH treatment alone. (n=6 for control and n=7 for LTH). LTH indicates long-term hypoxia; L-NAME, nitro-L-arginine methyl ester; NOS, nitric oxide synthase; ACTH, adrenocorticotropic hormone.

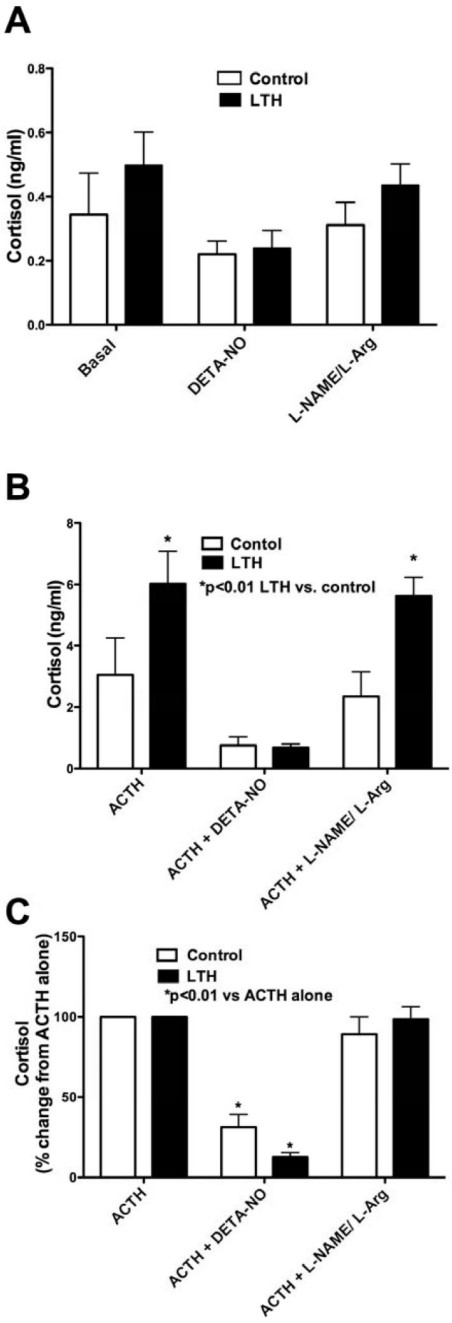

Figure 2.

Cortisol production in FACS from control and LTH ovine fetuses as described in the Methods section. A) untreated basal, treated with Diethyleneamine (DETA-NO 0.5 nM), or a combination of L-Arg (2mM) and L-NAME (1mM). B) treated with ACTH (10nM) alone or pre-treated with Diethyleneamine (DETA-NO 0.5 nM), or a combination of L-arginine (2mM) and L-NAME (1mM) followed by ACTH treatment. C) Cortisol data normalized to % change from ACTH treatment alone. (n=4 for control and 6 for LTH). LTH indicates long-term hypoxia; FACs, fetal adrenal cortical cells; L-Arg, L-arginine; L-NAME, nitro-L-arginine methyl ester; ACTH, adrenocorticotropic hormone.

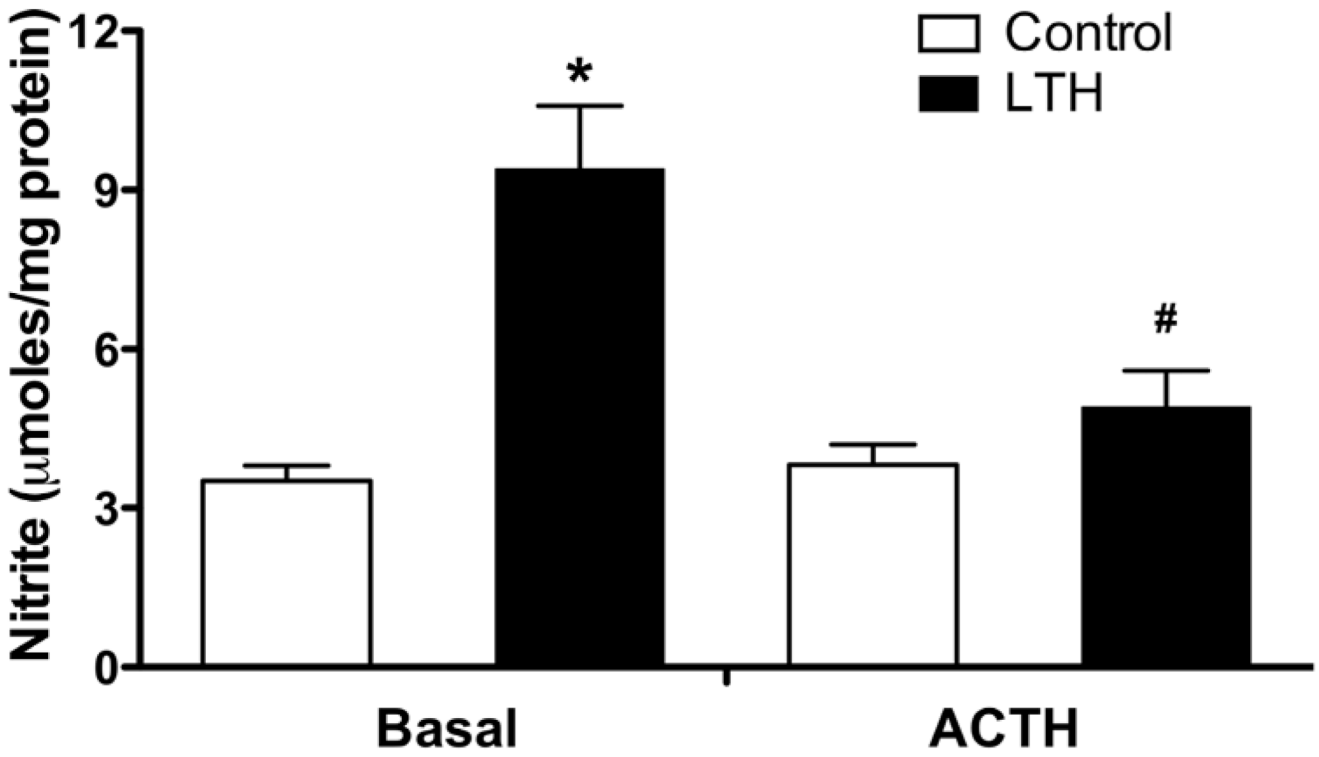

Figure 3.
NOS activity in control and LTH fetal adrenal cortical cells under basal and ACTH (10nM) stimulated conditions. Under basal conditions, LTH NOS activity was significantly greater than all other treatments (*$p<0.05$). Following ACTH treatment there was a significant reduction in NOS activity in the LTH group compared to basal ($^{\#}p<0.05$). (n=7 for each group). LTH indicates long-term hypoxia; ACTH, adrenocorticotropic hormone.