

THEMATIC REVIEW

eNOS activation and NO function: Differential control of steroidogenesis by nitric oxide and its adaptation with hypoxia

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Abstract

Nitric oxide (NO) plays a role in a wide range of physiological processes. Aside from its widely studied function in the regulation of vascular function, NO has been shown to impact steroidogenesis in a number of different tissues. The goal of this review is to explore the effects of NO on steroid production and further, to discern its source(s) and mechanism of action. Attention will be given to the

regulation of NO synthases in specific endocrine tissues including ovaries, testes, and adrenal glands. The effects of hypoxia on generation of NO and subsequent effects on steroid biosynthesis will also be examined. Finally, a potential model for the interaction of hypoxia on NO synthesis and steroid production is proposed.

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Introduction

Nitric oxide (NO) is a diatomic free-radical gas involved in a number of physiologic processes (Moncada & Palmer 1991, Moncada *et al.* 1991) and is synthesized from L-arginine by a family of NO synthases (NOS; Ignarro 2002). There are three isoforms including the constitutively expressed neuronal NOS (nNOS/NOS-I) and endothelial NOS (eNOS/NOS-III) and a third inducible isoform NOS (iNOS/NOS-II; see Daff (2010) for review). iNOS is Ca²⁺/calmodulin independent while eNOS and nNOS are regulated by Ca²⁺ and calmodulin (Michel & Feron 1997). In general, NO produced by eNOS and nNOS appears to regulate physiologic functions while iNOS tends to be involved in more pathophysiological situations.

The role of NO in the regulation of vascular function has been clearly defined (see Ignarro (2002) for review). Recently, it appears that this diatomic gas has profound inhibitory effects on steroidogenesis in a wide range of endocrine tissues and NOS are subject to regulation by hypoxia. As such, NO represents a potential mechanism for changes in steroidogenesis in response to hypoxia. This review will focus on the role of NO in the regulation of steroidogenesis and the potential interaction of NO production and hypoxia.

This paper is one of three papers that form part of a Thematic review section on eNOS activation and NO function. The Guest Editor for this section was Ian Bird, University of Wisconsin–Madison, USA.

NO and steroidogenesis

The initial rate-limiting step in steroidogenesis involves cholesterol transport to the inner mitochondrial membrane by steroidogenic acute regulatory protein (StAR) and its binding partner, peripheral benzodiazepine receptor. Within the mitochondria, StAR transfers cholesterol to CYP11A1 (P450 side-chain cleavage), which carries out a second rate-limiting step, conversion of cholesterol to pregnenolone (Pon *et al.* 1986, Arakane *et al.* 1997, Stocco 2001). NO has been clearly shown to have a profound effect on steroidogenesis in a wide range of endocrine tissues and CYP11A1 has been suggested as one potential target due to its fundamental role in steroidogenesis. Studies in multiple species and endocrine tissue types have demonstrated primarily an inhibition of steroid production by NO. The principal endocrine organs in which NO has been found to have an impact are the ovary, testes, and adrenal gland. The effects of NO on these tissues will be discussed separately below.

Ovary Using corpora lutea (CL) from pseudo-pregnant rabbits, Gobbetti *et al.* (1999) demonstrated that treatment with the NO donor nitroprusside resulted in a two- to fivefold decrease in progesterone production that was reversed with the NOS inhibitor N(G)-nitro-L-arginine methyl ester (L-NAME). Furthermore, the luteolytic activity of PGF2 α was inhibited with L-NAME treatment, suggesting that NO may mediate prostaglandin-induced functional luteolysis.

Additional studies in the rabbit also demonstrated that NO downregulates estradiol (E_2) biosynthesis during the follicular phase (Yamauchi *et al.* 1997). The same study further noted that NO also regulated prostaglandin synthesis, which in turn affected efficiency of follicular rupture.

In the rat ovary, iNOS and eNOS demonstrated distinct cell-specific expression patterns regulated differentially during follicular and luteal development (Jablonka-Shariff & Olson 1997). In porcine CL, studies revealed that NO not only inhibited progesterone and E_2 synthesis (Masuda *et al.* 1997, 2001) but also regulated steroidogenesis differently depending on the phase of follicular development. Using granulosa cells derived from both small (<3 mm) and medium (3–5 mm) follicles, they found that NO inhibited E_2 production by directly inhibiting aromatase activity in granulosa cells from follicles at both levels of maturity. However, in the presence of gonadotropin, NOS inhibition with methyl arginine (L-NMMA) increased production of E_2 and progesterone in granulosa cells, albeit to a lesser extent, from the less mature, smaller follicles than in the larger more mature follicles (Masuda *et al.* 2001). It was suggested that locally released NO in granulosa cells may serve a paracrine function by causing local vasodilatation and enhanced blood flow to the maturing follicle and CL. In contrast to earlier studies, Faes *et al.* (2009) found that NO positively regulated E_2 synthesis via cGMP during the first 24 h of culture in bovine granulosa cells while it inhibited progesterone synthesis in a cGMP-independent manner. This is a surprising finding that was not fully explained. The most reasonable explanation for the seemingly contradictory results was the use of a chemically defined medium replacing BSA with polyvinyl alcohol.

More recent studies implicated an inverse relationship between ovarian NOS activity and synthesis of StAR (Srivastava *et al.* 2007). This laboratory previously found that alcohol intake during adolescence in rhesus monkeys suppressed E_2 that, in turn, delayed development of regular menstrual cycles (Dees *et al.* 2000). With these data together, Srivastava *et al.* (2007) suggested that a combined action of alcohol enhancing ovarian NOS while suppressing StAR expression contributed to these abnormalities. The mechanisms of NO suppression of steroidogenic activity will be discussed later in this review.

Testes The earliest studies to recognize the potential effect of NO on steroidogenesis in the testes examined the effect of the NOS inhibitor L-NAME on serum and interstitial testosterone levels (Adams *et al.* 1992). Inhibition of NOS activity resulted in a significant enhancement of testosterone concentrations without affecting prolactin or LH levels. The authors concluded that NO exerted an inhibitory effect on testicular steroidogenesis. However, the specific site of action or mechanism was not defined. In an effort to determine the potential mechanism and site of action of NO in the testes, Welch *et al.* (1995) treated enriched Leydig cells *in vitro* with L-NMMA or L-NAME. This resulted in an increase in rat Leydig cell testosterone synthesis with a concomitant decrease

in cGMP accumulation in Leydig cell-enriched cultures. D-NMMA, the inactive enantiomer however, had no effect on testosterone levels. Furthermore, the observed inhibition of testosterone was not associated with a change in specific human chorionic gonadotropin (hCG) binding or cAMP generation. Thus, it was suggested that cGMP is involved in the regulation of Leydig cell testosterone biosynthesis.

Another study using NO donors also found a biphasic effect of NO on testosterone production (Valenti *et al.* 1999). With low levels of NO, they observed a transient stimulatory effect that appeared to correlate with increased cGMP levels. On the contrary, high levels of NO were associated with testosterone inhibition with no change in cGMP production. Additional studies found that immobilization stress increased NO in rat testis with a concomitant reduction in testosterone production (Kostic *et al.* 1998) and that injection of an NO donor into the testis mimicked this effect (Kostic *et al.* 2000). Furthermore, inhibiting NOS increased testosterone production in Leydig cells (Dobashi *et al.* 2001).

Steroidogenesis in the testes appears to be dependent on cGMP (Andric *et al.* 2007), which is in marked contrast to adrenal steroid production, which has been shown to be cGMP independent (Hanke *et al.* 1998). Recent studies (Andric *et al.* 2010) have provided evidence for the role of testosterone itself in the reduction in steroidogenesis by Leydig cells through regulation of the NO–cGMP signaling cascade. Testosterone enanthate treatment of rats for 2 weeks resulted not only in decreased serum LH but reduced the ability of isolated Leydig cells to produce testosterone. This was attributed to the observed upregulation of iNOS protein and NO production.

It appears that in the testes, the inhibitory effect of NO is mediated, at least in part, through inhibition of the heme-containing steroidogenic enzyme CYP17 (P450c17). When progesterone or pregnenolone was added to cultured rat Leydig cells, NO donors inhibited testosterone production (Del Punta *et al.* 1996, Pomerantz & Pitelka 1998). However, NO donors did not inhibit testosterone production from exogenous dehydroepiandrosterone or androstenedione (Pomerantz & Pitelka 1998).

At higher concentrations *in vitro*, NO also suppressed Leydig cell function through inhibition of CYP11A1 (Del Punta *et al.* 1996, Drewett *et al.* 2002). On the contrary, at lower concentrations, NO appeared to stimulate steroidogenesis in Leydig cells by activating soluble guanylate cyclase–cGMP signaling, which appears to be through activation of cGMP-dependent protein kinase 1 (PRKG1) and phosphorylation of StAR protein (Andric *et al.* 2007, 2010). Interestingly, all three NOS isoforms have been identified in testes (Kim *et al.* 2007) and NO inhibition of testosterone synthesis is conserved across species as diverse as teleost fish (nee Pathak & Lal 2008) and birds (Di Fiore *et al.* 2008).

Adrenal The early work of Palacios *et al.* (1989) suggested that the adrenal gland has an active NOS system and that adrenal NOS was NADPH and calcium dependent with

inhibition by L-arginine. These studies were performed by whole rat adrenals and bovine adrenal cortical and medullary cytosol. Multiple studies later demonstrated immunohistochemical localization of nNOS in the adrenal medulla (Dun *et al.* 1993, Afework *et al.* 1994, Heym *et al.* 1994, Marley *et al.* 1995, Peterson *et al.* 2001, Monau *et al.* 2009).

Like the ovary and testes, steroidogenesis in the adrenal gland also appears to be influenced by NO. This occurs in multiple steroidogenic regions of the adrenal cortex. In adult rat zona glomerulosa, NO clearly inhibited not only basal, but also ACTH and angiotensin II-induced aldosterone production (Hanke *et al.* 1998). Interestingly, later studies by the same group observed that NO inhibition of aldosterone biosynthesis was cGMP independent, and reversed by the NOS inhibitor thiocitrulline (Hanke *et al.* 2000). *In vivo* studies in human subjects also showed that inhibition of NOS with L-NAME increased aldosterone levels (Muldowney *et al.* 2004).

At the level of the zona fasciculata, NO donors decreased both basal and ACTH-stimulated corticosterone production while NOS inhibition enhanced glucocorticoid output (Cymeryng *et al.* 1998). Other studies on the zona fasciculata found NO to act as an inhibitor of corticosterone biosynthesis (Adams *et al.* 1992). Together, these data suggested that NO-mediated inhibition of key rate-limiting steps in the steroidogenic pathway (Drewett *et al.* 2002) that will be discussed below. The results of these studies are in marked contrast to studies by Mohn *et al.* (2005) who found that NO enhanced corticosterone release from adrenals *in vitro*. They postulated that ACTH treatment activated NOS and the resulting increase in NO-activated cyclooxygenase yielding increased PGE₂ production. PGE₂ in turn was hypothesized to facilitate release of corticosterone from microvesicles or other cellular organelles within the adrenal cortex. The variance of this study with those previously described has been suggested to be due to the difference in the *in vitro* preparation. Whereas previous studies have used dispersed cell preparations, the study by Mohn *et al.* (2005) utilized a preparation in which an entire half of a bisected adrenal gland was incubated intact. The authors suggested that the loss of the cell-cell interactions might be responsible for the differences in responsiveness to NO. Whether this is the case has yet to be established.

In our laboratory, we studied the effect of NO on cortisol biosynthesis using adrenal cortical cells collected from near-term sheep fetuses under normoxic conditions as well as from fetuses that were moderately hypoxic throughout most of gestation (long-term hypoxic (LTH)). We found that enhancement of NO with either sodium nitroprusside or L-arginine resulted in an inhibition of ACTH-induced cortisol synthesis, but only in adrenocortical cells from LTH fetal adrenals. L-NAME also had an effect on the LTH group with a significant increase in cortisol compared with ACTH treatment alone. A similar trend was noted in the control but was not significant. L-NAME also counteracted the inhibitory

effect of L-arginine on cortisol synthesis (Monau *et al.* 2010). These data clearly illustrate that NO plays a significant role in the regulation of cortisol synthesis in the LTH fetal adrenal. The interaction of NO and hypoxia on steroidogenesis will be discussed later in this review.

Cellular localization of NOS in endocrine tissue

Although the potent inhibitory effects of NO on steroidogenesis are clear, the source of NO in endocrine tissue is controversial. Endocrine tissues are composed of an intricate arrangement of steroidogenic cells as well as other non-steroidogenic components such as macrophages, endothelial cells, fibroblasts, and neuronal cells (Mohn *et al.* 2005). The short half life of NO dictates that the site and source of NO production be in close proximity to the target cells for inhibition to occur. The source of NO remains unclear but appears to be dependent on tissue type as well as species. To determine the origin of NO, numerous studies have focused on the isolation of NOS isoforms in endocrine tissue.

In the human ovary, NOS was localized in granulosa-luteal cells by immunocytochemistry (Van Voorhis *et al.* 1994). Further studies using *in situ* hybridization localized iNOS to granulosa cells of secondary follicles and small antral follicles of the rat ovary (Van Voorhis *et al.* 1995). On the contrary, eNOS could only be localized in the vascular portion of the ovarian stroma and not in the granulosa cells themselves. In porcine granulosa cells both iNOS and eNOS were localized in granulosa cells (Masuda *et al.* 1997).

Cellular localization of NOS isoforms in the testes has been less documented. However, multiple studies using immunohistochemical methods have demonstrated the presence of NOS not only in Leydig cells but also to a lesser extent in a wide range of testicular tissue, including Sertoli, peritubular, endothelial, and macrophage cells (Zini *et al.* 1996, Davidoff *et al.* 1997). More recent studies by Lamanna *et al.* (2007) also utilizing immunohistochemistry demonstrated the presence of nNOS in Leydig cells of boar testes that in some cases colocalized with D-aspartic acid.

The majority of studies examining localization of NOS have focused on the adrenal gland. Early experiments found immunoreactive NOS proteins or NOS activity in acutely isolated adrenal cortical tissue (Afework *et al.* 1994, Marley *et al.* 1995). nNOS also increased in rat adrenal cortex after immobilization stress (Kishimoto *et al.* 1996, Natarajan *et al.* 1997), and eNOS expression has been shown in rat zona glomerulosa (Natarajan *et al.* 1997, Cymeryng *et al.* 2002) and fasciculata (Cymeryng *et al.* 2002) and in adult sheep fasciculata (Peterson *et al.* 2001). Hanke & Campbell (2000), however, were unable to demonstrate the presence of NOS in cultured bovine adrenal zona glomerulosa cells by western analysis or NOS enzymatic activity. On the contrary, adrenal endothelial cells showed both enzymatic and immunoreactive eNOS. It was suggested that these disparities could be the result of species differences or cell culture conditions. However, it is clear that cortical NOS expression is regulated, since immobilization stress increased nNOS

mRNA in the adrenal cortex of rats (Natarajan *et al.* 1997, Cymeryng *et al.* 2002). It is important to note that these data were derived from the study of adult adrenal tissue.

A recent study from our laboratory utilized both qRT-PCR and western blot to show that although mRNA for all three NOS isoforms were present in the ovine fetal adrenal cortex, there was no detectable protein expression for iNOS and that eNOS was the predominant isoform (Monau *et al.* 2009). This information was derived from a whole adrenal cortex preparation, however, and did not reveal the actual cellular localization of the eNOS. Additional studies using standard immunohistochemistry demonstrated that eNOS appeared to be localized not only within the vasculature but also in the adrenal cortical cells themselves. The intensity of immunohistochemical staining for eNOS was greatest in the zona fasciculata region of the cortex which is similar to the distribution of eNOS staining reported in the adult ovine adrenal (Peterson *et al.* 2001). In that study, however, staining for nNOS was not apparent. We suggested that the differences in nNOS expression between adult and fetus were due to developmental changes (Monau *et al.* 2009). We also demonstrated very low expression levels of iNOS mRNA, but iNOS protein was undetectable with either western analysis or immunohistochemistry. A similar lack of protein expression was noted in earlier studies of rat and human adrenal cortex (Natarajan *et al.* 1997), bovine zona glomerulosa cells (Hanke & Campbell 2000), and adult ovine adrenal (Peterson *et al.* 2001). The absence of iNOS seems logical from a regulatory standpoint since iNOS activation is calcium independent and is only regulated at the level of expression unlike the other NOS isoforms. This would make iNOS a relatively ineffective mechanism in controlling steroidogenesis.

Further evidence for localization of eNOS within adrenal cortical cells was provided by immunofluorescent dual labeling studies. eNOS clearly colocalized with CYP17, a key enzyme involved in cortisol synthesis that is expressed in cells of the zona fasciculata (Monau *et al.* 2009). Together, these data further reinforce the concept that adrenal cortical cells express eNOS and that NO produced locally may play a role in local regulation of cortisol synthesis. Other studies have also suggested that endogenous generation of NO from adrenal cortical cells occurs through constitutively expressed eNOS in the rat (Cymeryng *et al.* 2002).

As described above there still remains some controversy over the exact source of NO in various endocrine tissues. However, irrespective of differences in species, stage of development or actual cellular localization of NOS, it is likely that steroidogenic tissues such as the ovary, testes, and adrenal are exposed to NO in response to various physiological and pathological situations.

Mechanism(s) of NO disruption of steroidogenesis

As previously described, endocrine tissues are exposed to NO and in general, NO has an inhibitory effect on

steroidogenesis. The potential mechanisms of NO action will be examined in the context of both direct and potentially indirect effects.

Direct effects

Soluble guanylate cyclase and cGMP. The classic mechanism by which NO mediates cellular signaling is activation of soluble guanylate cyclase. NO activates guanylate cyclase by altering its conformation via binding to the heme group of this enzyme, leading to elevation of intracellular cGMP with subsequent activation of PKG (Ignarro 1990). This has been best studied in vascular relaxation. However, the vasorelaxant actions of NO are not fully blocked by inhibitors of guanylate cyclase, indicating that a significant component of NO-mediated vasorelaxation is cGMP independent (Denninger & Marletta 1999). Indeed, many of the known effects of NO now appear to be independent of intracellular cGMP. The inhibitory effect of NO on steroidogenesis has also been shown to be cGMP independent in the adrenal gland (Hanke *et al.* 2000). The effects of NO on steroidogenesis in granulosa cells also appears to be cGMP independent since treatment with 8-bromo-cGMP did not inhibit steroid biosynthesis (Masuda *et al.* 1997). Later studies by Basini *et al.* (2000), also demonstrated that NO inhibits steroidogenesis in granulosa cells without involving cGMP.

Effects on heme binding. Similar to guanylate cyclase, NO is capable of competitively interacting with the heme oxygen-binding site of key steroidogenic enzymes such as CYP11A1 and CYP17 (Tsubaki *et al.* 1987). Studies using purified or semi-purified cytochrome P450 enzymes confirmed these earlier observations (Wink *et al.* 1993, Hanke *et al.* 1998). Since these enzymes use several rounds of attack of the heme-oxygen complex on the steroid substrate, Peterson *et al.* (2001) suggested that such multi-step targets would be more sensitive to NO inhibition than other enzymes such as CYP21 or CYP11B1. Hanke & Campbell (2000) also clearly demonstrated the relationship between oxygen levels, NO and steroidogenesis by showing that decreasing oxygen concentrations resulted in a lower threshold for NO-mediated inhibition of aldosterone synthesis in adult rat adrenals. Overall, this competitive relationship makes NO an effectual inhibitor of steroid biosynthesis.

Potential indirect effects on NO

Cysteine S-nitrosylation and tyrosine nitration. Maintaining the proper redox status of cysteine residues is critical for the structure and function of numerous enzymes, transcription factors and receptors. Free sulfhydryl groups of cysteine are subject to oxidation by reactive oxygen species (widely used as a marker for oxidative stress) as well as nitrogen species such as NO. It is becoming recognized that NO-mediated S-nitrosylation of reactive cysteine sulfhydryl residues is reversible and as such, an important post-translational modification regulated in a manner akin to phosphorylation. NO reacts with O₂ to form the more reactive

NO-derived species including NO₂ and N₂O₃ that S-nitrosylate cysteine thiols in target proteins. *In vivo*, cysteine S-nitrosylation is dynamically controlled, with steady-state levels regulated by rates of NO formation via one of the NOS isoforms versus de-nitrosylation of the targeted cysteine residues.

A large number of the physiological actions of NO are mediated via reversible post-translational modification of proteins critical for cellular function (Stamler *et al.* 2001). There are two distinct post-translational modifications of proteins mediated by NO and NO-derived species: S-nitrosylation of cysteine residues and nitration of tyrosine residues. Cysteine S-nitrosylation is generally considered to mediate physiological actions of NO (Stamler *et al.* 2001) while tyrosine nitration is most often implicated in pathological states (Greenacre & Ischiropoulos 2001). Indeed Stamler *et al.* (2001) supplied a table of over 100 proteins known to be targeted by NO-mediated cysteine S-nitrosylation. These include key signaling proteins such as the NMDA receptor, RAS, dexRAS, SRC, JNK, and FOS/JUN (Hess *et al.* 2005). S-nitrosylation has also been demonstrated to inactivate protein-tyrosine phosphatases (PT-1, 2) thus prolonging signaling. In particular those downstream from tyrosine kinases, including SRC and growth factor receptors, are affected since the latter rely on their receptor tyrosine kinase activity in transducing their signal. Cysteine S-nitrosylation is reversible by either glutathione or thioredoxin thus representing potential rapid regulatory steps. Thioredoxin is also subject to regulation by thioredoxin interacting protein (TXNIP) that inhibits its activity (Patwari *et al.* 2006). TXNIP expression and activity is increased by p38 MAPK (Li *et al.* 2009).

Given the previous widespread demonstration of cysteine S-nitrosylation on protein function, S-nitrosylation of CYP11A and/or CYP17 is a potential mechanism by which NO could suppress steroidogenesis. CYP enzymes have critical cysteine residues in the active site and modification of these residues could play a crucial role in their activity. Indeed CYP S-nitrosylation has been reported in liver (Lee *et al.* 2008). Data from our laboratory have preliminarily confirmed S-nitrosylation of CYP17 in the fetal adrenal cortex (Ducsay & Myers, unpublished).

Zinc-finger transcription factors, including nuclear receptors such as the estrogen receptor (Garban *et al.* 2005) are also targets of S-nitrosylation, resulting in the loss of Zn⁺ from the DNA-binding pocket disrupting their function as transcriptional activators (Kroncke 2001). Of particular relevance, steroidogenic factor-1 (SF-1) is a member of the Zn⁺ finger family. SF-1 is a major transcription factor governing not only formation of the adrenal cortex but the expression of CYP11A, CYP17, CYP21, and StAR as well (Sewer & Waterman 2003, Zhao *et al.* 2007). As such, S-nitrosylation of SF-1 could also serve as another mechanism via which expression of CYP17 and CYP11A1 could be regulated.

Effects of hypoxia on NO production and steroidogenesis Hypoxia has a wide range of effects on NO production (and/or expression of NOS isoforms) in different animal models and tissues. Hypoxia increases eNOS expression and NO production in microvessels in the heart of pigs (Justice *et al.* 2000). Our group has shown that LTH enhances eNOS expression in ovine uterine arteries (Xiao *et al.* 2001). On the contrary, in cerebral arteries, hypoxia reduced expression of eNOS yet increased some components of the NO/cGMP/PKG pathway, thus increasing vascular sensitivity to NO (Williams *et al.* 2006). Murata *et al.* (2001) observed a reduction in eNOS expression and function in cultured pulmonary arteries following hypoxia (5% O₂ for 7 days). The same group also found that hypoxia-induced pulmonary hypertension reduced NO production by impairing the interaction of eNOS with its regulatory proteins (Murata *et al.* 2002). Hypoxia may also have divergent effects on eNOS expression with decreased fetal eNOS expression and increased adult expression in guinea pig hearts (Thompson & Dong 2005). Hypoxia stimulates eNOS-HSP90 interaction and activates the PI3-AKT pathway leading to eNOS phosphorylation, thus increasing NO production (Chen & Meyrick 2004).

5' AMP-activated protein kinase (AMPK) plays a key role in the regulation of a wide range of intracellular systems and is regulated by hypoxia. With decreased oxygen there is an associated decreased oxidative phosphorylation leading to a reduction in ATP and subsequent increase in AMP, the activator of AMPK. The interaction of hypoxia and the AMPK, AKT signaling of eNOS activation was clearly demonstrated in endothelial cells. Hypoxia activated AMPK, which in turn promoted mitogen-stimulated AKT activation and angiogenesis. AKT was found to be the predominant kinase involved in eNOS phosphorylation at Ser1177 in hypoxic endothelial cells. In addition, however, direct eNOS phosphorylation by AMPK was suggested to become appreciable under conditions of prolonged hypoxia (Nagata *et al.* 2003). Recently, Chen *et al.* (2009) showed that AMPK phosphorylates eNOS at Ser633 and is a functional signal of NO bioavailability.

Hypoxia activated ERK, while administration of a NOS inhibitor prevented hypoxia-induced phosphorylation in neuronal nuclei of newborn piglets, suggesting that the hypoxia-induced ERK phosphorylation is NO mediated (Mishra *et al.* 2004). In endothelial cells, hypoxia also enhanced ERK phosphorylation (Zhu *et al.* 2003). Data from our own group showed that resting PKA activity in fetal hearts was significantly higher in LTH fetuses compared with normoxic controls (Onishi *et al.* 2003) and that LTH upregulated eNOS gene expression in ovine uterine arteries (Xiao *et al.* 2001).

Together, the studies previously described demonstrate that hypoxia can regulate NOS expression/activity. It is also clear that there is both tissue and species specificity regarding NOS expression. However, all of these studies were focused

on non-steroidogenic tissue. In reference to hypoxia and steroid biosynthesis, Raff *et al.* (1989) originally showed that adrenal steroidogenesis is regulated by oxygen that was further confirmed in a more recent study (Raff & Bruder 2006). Hanke & Campbell (2000) confirmed the relationship between oxygen levels, NO and steroidogenesis by demonstrating that decreasing oxygen concentrations resulted in a lower threshold for NO-mediated inhibition of aldosterone synthesis in adult rat adrenals.

In our model of LTH in which pregnant sheep are exposed to high-altitude hypoxia for the duration of gestation, the fetal adrenal exhibits specific adaptive endocrine responses, which appear to be related to the regulation of NO. We recently showed that LTH upregulates fetal adrenal expression of eNOS, both mRNA and protein, and that eNOS was located in the cortisol-producing cells of the zona fasciculata (Monau *et al.* 2009). We postulated that the enhanced ability to produce NO under conditions of LTH prevents premature maturation of the fetal adrenal cortex that if left unchecked could result in excess cortisol production in the face of the observed elevated basal ACTH levels (Myers *et al.* 2005).

We also found that in the LTH adrenals, enhancement of NO with either SNP or L-arginine resulted in a significant reduction in ACTH-stimulated cortisol synthesis by adrenal cortical cells *in vitro*, and inhibition of NOS with L-NAME resulted in increased cortisol production compared with ACTH treatment alone. None of these effects were observed in normoxic control cells (Monau *et al.* 2010). Fetal adrenal cortical cells display a number of differences from adult adrenocortical cells that may account for the lack of major effects of NO on normoxic fetal adrenal cortical cells *in vitro*. Our finding that normoxic cells do not respond to NO while those from hypoxic fetuses do respond indicates an increased sensitivity to NO, or perhaps an advanced maturation of these cells to a more adult-like phenotype. Furthermore, basal NOS activity was enhanced in the LTH that is in agreement with the earlier observation that LTH adrenals had enhanced eNOS expression (Monau *et al.* 2009). Surprisingly, treatment with stress levels of ACTH decreased NOS activity in the LTH group.

One of the key factors that may link ACTH stimulation of cortisol production and a decrease in NOS activity is ERK1/2. Although the role of ERK1/2 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 but inhibited ATP-stimulated activity in COS-7 cells (Cale & Bird 2006). MEK/ERK1/2 inhibition also enhanced eNOS activity in porcine pulmonary arteries (Chen & Meyrick 2004). Therefore, studies suggest that stimulation of ERK1/2 could inhibit NOS activation. ERK1/2 stimulation plays an important role in adrenal steroidogenesis by upregulation of StAR expression (Gyles *et al.* 2001). We have recently shown that the ERK1/2 signaling pathway plays a key role in ACTH- and cAMP-induced cortisol synthesis in the ovine fetal adrenal cortical cells (Vargas *et al.* 2009). Furthermore, ERK inhibition had a

greater effect and was more pronounced in the LTH group, consistent with our preliminary data that ERK1/2 are upregulated in response to LTH (Ducsay & Myers, unpublished observations). Although the direct effect of NO on StAR activity expression is controversial (Dobashi *et al.* 2001, Cymeryng *et al.* 2002, Andric *et al.* 2007), this is another potential pathway of interaction of NO, and fetal adrenal steroidogenesis. ACTH has also been shown to inhibit iNOS mRNA in macrophages (Altavilla *et al.* 2000).

In our study, the locus of the ACTH effect on NOS activity in the LTH was not established (Monau *et al.* 2010). 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(s) 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. The previously described upregulation of adrenal eNOS in the LTH group may be responsible for enhanced basal NOS activity. This would exert an inhibitory effect on basal cortisol production overcoming the low level of stimulation by elevated basal ACTH. Stimulation with high levels of ACTH *in vitro* (Monau *et al.* 2010) or those observed *in vivo* in response to a secondary stressor (Adachi *et al.* 2004, Imamura *et al.* 2004) would decrease NO activity, thus enhancing cortisol output. We propose that this can occur through ACTH interaction with the MEK/ERK1/2 and/or the AKT/IP₃. A preliminary investigation of expression of both the native and phosphorylated forms of these members of their respective signaling pathways revealed that there is an enhancement of protein expression following LTH. Phosphorylation of SF-1, the major transcription factor governing CYP11A1 and CYP17 expression is also enhanced (Ducsay & Myers, unpublished observations).

Another mechanism activating eNOS is the interaction with CAV-1 and HSP90. Gratton *et al.* (2000) showed that eNOS interacts with both CAV-1 and HSP90 during activation/inactivation translocation cycle, binding in a trimeric complex. CAV-1 keeps eNOS in an inactive state at the caveolae while also inhibiting eNOS translocation and/or the stimulatory effect of calmodulin binding. On the contrary, HSP90 not only binds free eNOS but may also disrupt the CAV-1/eNOS complex. Furthermore, hypoxia has been shown to alter CAV-1 expression and increase HSP90 binding to eNOS (Shi *et al.* 2002, Chen & Meyrick 2004). Our studies on the effects of LTH on CAV-1 and HSP90 showed that in the LTH fetal adrenal cortex, higher levels of mRNA and density of HSP90 and lower levels of CAV-1 correlated with our previously observed difference in eNOS expression and activity. These data support the hypothesis that LTH alters the distribution and expression of HSP90 and CAV-1 in the ovine adrenal and suggest that these proteins play a regulatory role in eNOS activity in the LTH fetal adrenal (Furuta *et al.* 2010).

Consistent with the known effects of hypoxia reported in literature and our published and preliminary data in the fetal adrenal cortex of the LTH sheep fetus, we propose that LTH results in increased activation of PI3K, AMPK, and RAS signaling pathways. Activation of PI3K and/or AMPK results in elevated pAKT/AKT which we hypothesize results in increased activity of eNOS via phosphorylation of eNOS at Ser1179 and dissociation of CAV-1 and recruitment of the eNOS activator, HSP90. Activated eNOS results in NO generation leading to Cys S-nitrosylation of critical target proteins governing cortisol synthesis (CYP11A1 and CYP17), SF-1, which regulates CYP11A1 and CYP17 transcription. We hypothesize that S-nitrosylation of cysteine residues in the DNA-binding domain of SF-1 reduces its capacity to interact with specific SF-1 *cis* elements in the promoter regions of CYP17 and CYP11A1 while nitrosylation of CYP11A1 and CYP17 decreases enzymatic activity. These hypotheses are consistent with the decreased cortisol synthesis and CYP expression observed in the adrenal cortex of LTH fetal sheep in spite of elevated basal plasma ACTH. However, under conditions of a secondary stressor, large increases in ACTH (approximately 10- to 20-fold over basal

levels) override the inhibitory effects of NO and result in enhanced cortisol production compared with that observed in control adrenals. Together, our hypotheses for the role of NO in the regulation of adrenal cortisol synthesis under hypoxic conditions are summarized in Fig. 1.

In addition to the schema outlined in Fig. 1, another novel mechanism involved in hypoxic regulation of NO-induced regulation of steroidogenesis is leptin. Leptin has been shown to enhance eNOS activity in a number of tissues (Mehebig *et al.* 2005, Fruhbeck 2006, Procopio *et al.* 2009) and also appears to be a hypoxia-inducible gene (Tschop *et al.* 1998, Ip *et al.* 2000, Tschop *et al.* 2000). Although we have shown that the LTH fetal adrenal has enhanced eNOS expression (Monau *et al.* 2009), the mechanism responsible for the upregulation of eNOS with its subsequent inhibitory effects of cortisol biosynthesis remains undefined. We have shown that under conditions of LTH, leptin also appears to be a hypoxia-inducible gene in the ovine fetus with enhanced expression of leptin in fetal adipose tissue and increased plasma leptin levels (Ducsay *et al.* 2006). Elevations in leptin in response to hypoxia could therefore also play a role in upregulation of NO in steroidogenic tissues.

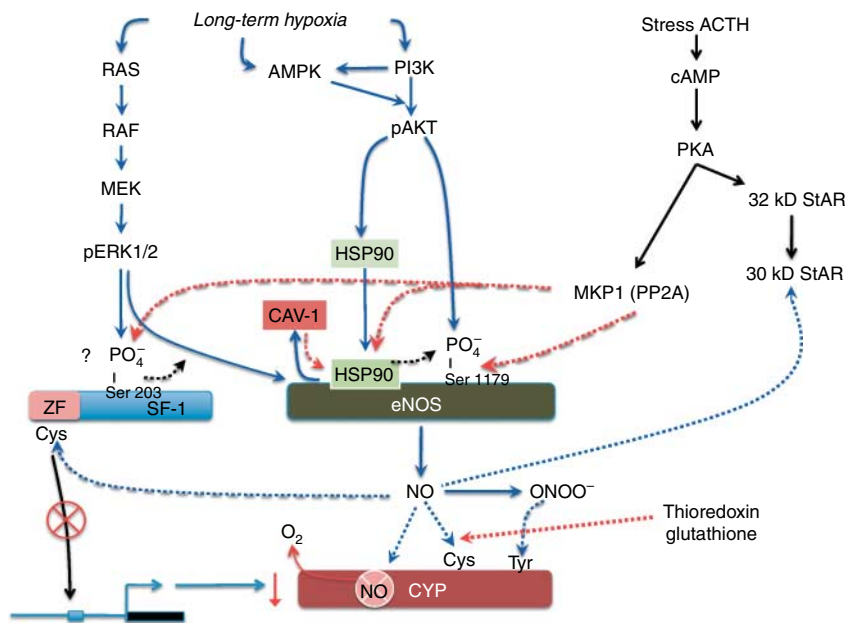


Figure 1 Proposed model for mechanism(s) via which development under sustained moderate hypoxia (long-term hypoxia (LTH)) attenuates adrenocortical glucocorticoid production despite elevated plasma ACTH. We propose that LTH activates eNOS via the PI3K, AMPK signaling pathways. AKT has been demonstrated to activate eNOS via phosphorylation of Ser1179. AKT also targets HSP90 resulting in its association with eNOS (and accompanying dissociation of CAV-1 from eNOS) resulting in cellular translocation of eNOS and enhancing eNOS activity. Activated eNOS results in NO generation and potentially peroxynitrite formation leading to cysteine nitrosylation (and potentially, tyrosine nitration) of rate limiting P₄₅₀ enzymes governing cortisol synthesis (CYP11A1 and CYP17). NO has also been shown to displace oxygen in the heme group of heme-containing proteins, such as P450 enzymes, inhibiting their activity. SF-1, a key zinc-finger transcription factor regulating CYP11A1 and CYP17 transcription, is a potential target for cysteine nitrosylation which could contribute to the lowered expression of CYP11A1 and CYP17 reported in the LTH adrenal cortex. In addition, long-term hypoxia activation of the RAS signaling pathway, via ERK1/2 activation may alter SF-1 function via phosphorylation of this transcription factor as well as affecting eNOS activity. Stress-induced elevation in ACTH may overcome the NO inhibition of steroidogenesis via induction of protein phosphatases as well as by increasing levels of thioredoxin or glutathione resulting in de-nitrosylation.

Conclusions

In summary, independent work from numerous laboratories on a wide variety of species supports the concept that NO exerts powerful, physiological, effects on steroidogenesis in key endocrine tissues such as the ovary, testes, and adrenal gland. Despite the general agreement of a role for NO in modulating steroidogenesis, the mechanisms via which NO regulates steroidogenesis may be tissue, and perhaps situation specific. The cell type specificity observed between adrenal cortex and gonads may also reflect differences in NO mechanisms. For example, direct effects on the enzyme via heme binding versus those mediated by cysteine or tyrosine nitration or upstream actions on other signaling cascades that mediate steroidogenesis. However, the noted actions may also reflect differences in culture conditions, age of cells (fetal versus adult), species, etc.

As hypothesized by Repetto *et al.* (2006) NO may serve as an autocrine/paracrine modulator of steroidogenesis involved in the refinement of the cellular responses to hormones. Such fine-tuning would provide a mechanism for the endocrine tissue to avoid an 'all or none' type of response. For example, in the ovary, NO regulation of blood flow may provide a key means of altering steroid formation, while in the adrenal gland, NO may alter steroid production by acting directly on the steroidogenic enzymes themselves or altering their expression via effects on transcription factor function. Studies from our laboratory and others supports that hypoxia modulation of steroidogenesis is profound and appears to enhance the effect of NO on steroidogenesis as well as upregulating expression of eNOS. In the oxygen compromised fetus, NO may provide a key potential mechanism for maintaining normal cortisol levels to allow for continued fetal growth and development in the face of this adverse situation and may implicate the NO system as a key target for treatment of preterm or compromised neonates in the neonatal intensive care unit.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

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