

# Nitric oxide and the ovary<sup>1</sup>

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**ABSTRACT:** Nitric oxide (NO) is synthesized from L-arginine by NO synthase (NOS), an enzyme with three isoforms. Two of them, neuronal and endothelial (nNOS and eNOS, respectively), are constitutive, whereas the third one, iNOS, is inducible. Nitric oxide is effective in mediating multiple biological effects, in part through the activation of soluble guanylate cyclase. Among these effects are smooth muscle cell tone, platelet aggregation and adhesion, cell growth, apoptosis, and neurotransmission. Because these mechanisms are associated with the pathophysiology of several reproductive processes, it has become clear that NO could play a key role in reproduction. Apart from its effects through the modulation of luteinizing hormone releasing hormone release, NO has been proven to act directly at the ovarian level, where it is produced by the vasculature and neurons, as well as by various cell types, including granulosa, theca, and luteal cells. Nitric oxide production is modulated by several hormones (estradiol 17 $\beta$ , luteinizing hormone, follicle-stimulating hormone, and human chorionic gonadotropin) and cytokines that in-

terfere either with eNOS or iNOS expression and activity. Experiments performed with NO donors and/or NO synthase inhibitors have demonstrated that NO decreases apoptosis and inhibits both estradiol 17 $\beta$  and progesterone production by granulosa cells (at least in part via guanylate cyclase). Nitric oxide is possibly involved in follicle growth; it is a potent mitogen in the presence of basic fibroblast growth factor, it increases the receptors for epidermal growth factor on granulosa cells, and it regulates programmed cell death, which is an important part of folliculogenesis. Gonadotropin-stimulated eNOS and iNOS expression, as well as the inhibition of ovulation by NOS inhibitors, suggest that NO participates in the ovulatory process. After ovulation, iNOS is expressed in luteal cells, but its activity diminishes with corpus luteum development. During the luteolysis phase, NO stimulates PGF<sub>2 $\alpha$</sub>  synthesis while decreasing progesterone secretion. Overall data provide convincing evidence that NO plays a critical role in ovarian physiology with regard to follicle growth, ovulation, and corpus luteum function, but its clinical implications have not yet been clarified.

Key Words: Corpus Luteum, Nitric Oxide, Ovaries, Ovarian Follicles, Ovulation

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## Introduction

Nitric oxide (NO) is an inorganic, short-lived (a few seconds) free radical gas that, due to its high solubility, freely diffuses through biological membranes. It is synthesized from L-arginine via an oxygen- and NADPH-dependent reaction that yields NO and L-citrulline (Bush et al., 1992; reviewed by Wu and Morris, 1998). Nitric oxide synthesis depends on the action of a NO synthase (NOS), an enzyme that exists in three iso-

forms that have been classified depending on tissue of origin as well as on functional properties. Two of them (neuronal [nNOS] and endothelial [eNOS]) are constitutive and seem to be responsible for the continuous basal release of NO; the third one is inducible (iNOS) and is expressed in response to inflammatory cytokines and lipopolysaccharides (Morris and Billiar, 1994). Both nNOS and eNOS are calcium/calmodulin dependent for their activation (Snyder, 1995), whereas iNOS is calcium independent. The three isoforms have been found in a variety of cell types, including neurons, gastric and bronchial epithelium, skeletal muscle, macrophages, cardiomyocytes, hepatocytes, and chondrocytes; the production of NO is therefore almost ubiquitous. Nitric oxide is involved in a wide range of functions: It determines vasodilation, inhibits platelet aggregation and neutrophil adhesion to endothelial

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cells, reduces smooth-muscle cell proliferation and migration, controls apoptosis, sustains endothelial cell barrier function (Rosselli et al., 1998), and acts as a neurotransmitter. Nitric oxide is generated by neurons, blood vessels, and cells of the immune system (which are structural and functional parts of the hypothalamus-pituitary-gonads axis), as well as by other cell types of this axis. Because NO plays an important role in the function of this system, its involvement in the mechanisms regulating the reproductive processes is quite obvious (Rosselli et al., 1998; Dixit and Parvizi, 2001).

### *Nitric Oxide Generation and Mechanisms of Action*

Availability of L-arginine is essential for NO generation in that it is the only physiological nitrogen donor for NOS-catalyzed reactions. Competitive inhibition of arginine uptake by other naturally occurring amino acids, such as L-lysine and L-ornithine, reduces NO synthesis (Inoue et al., 1993). Nonphysiological substances derived from arginine (nitro-L-arginine-methyl ester [**L-NAME**], Ng-monomethyl-L-arginine [**L-NMMA**]) are being used to determine the effects of NO deprivation. In the same way, NO donors (sodium nitroprusside [**SNP**], *S*-nitroso-L-acetyl-penicillamine, [**SNAP**]) are also used to evaluate the involvement of NO in biological functions.

Many of the biological effects of NO may result from the alteration of multicomponent signal transduction pathways and are exerted via different mechanisms (for a review, see Schindler and Bogdan, 2001), three of which seem to be the most important. The first one involves NO binding to the heme iron of soluble guanylate cyclase, thus activating guanosine 3',5'-cyclic monophosphate (**cGMP**), which mediates most of the effects on vessel and intravessel functions (Murad, 1994). Nitric oxide also induces the *S*-nitrosylation of thiol groups of free amino acids, peptides, and proteins (Kelly et al., 1996) and can react with other radicals, resulting in the formation of peroxynitrite, a potent oxidant effective in inducing cytotoxicity (for a review, see Droge, 2002). Taken together, the available information suggests that the effects of NO are strictly dependent on its concentration, as well as on the presence of metals, proteins, and low-molecular-weight thiols in a given cell (Davies et al., 1995).

### *Effects of Nitric Oxide at the Hypothalamic-Pituitary Level*

Nitric oxide has been shown to modulate reproductive activity by acting at both the hypothalamic and pituitary levels (for a review, see Dixit and Parvizi, 2001). Experimental results (Bhat et al., 1995) suggest that NO is an important mediator of basal GnRH production since NO neurons are present in hypothalamic sites involved in GnRH secretion. Experiments with an NO donor (SNP) and with the NO scavenger hemoglobin

demonstrated that NO stimulates GnRH release in rats; the effect may be induced both by activation of guanylate cyclase (Moretto et al., 1993) and through the activation of neuropeptide Y (Bonavera and Kalra, 1996). Nitric oxide has also been shown to promote LH secretion in the cow (Honaramooz et al., 1999); the positive effect on LH secretion is likely to be exerted via a cGMP-independent mechanism (Pinilla et al., 1998). An interaction between NO and opioids has also been proposed. The administration of naloxone (an opioid antagonist) enhances NOS activity (Bhat et al., 1996), whereas naltrexone blocks the inhibitory effect of  $\beta$ -endorphin on LHRH release and NOS activity in the rat (Faletti et al., 1999a).  $\beta$ -endorphin also blocks the positive effect of NO on PGE<sub>2</sub>, and therefore on GnRH release. Leptin-induced LH release may be mediated via nitric oxide (Yu et al., 1997). Nitric oxide also seems to be involved in controlling the preovulatory LH surge (for a review, see Dhandapani and Brann, 2000) because NOS inhibitors have been demonstrated to reduce LH release in rats (Bonavera et al., 1994). The expression of nNOS in the preoptic area increases concomitantly with LH peak in the same species (Lamar et al., 1999). The generation of NO by hypothalamic and pituitary cells facilitates sexual behaviour in females; administration of NOS inhibitors and NO donors to conscious female rats prevents or stimulates lordosis behavior, respectively (Mani et al., 1994).

The overall data on the effects of NO in the modulation of reproductive activity at the hypothalamic-pituitary level strongly indicate that NO exerts a positive action and is effective in stimulating GnRH and LH secretion, as well as estrous behavior; nevertheless, because contrasting results have been reported in the rat (Ceccatelli et al., 1993; Chatterjee et al., 1997), further studies are required to definitely confirm the role of NO at this level.

### *Nitric Oxide and Ovarian Function*

The involvement of NO in the modulation of ovarian function is documented by several studies aimed at demonstrating its production within the ovary and at clarifying its role in the regulation of steroidogenesis, follicle development, ovulation, luteal function, and luteal regression.

### *Regulation of Nitric Oxide Production within the Ovary*

Nitric oxide is generated by several ovarian cells and within the ovarian vasculature; resident macrophages have also been indicated as a possible source of NO in the rat (Dave et al., 1997). Both eNOS and iNOS seem to be involved, although their expression and activity greatly depend on cell type and animal species and vary throughout the different ovarian processes (Rosselli et al., 1998). In particular, data on iNOS expression (which is usually influenced by inflammatory condi-

tions) in granulosa cells are still conflicting. Van Voorhis et al. (1994) reported that human granulosa-luteal cells express eNOS. The presence of eNOS also has been confirmed in rat mural granulosa cells (Jablonka-Shariff and Olson, 1997), in blood vessels (Van Voorhis et al., 1995), in rat stroma, thecal, and luteal cells (Zackrisson et al., 1996), and in pig granulosa cells (Ponderato et al., 2000; Takesue et al., 2001). Results from different studies indicate that rat granulosa cells from primary, secondary, and small antral follicles (Van Voorhis et al., 1995; Matsumi et al., 1998a) and rat stroma, thecal, and luteal cells (Zackrisson et al., 1996) also express iNOS. This isoform, on the other hand, has not been detected in either rat (Jablonka-Shariff and Olson, 1997) or in porcine (Ponderato et al., 2000) granulosa cells. Nitric oxide production also has been demonstrated in bovine granulosa cells (Basini et al., 1998; Basini et al., 2000); these studies, however, were not aimed at defining which isoform is responsible for NO synthesis. The expression of both iNOS and eNOS is regulated by gonadotropins (Jablonka-Shariff and Olson, 1997) since both PMSG and hCG have been shown to influence eNOS and iNOS concentrations, thus confirming that both isoforms participate in the ovarian functions. Nitric oxide production in rat ovarian cells is actively stimulated by interleukin-1, as well as by several proinflammatory cytokines (Ellman et al., 1993; Ben-Shlomo et al., 1994; Matsumi et al., 1998a); iNOS has been demonstrated to be induced in bovine thecal cells by tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), thus stimulating cGMP accumulation (Brunswig-Spickenheier and Mukhopadhyay, 1997). In addition, NO generation (measured on the basis of nitrite and nitrate concentration) increases with both estradiol levels in human follicular fluid and with follicular size (Rosselli et al., 1994; Anteby et al., 1996). These observations suggest a possible causal relationship between these characteristics, even though an inverse relationship between estradiol and nitrite concentrations has been observed in swine (Grasselli et al., 1998) and in bovine (Basini et al., 1998) follicular fluid. However, the increase of nitrite and nitrate levels in the serum of postmenopausal women subjected to E2 substitution therapy substantiates the positive effect of estrogens on NO production (Rosselli et al., 1994).

#### *Nitric Oxide and Steroidogenesis*

Nitric oxide has been shown to exert negative effects on steroidogenesis, possibly through a direct action on steroid-secreting cells rather than via an effect on local ovarian blood flow (Dave et al., 1997). The impairment of steroid production by NO has been demonstrated in different species and in different conditions (rat, Dave et al., 1997; human, Van Voorhis et al., 1994 and Rosselli et al., 1998; porcine, Masuda et al., 1997; Matsumi et al., 2000; Ponderato et al., 2000, and Grasselli et al., 2001; bovine, Basini et al., 1998 and Basini and Tamanini, 2000). The negative effect of NO on steroid

production has been demonstrated by treating cultured granulosa-luteal cells with SNAP, an NO donor, or with L-NAME, an NOS inhibitor, which markedly decrease or stimulate, respectively, both estradiol and progesterone release. This effect seems to be cGMP independent (human, Van Voorhis et al., 1994; Rosselli et al., 1998; bovine, Basini et al., 2000), even though different conclusions have been drawn in other species (swine, Grasselli et al., 2001; rat, Ishimaru et al., 2001). The negative effect of NO on both basal- and gonadotropin-stimulated estradiol production in the rat may be, at least in part, exerted through an inhibition of androstenedione secretion (Dunnam et al., 1999); in addition, the cytochrome P450 aromatase, responsible for estradiol production, has been shown to be possibly inhibited (Van Voorhis et al., 1994). This inhibition may be exerted through a reduction of aromatase messenger RNA (mRNA) levels and/or of enzyme effect (human, Snyder et al., 1996; Kagabu et al., 1999).

#### *Nitric Oxide and Folliculogenesis*

It is well known that both folliculogenesis and ovulation are regulated by a variety of factors, such as cytokines, growth factors, and locally produced substances, among which NO seems to play an important role. Nitric oxide levels have been shown to change during follicular growth (Rosselli et al., 1998). Follicular development, induced by PMSG in immature rat, is associated with an increase in eNOS (but not iNOS) expression (Van Voorhis et al., 1995; Jablonka-Shariff and Olson, 1997), whereas a subsequent stimulation with hCG induces an increase of both isoforms (Jablonka-Shariff and Olson, 1997). On the other hand, Matsumi et al. (1998b) observed a decrease in iNOS mRNA levels induced by PMSG in granulosa cells from immature rat follicles; on this basis, they suggested that NO could possibly represent a cytostatic factor. This hypothesis has been reinforced by results from a more recent study in the rat (Matsumi et al., 2000), which also show a GnRH- and endothelial growth factor (EGF)-induced reduction in iNOS mRNA levels. On the other hand, NO has been shown to act as an antiproliferative agent (Kuzin et al., 1996) and to inhibit mitosis (Takagi et al., 1994) in other mouse cell types. In contrast, a growth-promoting effect of NO is supported by the observation (Hattori et al., 1996) that NO increases EGF receptors in rat granulosa cells and IL-1 $\beta$ -stimulated NO production is effective in promoting muscle cell growth in presence of basic fibroblast growth factor (Dubey et al., 1997). However, treatment of bovine granulosa cells from different size follicles with the NO donor SNAP does not influence proliferation (Basini et al., 1998). The above findings provide evidence that the exact role played by NO in the regulation of cell growth is yet to be elucidated. It is feasible that effects of NO are strongly dependent on interactions with other growth modulatory factors acting within the ovary.

A further mechanism through which NO may be involved in the control of follicular development is its effects on apoptosis, the programmed cell death by which the majority of ovarian follicles are lost during postnatal life (Kiess and Gallaher, 1998; Li et al., 1998). High NO levels have been shown to reduce apoptosis in both swine (Ponderato et al., 2000) and bovine (Basini et al., 1998) granulosa cells, whereas an opposite effect has been induced by low NO levels in more differentiated granulosa cells (from large follicles). A protective NO effect has been also observed in rat granulosa cells from immature (Matsumi et al., 1998b; 2000) and pre-ovulatory (Yoon et al., 2002) follicles. In addition, the IL-1 $\beta$ -induced antiapoptotic effects have also been reported to be NO-mediated (Chun et al., 1995). Different data have been reported by Sugino et al. (1996), whose findings in human granulosa cells do not clearly confirm NO involvement in the regulation of apoptosis. Pro- and antiapoptotic properties have also been attributed to NO in other cell types (Mannick et al., 1994; Kim et al., 1999), and possibly depend on its concentrations as well as on its possible interactions with a variety of molecules (irons, thiols, proteins, etc.) (Chung et al., 2001).

NO may also influence follicle development by mediating the effects of gonadotropins on the blood-follicle barrier, thus influencing its permeability to different substances (Powers et al., 1995).

The overall results on the effects of NO on folliculogenesis suggest that locally produced NO contributes to modulate follicle development and possibly prevents apoptosis, at least at low concentrations, whereas high levels may promote cell death via peroxynitrite formation.

### *Nitric Oxide and Ovulation*

The ovulatory process depends on a coordinated activity of gonadotropins and steroid hormones, as well as mediators involved in inflammatory reaction, such as cytokines, prostaglandins, leukotrienes, and so forth. Results from recent studies suggest an involvement of the NOS/NO system in ovulatory mechanism(s), mainly via its effects on vasculature and prostaglandin production. Local administration of iNOS inhibitors has been reported to suppress the ovulatory process in rat, an effect reversed by sodium nitroprusside (Shukovski and Tsafiri, 1994). Similar results have been reported in hCG-treated rabbits (Hesla et al., 1997) and the systemic administration of NO blockers inhibits ovulation and suppresses the positive effect of IL-1 on LH-induced ovulation rate (Bonello et al., 1996). The role of eNOS in ovulation seems more important than that of iNOS (Mitsube et al., 1999), even though the results are still conflicting (Faletti et al., 1999b). In fact, both rat thecal and stromal compartments present high eNOS levels around ovulation (Zackrisson et al., 1996). Furthermore, eNOS deficiency in the mouse has been shown to be associated with reduced ovulatory potential after

a superovulatory treatment (Hefler et al., 2002) and eNOS knockout females showed a significant reduction in hCG-induced ovulation (Jablonka-Shariff and Olson, 1998). A possible mechanism by which NO stimulates the ovulatory process involves the production of prostaglandins (which contribute to enhancing the inflammatory process in the periovulatory period) by a direct activation of cyclooxygenase (Salvemini, 1997). A cross talk between the NO and PG biosynthetic pathways, as well as a stimulatory effect of NO on PGF<sub>2 $\alpha$</sub>  production by large bovine follicles, has been recently reported (Basini and Tamanini, 2001). It has been suggested that NO might contribute to follicle rupture by also increasing the intrafollicular pressure (Matousek et al., 2001), either by increasing the vascular flow and the transudation of fluid to the follicular antrum or by stimulating the contractile elements of the ovarian follicle.

Nitric oxide synthesis seems to also be important for oocyte maturation because eNOS knockout mice exhibited a reduced number of oocytes in metaphase II of meiosis—a high percentage of oocytes remained in metaphase I or were atypical compared to controls (Jablonka-Shariff and Olson, 1998; 2000). Furthermore, in the same species, SNP has been demonstrated to stimulate meiotic maturation to metaphase II stages in cumulus enclosed oocytes (Sengoku et al., 2001). Conflicting data have reported a possible relationship between NO concentration in follicular fluid and oocyte quality and developmental competence (Barroso et al., 1999; Lee et al., 2000).

### *Nitric Oxide, Luteal Function, and Luteal Regression*

Much evidence suggests that NO is involved in the regulation of corpus luteum (CL) function and lifespan, but opposing actions have been reported, depending on the stage of CL development. Motta et al. (2001) observed that in the midstage CL in the rat, NO stimulates both glutathione, a major antioxidant, and progesterone production, thus favoring the maintenance of CL; NO, together with PGE, seems to act through its effects on vasculature and proteolytic processes (Hurwitz et al., 1997). Recent findings indicate that iNOS-mediated NO secretion stimulates PGE synthesis, which is effective in increasing progesterone production (Hurwitz et al., 2002). Prostaglandin E has been demonstrated to enhance basal progesterone secretion also in newly formed CL from pseudopregnant rabbits (Boiti et al., 2000). A positive effect of NO on progesterone synthesis and luteal support also has been suggested in the rat by Dong et al. (1997; 1999); they speculated that NO could reduce or prevent luteolytic effects of prostaglandins, thus maintaining adequate progesterone, but the precise mechanisms by which it exerts its effects remain to be elucidated. Nitric oxide is also possibly involved in the control of luteal vascularization. In fact, NO produced by endothelial luteal cells increases blood flow by stimulating arteriolar smooth muscle relaxation and favours angiogenesis through an

increase in vascular endothelial growth factor production by capillary pericytes (Reynolds et al., 2000). The expression of eNOS, as well as total NOS activity, diminishes with CL aging (sheep, Reynolds et al., 2000; rabbit, Gobbetti et al., 1999; Boiti et al., 2002; rat, Motta and Gimeno, 1997), even though different findings have been reported in humans (Devoto et al., 2002).

As above mentioned, NO is also involved in luteolysis, which depends on an oxytocin-mediated prostaglandin release. Evidence exists that shows that oxytocin acts by enhancing NOS activity (Motta and Gimeno, 1997; Motta et al., 1997) and NO stimulates the synthesis of PGF<sub>2α</sub> (human, Friden et al., 2000; bovine, Skarzynski et al., 2000), which in turn increases NOS activity, thus activating a positive feedback mechanism (rabbit, Boiti et al., 2000; rat, Estevez et al., 1999; Motta et al., 2001). At the same time, NO decreases progesterone production (rat, Motta et al., 1999; rabbit, Gobbetti et al., 1999; Boiti et al., 2000; bovine, Skarzynski and Okuda, 2000). Alternative mechanisms by which NO participates in luteal regression involve lowering estradiol production, resulting in the subsequent demise of the CL (Olson et al., 1996), and increasing apoptosis (Vega et al., 2000). In fact, the large amounts of NO induced by iNOS during the late stage of CL are likely to exert a proapoptotic effect.

### Implications

This review provides convincing evidence that nitric oxide is involved in all the ovarian functions and plays a crucial role in reproductive processes, even though most studies have been carried out on rats and humans and very little is known about livestock. Fine-tuning of nitric oxide generation seems to be essential for ovarian physiology; however, the precise mechanisms by which it exerts its effects are not clearly understood and need further investigation. Future studies should also be aimed at verifying whether ovarian dysfunctions are associated with an altered nitric oxide production in order to clarify whether these defects can be corrected by nitric oxide.

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