REPRODUCTION

REVIEW

Nitric oxide in follicle development and oocyte competence

Giuseppina Basini and Francesca Grasselli

Dipartimento di Scienze Medico-Veterinarie, Università degli Studi di Parma, Via del Taglio 10, I-43126 Parma, Italy

Correspondence should be addressed to G Basini; Email: basini@unipr.it

Abstract

Apart from its well-known role in regulating endothelial function, in mammals, nitric oxide (NO) is an important signaling molecule involved in many processes, regulating different biological functions. It has been demonstrated that NO plays a role in the physiology of the reproductive system, where it acts in controlling the activity of reproductive organs in both sexes. In the female of several animal species, experimental data suggest the presence of an intraovarian NO-generating system, which could be involved in the control of follicular development. The role of NO in regulating follicular atresia by apoptosis is still controversial, as a dual action depending mostly on its concentration has been documented. NO also displays positive effects on follicle development and selection related to angiogenic events and it could also play a modulatory role in steroidogenesis in ovarian cells. Both in monovulatory and poliovulatory species, the increase in PGE_2 production induced by NO via a stimulatory effect on COX-2 activity appears to be a common ovulatory mechanism. Considerable evidence also exists to support an involvement of the NO/NO synthase system in the control of meiotic maturation of cumulus–oocyte complexes.

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Introduction

In the late 1970s, it was recognized that the endothelium releases a factor that relaxes vascular smooth muscle cells, thereby causing vasodilatation (Furchgott & Zawadzki 1980), and it was named endothelium-derived relaxing factor (EDRF). Later on, EDRF was simultaneously identified by Ignarro *et al.* (1987) and Palmer *et al.* (1987) as nitric oxide (NO), a colorless and odorless gas. Several lines of evidence have shown that this gas is a fundamental messenger involved in numerous biological processes and the journal 'Science' entitled NO as 'Molecule of the Year' in 1992 while in 1998 NO discovery merited the Noble prize.

This short-lived radical molecule is synthesized by a complex family of NO synthase (NOS) enzymes. It is produced by the oxidation and cleavage of one of the terminal nitrogen atoms of the amino acid L-arginine. Mammalian cells are endowed with three genes encoding distinct isoforms of NOS, NOS1, NOS2, and NOS3, which share a 51–57% homology. These isoforms show different tissue localizations, regulation, and inhibitor sensitivity. NOS1, also known as neuronal NOS (nNOS) (first isolated from neuronal tissue), and NOS3 or endothelial NOS (eNOS) (first found in endothelium) are constitutive and activated by an increase in calcium, thus producing low transient concentrations of NO. On the contrary, NOS2 is an inducible NOS (iNOS) and calcium-independent isoform (Daff 2010). In addition, a mitochondrial NOS was described by Kobzik *et al.* (1995). Since then, several studies were addressed in order to characterize the isoform identity, its regulation, and its involvement in physiological or pathological events (Finocchietto *et al.* 2009, Zaobornyj & Ghafourifar 2012, Geary *et al.* 2014).

NO can be generated independently from NOS by reduction of nitrite, which can occur spontaneously under hypoxic and/or acidic conditions (Cortese-Krott *et al.* 2015). Enzymes such as xanthine oxidase and cytochrome oxidase c can also mediate reduction of nitrite (Zweier *et al.* 1995, Godber *et al.* 2000).

Differently from conventional biosignaling molecules, NO activity is not mediated by its binding to receptors. Instead, NO easily diffuses into cells and exerts its bioactivity directly acting on many signaling pathways (Moncada *et al.* 1991).

Owing to its unpaired electron, NO displays a high reactivity with many biological components (Grisham *et al.* 1999). One of the most physiologically relevant reactions is that with heme proteins, which can result in the formation of stable chemical species. This is particularly important as the activation of guanylate cyclase, the main effector for NO activity, is due to NO binding to heme moiety in the enzyme, thus resulting in an increased cGMP production (Denninger & Marletta 1999).

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NO itself has a short life *in vivo* because of its reactivity with hemoglobin and a broad spectrum of other biological components (Grisham *et al*. 1999). As a free radical, NO can react with other molecules. Moreover, NO may be formed and/or bioactivated as nitroxyl or nitrosonium, which can be stabilized in biological complexes with thiols, nitrite and other intermediates. In addition, several biomolecules can react with NO, thus resulting in nitration (addition of NO_2), nitrosation (addition of NO^+), and nitrosylation (addition of NO) (Moncada *et al*. 1991).

NO acts as an important intra- and inter-cellular messenger adjusting numerous functions, primarily that of the vascular endothelium. Its role in the maintenance of small arteries and basal tone of arterioles is supported by experimental observations documenting an increase in blood pressure resulting from the administration of the NOS inhibitor in different animal species (Chatterjee *et al*. 2008).

The presence of a 'nitrergic' nervous system, composed of NO-releasing nerves previously classified as non-adrenergic, non-cholinergic, has been hypothesized in the cardiovascular, respiratory, and urinary system. In the CNS, NO displays different effects, being involved in the mechanisms of memory formation (Katusic & Austin 2014).

The sustained NO production by iNOS in activated macrophages is important in host defense against infection (Wink *et al*. 2011).

Moreover, on the basis of animal and *in vitro* studies, it has been shown that NO may modulate endocrine system function (Vargas *et al*. 2007).

Therefore, as hormones, neurons, blood vessels, and cells of the immune systems are integral parts of the reproductive organs, it is likely that NO functions (Fig. 1) as an important regulator of the biology and physiology of the reproductive system, where it acts in regulating multiple functions within the female as well as the male reproductive organs.

First of all, NO is involved in the control of gonadotropin secretion both with a direct effect and via a regulatory effect on hypothalamic GnRH release (Bellefontaine *et al*. 2011).

NO is a physiological mediator of erectile function (Yetik-Anacak *et al*. 2014) and testicular cells are well equipped with a NO–cGMP pathway, which may participate in the regulation of testicular functions, such as spermatogenesis and steroidogenesis (Ducsay & Myers 2011). A physiological role for NO in regulating oviduct function and biology of the uterus that has gained intense attention (Toda *et al*. 2013) has been

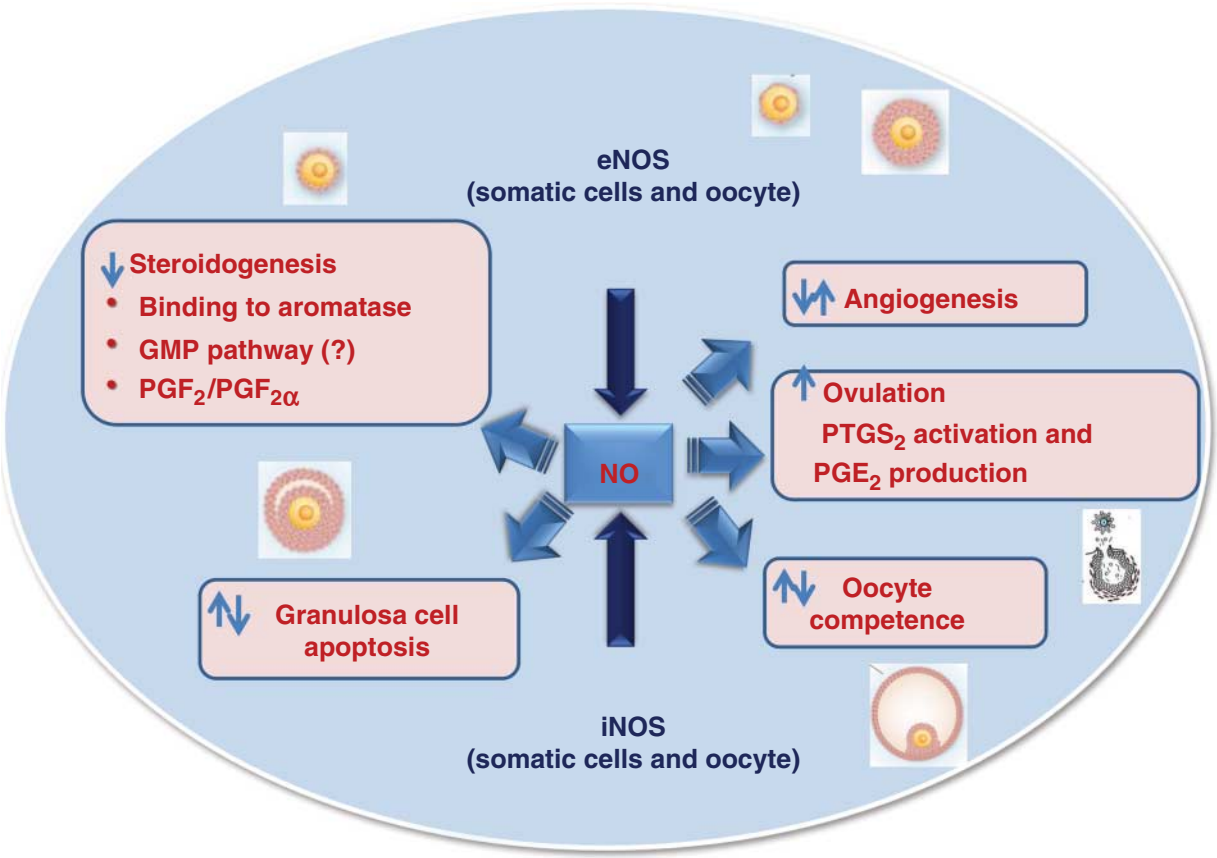

Figure 1 Follicular events controlled by NO.

proposed (Chang & Hsu 2013). As for the ovary, it is still unclear whether these effects are due to NO generated in the vasculature and neurons within the ovary or directly attributable to NO generated by various cells within the ovary. In order to get an insight into these findings, in this review, we will concentrate on the role of NO in the control of ovarian follicle development and oocyte competence.

NO in follicle development

Regulation of NO production

Pituitary gonadotropins are well recognized as key regulators of the final stages of follicular development, but a growing body of evidence underlines the importance of the intrafollicular balance of autocrine or paracrine factors in driving normal follicular growth.

The presence of NO in follicular fluid has been confirmed in several animal species, and the demonstration of NOS expression suggests the presence of an intraovarian NO-generating system and emphasizes its role in the control of follicular development (Fig. 1).

The major regulator of NO production is NOS, which appears in three isoforms: nNOS, eNOS, and iNOS. In the ovary, NO can be generated by several ovarian cells and within the ovarian vasculature; resident macrophages have also been indicated as a possible source of NO (Dave *et al.* 1997). As for the ovarian cells, many studies have been carried out to examine the expression and localization patterns of NOS isoforms in the ovary of different species.

The localization of NOS isoforms in several mammalian ovaries was reported, but the results were not consistent. In the rat, Zhang *et al.* (2011) demonstrated cellular expression and immunolocalization of three different NOS isoforms in the ovary before puberty. In adult rats, several authors assessed the expression of eNOS in granulosa cells, thecal layer, and ovarian stroma (Zackrisson *et al.* 1996, Jablonka-Shariff & Olson 1997, Jablonka-Shariff *et al.* 1999, Nakamura *et al.* 1999, Yamagata *et al.* 2002), while iNOS was localized only in somatic cells of follicle and luteal cells (Jablonka-Shariff & Olson 1997, Tao *et al.* 1997, Yamagata *et al.* 2002). Even though eNOS has been considered a constitutively expressed enzyme isoform, many experimental works document that the pattern of protein and mRNA expression within the ovary is subjected to changes during follicular and luteal phases of the estrous cycle. Gonadotropin stimulation induces an increase in *eNOS* mRNA levels, which are highest during the periovulatory period (Van Voorhis *et al.* 1995), as well as an enhanced protein expression (Jablonka-Shariff & Olson 1997). iNOS expression has also been documented in the rat ovary, but different regulatory mechanisms have been proposed for this isoform.

iNOS mRNA is undetectable in gonadotropin-stimulated ovulatory follicles (Van Voorhis *et al.* 1995) and cannot be induced by follicle-stimulating hormone (FSH) in granulosa cells; in fact, both its expression and activity seem to require IL1 beta stimulation (Tabraue *et al.* 1997). In accordance with these findings, Matsumi *et al.* (2000) documented NO production by iNOS only in immature follicles as well as a decrease in *iNOS* mRNA levels induced by gonadotropin administration. These data would support the hypothesis of a role played by iNOS as a cytostatic factor in the earlier stages of rat follicular development.

In the mouse, Mitchell *et al.* (2004) found that both eNOS and iNOS were expressed in theca and granulosa cells where iNOS occurred predominantly.

Among monovulatory species, NOS expression has been scarcely investigated in humans but eNOS was demonstrated within granulosa–lutein cells (Van Voorhis *et al.* 1994).

In the bovine, Pires *et al.* (2009) demonstrated that eNOS is detectable in theca, granulosa, surface epithelium, and corpus luteum, and that NO is necessary for follicle development. In addition, Moonmanee *et al.* (2013) pointed out a relationship among eNOS expression, vascularization, and mitotic activity in the first follicular wave, thus suggesting a role for eNOS in selection of nonovulatory dominant follicles. Herath *et al.* (2007) did not detect *iNOS* mRNA in ruminant granulosa cells, while Zamberlam *et al.* (2011) documented the expression of iNOS in the same cells and its regulation by FSH and insulin-like growth factor 1, probably mediated by estradiol. These observations would support the hypothesis that, in this species, endogenous NO production could be involved in follicle selection. NO has been detected in ruminant follicular fluids (Basini *et al.* 1998, Khan & Das 2011, EL-Sherry *et al.* 2013), with higher concentrations in small follicle (Basini *et al.* 1998, Khan & Das 2011). Different results were documented by Pancarcı *et al.* (2011) who measured the lowest NO levels in bovine dominant follicles.

Grazul-Bilska *et al.* (2006) reported an increase in eNOS protein expression around ovulation in ewes, suggesting a regulatory role of NO in the ovulatory process. Perifollicular blood flow is positively related to NO concentrations both in the bovine and ovine (Pancarci *et al.* 2012, EL-Sherry *et al.* 2013) ovaries.

A preliminary study on the buffalo (Dubey *et al.* 2012) demonstrated the presence of all the NOS isoforms in the different stages of ovarian follicles, from preantral to ovulatory. NO appears to be involved in follicular development in this species, but the exact definition of its role requires further research.

In the horse, Pinto *et al.* (2003) found that NO is detectable in preovulatory follicular fluids and its concentration increases after administration of hCG, thus suggesting its involvement in the ovulatory cascade.

It appears that, in porcine ovary, eNOS is found more frequently than iNOS. Porcine granulosa cells represent a site of NO production (Grasselli *et al.* 2002); the presence

of *iNOS* mRNA cannot be confirmed in granulosa cells from swine antral follicles (Grasselli *et al*. 2001, Takesue *et al*. 2001); *eNOS* mRNA expression in cultured porcine granulosa cells has been shown to depend upon FSH stimulation (Takesue *et al*. 2001). Physiological hypoxia taking place during follicle growth could be an important factor inhibiting NO synthesis in swine granulosa cells (Basini *et al*. 2004). In addition, it has been demonstrated that NO production by swine granulosa cells is modulated by physiological peptides (Basini *et al*. 2011, 2014) as well as by endocrine disruptors (Santini *et al*. 2009, Basini *et al*. 2012).

Role of NO in the control of follicular growth and atresia

In the mammalian ovary, more than 99% of ovarian follicles undergo a degenerative process called atresia. Follicular atresia is a selective process during follicular growth that involves granulosa cell death by apoptosis. Many researchers described mechanisms that regulate apoptotic cell death during follicular atresia. An involvement of NO in modulating these events has been postulated as this molecule appears to be involved in controlling cell growth and death in several cell types. In the follicle, the role of NO in these events is controversial as it can be toxic or protective mostly depending on its concentration. This dual effect has been clearly evidenced in the buffalo by Dubey *et al*. (2011) and in the bovine by Basini *et al*. (1998). Several studies (Matsumi *et al*. 1998, Yoon *et al*. 2002, Chen *et al*. 2005) point out a protective effect of NO vs apoptosis in rat follicles. This finding has also been reported in human granulosa cells (Dineva *et al*. 2008). Sugino *et al*. (1996) postulated that internucleosomal DNA cleavage resulting in DNA fragmentation could be mediated by NO in small follicles but not in large ones. Large follicles probably possess a tonic inhibitory system suppressing apoptotic DNA cleavage.

Data supporting NO antiapoptotic effects have been documented by Zamberlam *et al*. (2011), who hypothesized that increased NO levels in the bovine dominant follicle would inhibit FasL-mediated apoptosis.

On the contrary, in the chicken, NO appears to be involved in follicle regression (Sundaresan *et al*. 2007).

Role of NO in the control of follicular steroidogenesis

The steroidogenic pathway within the follicle gives rise to progestins, androgens, and estrogens, all of them acting via specific nuclear receptors to regulate reproductive functions and to maintain fertility. As sex steroids play an important role in the growth and differentiation of reproductive tissues, different factors that impair their production usually compromise fertility.

NO has been demonstrated to inhibit follicular steroidogenesis in rats (Dave *et al*. 1997, Shahpar *et al*. 2007), human (Van Voorhis *et al*. 1994, Rosselli *et al*. 1998, Tobai & Nishiya 2001), bovine (Basini *et al*. 1998, Basini & Tamanini 2000, Faes *et al*. 2009), buffalo (Dubey *et al*. 2011), and swine (Masuda *et al*. 1997, Matsumi *et al*. 2000, Ponderato *et al*. 2000, Grasselli *et al*. 2001). NO exerts its effects by binding to the prosthetic heme group of enzymes. Thus, NO may directly bind to P450 aromatase, a key enzyme in the steroidogenic pathway (Hanke *et al*. 1998). The activation of soluble guanylate cyclase is another mechanism of steroid inhibition mediated by NO. However, conflicting results have been obtained in different species, possibly owing to different culture conditions. In fact, in cultured granulosa cells from mice (Ishimaru *et al*. 2001) and pigs (Grasselli *et al*. 2001), the NO/cGMP pathway has been suggested as one of the mechanisms used by NO to inhibit steroidogenesis, while this effect appear to be cGMP independent in human (Van Voorhis *et al*. 1994) and bovine (Basini *et al*. 2000). In addition, Basini & Tamanini (2001) suggest that the inhibitory effect of NO on bovine granulosa cell steroidogenesis could be at least partially mediated by PGE_2 and $PGF_{2\alpha}$ and a crosstalk between NOS and COX metabolites can be hypothesized.

Role of NO in the control of follicular angiogenesis

Follicle development is dependent on the establishment and continual remodeling of a complex vascular system. This enables the follicle to receive the required supply of nutrients, oxygen, and hormonal support as well as facilitating the release of steroids. During the transition from avascular primary follicle to a vascular secondary follicle, angiogenesis occurs in the theca layer but the mechanisms by which the secondary follicle becomes endowed with vasculature remain unclear. This transition may be due to local transformation of mesenchymal cells into endothelial cells or active migration of endothelial cell precursors from preexisting blood vessels. Apart from the effect of NO on follicular blood flow demonstrated in the rat (Griffith 1994, Zackrisson *et al*. 2000, Mitsube *et al*. 2002) and human (Zackrisson *et al*. 1996), suppressive effects of NO on angiogenesis in the bovine follicle have been postulated by observing a negative relationship between VEGF and NO levels in porcine granulosa cells (Grasselli *et al*. 2002).

The modulatory role of NO on the follicular angiogenic process appears still controversial at the present time, as positive effects of NO on follicle development and selection related to angiogenic events have been instead demonstrated in bovine (Grazul-Bilska *et al*. 2007, Tessaro *et al*. 2011), horse (Pinto *et al*. 2003), and sheep (Seekallu *et al*. 2010).

Role of NO on follicular prostaglandin secretion and ovulation

The ovulatory cascade is triggered by a surge of luteinizing hormone (LH), which induces a molecular machinery in which NO exerts a crucial role at least in rodents (Shukovski & Tsafriri 1994) and rabbits (Hesla *et al*. 1997), mainly increasing prostaglandin production. The prostaglandin-endoperoxide synthases (PTGS) are the key enzymes that mediate the synthesis of prostaglandins, PTGS1, constitutively expressed in most cells involved in maintaining homeostatic functions, and PTGS2, very low under normal physiological conditions but rapidly induced by several stimuli and known to exert a pivotal role in ovulation (Sugimoto *et al*. 2015). NO is able to induce PTGS2 expression in several cell types.

The involvement of NO in monovulatory species ovulation was demonstrated for the first time in sheep (Grazul-Bilska *et al*. 2006). More recently, both in bovine (Zamberlam *et al*. 2014) and human (Fang *et al*. 2015) the central role of NO in stimulating PTGS2 activation and PGE_2 production has been confirmed.

Therefore, taken together, the increase in PGE_2 production induced by NO via a stimulatory effect on PTGS2 activity appears to be a common mechanism in both monovulatory and poliovulatory species.

NO in oocyte competence

The mechanisms involved in the regulation of meiotic cell cycle in oocytes are not fully understood yet, but considerable evidence exists to support an involvement of the NO/NOS system in the control of meiotic maturation of cumulus–oocyte complexes. NO is now thought to represent a vital component of the oocyte microenvironment as it plays a physiological role during oocyte maturation, fertilization, and beginning of embryo development (Jablonka-Shariff & Olson 1998, Sengoku *et al*. 2001, Bergandi *et al*. 2014).

eNOS and iNOS expression has been documented in mammalian oocytes (mice: Mitchell *et al*. (2004); rat: Jablonka-Shariff & Olson (1998); cattle: Tesfaye *et al*. (2006); Pires *et al*. (2009); pig: Chmelíková *et al*. (2009)) and their presence was confirmed throughout folliculogenesis and follicle maturation (Chmelíková 00000000000*et al*. 2009, Pires *et al*. 2009). As for nNOS, its presence in oocytes has been documented only in the pig (Chmelíková *et al*. 2009), and its mRNA in the mouse (Abe *et al*. 1999).

Further confirmation of NO role in oocyte maturation comes from studies on *eNOS* knockout mice (Jablonka-Shariff & Olson 1998), which display an impairment of ovulation and a higher percentage of atypical oocytes. Recently, Goud *et al*. (2014) have confirmed their previous observations about the role played by NO in delaying mouse oocyte aging. The inhibition of NO synthesis during IVM decreases the number of blastocysts (Matta *et al*. 2009) and increases apoptosis in embryos (Schwarz *et al*. 2010). On the other hand, high NO levels have been reported to impair meiotic progression and embryonic development in cattle (Schwarz *et al*. 2008). Other evidence exists that NO donors would prevent or delay meiotic resumption in the rat (Nakamura *et al*. 2002, Bu *et al*. 2004, Sela-Abramovich *et al*. 2006), cattle (Schwarz *et al*. 2014), and pigs (Tao *et al*. 2005), while iNOS-specific inhibitors induce meiotic resumption (Nakamura *et al*. 2002). Recently, Goud *et al*. (2014) have confirmed that NO plays a significant role in maintaining oocyte quality. The controversial effects reported in these studies would suggest that NO can play a dual function in oocyte maturation, as already highlighted with regard to its modulatory role on follicular function (Tamanini *et al*. 2003). Bu *et al*. (2003) documented NO paradoxical effects on mouse oocyte maturation depending on its concentration: eNOS-derived NO from cumulus cells stimulates meiotic maturation of mouse oocytes at low doses, while a milieu of high concentrations of NO would maintain the meiotic arrest of oocytes (Nakamura *et al*. 2002). The decrease in NO after LH preovulatory surge may be a key factor for meiosis resumption: as a consequence of the activation of MAPK, the disruption of gap junctional communication would stop the transfer of inhibitory substances from granulosa cells to the oocyte, enabling it to resume meiosis (Sela-Abramovich *et al*. 2008).

Different findings indicate that the oocyte itself possesses the ability to produce adequate NO levels through iNOS-mediated pathway required for the maintenance of meiotic arrest at diplotene stage (Tripathi *et al*. 2010).

NO levels thus appear as a critical factor in cell survival and physiology. In a study on the effect of varying NO concentrations on bovine oocyte nuclear maturation, Bilodeau-Goeseels (2007) reported that germinal vesicle breakdown in cumulus-enclosed oocytes was prevented or stimulated by high or low doses of the NO donor SNP respectively. These observations would suggest that NO reduction, possibly linked to transcript reduction in eNOS, is necessary for germinal vesicle breakdown (Tesfaye *et al*. 2006, Pires *et al*. 2009) and meiosis resumption.

As for NO signaling pathways, cGMP has been documented as a crucial factor in maintaining the meiotic arrest in oocytes (Nakamura *et al*. 2002), but the role played by NO in the cGMP/cAMP pathway during meiosis resumption is not completely known and still a matter of study. Schwarz *et al*. (2014) have recently confirmed that the progression of meiosis in bovine oocytes is linked to the inactivation of the NO/guanylate cyclase/cGMP pathway, but did not observe a significant involvement of cAMP levels in oocyte maturation. Different results have been reported by Bilodeau-Goeseels (2007) in the same species, who hypothesized that the NO-induced reduction of germinal vesicle

breakdown rate would not be exerted via sGC/sGMP. Abbasi *et al*. (2009) suggested that the stimulatory effect of NO on mice oocyte meiotic resumption would involve cAMP, while the cGMP pathway would be involved in mediating the inhibitory effect of NO. High cGMP levels produced by iNOS-derived NO could maintain meiotic arrest of preovulatory oocytes via two different pathways. One would involve inhibition of oocyte cAMP phosphodiesterase to maintain cAMP levels, and a second one the activation of cGMP-dependent protein kinase (Törnell *et al*. 1991).

Concluding remarks

After 1987, when EDRF was identified as NO, this simple molecule has been shown to be involved in many physiological functions. The demonstration of NO effects on neurons, endothelial cells, immune cells, and endocrine cells qualifies this molecule as a regulator of ovarian follicle, a structure that comprises all these cell types. In the follicle, NO controls the main functional activities such as growth or atresia, angiogenesis, steroidogenesis, and ovulatory events. In addition, NO has been shown to represent a vital component of the oocyte microenvironment, where it plays a physiological role during oocyte maturation and the acquisition of competence. Therefore, the ability to manipulate the players of the NO system could represent a promising tool to interfere with the follicular growth dynamics and ovulation.

Declaration of interest

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

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