The role of nitric oxide in parthenogenetic activation of pig oocytes: A review

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ABSTRACT: Parthenogenetic activation of mammalian oocytes with artificial stimuli is commonly applied in various reproductive biotechniques, e.g. cloning using nuclear transfer. For this reason, many studies focus on oocyte activation *in vitro*. Recently we have described the activation of pig oocytes using nitric oxide. This activating stimulus is very specific in many aspects. However, it does not provide an adequate stimulus for parthenogenetic development. It was shown that nitric oxide stimulated some signalling pathways which are inactive in conventional treatments for parthenogenetic activation, e.g. the cGMP-dependent signalling cascade. On the other hand, nitric oxide does not stimulate certain signalling pathways involved in oocyte activation after calcium ionophore, e.g. the PKC signalling cascade. The aim of this review is to characterize the complex processes induced in oocytes after treatment with nitric oxide. Perspectives for further research and the application of nitric oxide for parthenogenetic activation of oocytes are outlined.

Keywords: oocyte; parthenogenesis; activation; nitric oxide

Oocyte activation is crucial for many topics including cloning using nuclear transfer. Successful development of the cloned embryo depends, apart from other factors, on an adequate activating stimulus (Samiec, 2004; Samiec and Skrzyszowska, 2005a,b) which starts embryonic development. For this reason, there is a very urgent need to develop an activation treatment which perfectly matches the physiological events occurring in the oocyte after fertilization by the sperm. This is a very strong motive for an investigation of the mechanisms regulating parthenogenetic activation. New knowledge in this field is used for the development of new treatment protocols which could induce *in vitro* events in the oocytes that are typical of oocytes fertilized *in vivo*. However, this aim is still to be investigated.

Matured mammalian oocytes spontaneously block their meiotic maturation at the stage of metaphase II. Further progress in meiosis beyond this spontaneous block and oocyte activation is dependent on the activating stimulus. Without this stimulus, the mammalian oocytes underwent spontaneous death through the process referred to as ageing (Petrová et al., 2004, 2005). Under natural conditions, this stimulus is brought into the oocyte by the sperm (Yanagimachi, 1988). The nature of this activating stimulus is not known in detail. The sperm induces oscillations of intracellular levels of free calcium ions in a fertilized egg, and that is the reason why the activation is commonly presented as a calcium-dependent process (Homa et al., 1993; Swann and Ozil, 1994). Calmodulin (Lorca et al., 1991; Xu et al., 1996) and calmodulin-dependent kinase II (Lorca et al., 1993) is clearly involved in this process because it induces inactivation and disintegration of the molecules which are responsible for the matured oocyte remaining at the stage of metaphase II.

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The majority of the current systems for parthenogenetic activation of mammalian oocytes *in vitro* exploits the calcium-dependent signalling cascade. Treatments increasing intracellular calcium levels, e.g. electric stimulus (Procházka et al., 1992, 1993), ionophores (Jílek et al., 2000, 2001), ethanol (Petr et al., 1996) or chemicals modulating the intracellular turnover of calcium ions (Petr et al., 2000, 2002) are often used for this purpose. Some activating protocols are based on the inhibition of certain protein kinases which act downstream on calcium-dependent signalling pathways. These are for example protocols based on the inhibition of kinases inactivated during fertilization through calcium-dependent events (Mayes et al., 1995; Prather et al., 1997; Motlík and Kubelka, 2001).

On the other hand, Kuo et al. (2000) presented nitric oxide as an intracellular signal triggering the oocyte activation. Nitric oxide (NO) represents a very important signalling molecule. It is generated in various cells by nitric oxide synthase (NOS) from L-arginine (Kwon et al., 1990; Lamas et al., 1992; Herrero and Gagnon, 2001). NOS is expressed in three isoforms which are dimmers of identical subunits (Lamas et al., 1992; Xie et al., 1992). Constitutional isoforms of NOS, neuronal NOS (nNOS) and endothelial NOS (eNOS) are calcium- and calmodulin-dependent and produce small amounts of NO for a short period of time (Lamas et al., 1992; Nathan, 1992). An inducible isoform of NOS (iNOS) produces a stable amount of NO for a much longer period (Moncada et al., 1991; Nathan, 1992), and this NO-production is independent of calcium or calmodulin (Bian and Murad, 2003). The cells can express more than one isoform of NOS (Mehta et al., 1995).

NO plays a key role in many physiological processes including reproduction (Biswas et al., 1998). Besides many other events, NO is involved in the regulation of follicle growth and ovulation (Sengoku et al., 2001), spermatogenesis (Zini et al., 1996), and embryo implantation in the uterus (Moncada et al., 1991).

NO also plays an important role in oocyte maturation. The importance of NOS for meiotic maturation is confirmed by the expression of NOS isotypes in mouse and rat oocytes (eNOS, iNOS – Zackrisson et al., 1996; Jablonka-Shariff and Olson, 1997; Jablonka-Shariff et al., 1999; Nishikimi et al., 2001; Mitchell et al., 2004 or nNOS mRNA – Abe et al., 1999) or in pig oocytes (eNOS – Hattori et al., 2000, 2001; Takesue et al., 2001), and further confirmation of this comes from studies on mice with gene-knockout for eNOS. During maturation, oocytes of these mice exhibited blockage at the stage of metaphase I, or they exhibited various abnormalities (Jablonka-Shariff and Olson, 1998).

The role of the NO-dependent signalling cascade in the activation of mammalian oocytes is not clear and it is subjected to very intensive research. However, the NO-dependent signalling cascade does not represent a primary stimulus for oocyte activation. This was shown in studies which demonstrated a sharp increase in intracellular levels of free calcium ions preceding an increase in intracellular levels of NO in activated sea urchin eggs (Leckie et al., 2003) or activated tunicate and mouse oocytes (Hyslop et al., 2001). Nevertheless, it is concluded from the above-mentioned studies that the NO-dependent signalling cascade is functional in oocytes and is activated during oocyte activation after fertilization or parthenogenesis. This is not surprising with respect to data indicating the interconnection of both signalling cascades in somatic cells (Berridge et al., 2000).

However, is this really the case? To what extent are the two signalling cascades independent or interconnected? What are the potentials for exploiting the NO-dependent signalling cascade for artificial activation of mammalian oocytes? The present review focuses on these questions and summarizes current knowledge of the role of NO in mammalian oocyte activation.

Does nitric oxide activate mammalian oocytes?

The evidence for the involvement of NO-dependent signalling cascade in the activation of mammalian oocytes was given by Petr et al. (2005a), who successfully activated pig oocytes matured *in vitro* using NO-donors. Our observation of NO-dependent activation of pig oocytes was further supported by experiments during which oocytes were microinjected with eNOS and calmodulin. We observed oocyte activation only when eNOS was injected together with calmodulin. Calmodulin is a very potent activator of all three isoforms of NOS – eNOS, nNOS or iNOS, but its effect is more profound in the constitutive isoforms eNOS and nNOS (Alderton et al., 2001). In our experiments, microinjected calmodulin was unable to elicit a response through the activation of oocyte-endogenous NOS,

because when microinjected alone, calmodulin did not induce any oocyte activation. The specific effect of microinjected eNOS was demonstrated by its inhibition using L-NMMA, which inhibits all three isotypes of NOS (Alderton et al., 2001), or by inhibition using L-NAME, which also inhibits all NOS isoforms, but has the highest specificity to eNOS (Moore et al., 1990).

NOS inhibitors L-NMMA or L-NAME were able to block the activation of pig oocytes even after oocyte treatment with the NO donor SNAP (Petr et al., 2005a). This is a quite surprising observation because the blockage of endogenous NOS should be compensated by NO released from the NO donor SNAP. Moreover, elevated NO levels are able to suppress the activity of NOS (Cooper, 1999; Ravi et al., 2004). On the basis of data from the abovementioned experiments it is concluded that in addition to the presence of NO, the activity of NOS is also necessary for the activation of the NO-dependent signalling cascade. However, the precise role of NOS could be only speculated. One of the possible explanations is the involvement of NOS in effective intracellular NO-dependent signalling.

The NO-induced parthenogenetic activation of pig oocytes did not exhibit the full spectra of events which are typical of the oocyte fertilized by the sperm and even of the oocyte activated using other treatments (e.g. electrical stimuli or calcium ionophores). Oocytes treated with a NO-donor did not exhibit the exocytosis of cortical granules. The sperm penetration is followed by the exocytosis of cortical granules within 6 hours (Yoshida et al., 1993). After parthenogenetic activation, the exocytosis of cortical granules was weaker than after the sperm penetration (Wang et al., 1998b), but many types of artificial activating stimuli are able to induce the exocytosis of cortical granules (Sun et al., 1992; Machatý et al., 1996, 1997; Okada et al., 2003). Cortical granule exocytosis followed very quickly after the activating stimulus (Wang et al., 1999). The exocytosis of cortical granules does not always accompany oocyte activation by artificial activating stimuli, and this was the case, for example, when activation had been induced by the inhibition of protein kinases (Mayes et al., 1995). These observations are in agreement with those of Sun et al. (1997), who proved that the activation of oocytes and the exocytosis of cortical granules were independent processes. Quite a long exposure to an NO-donor (10 hours) is necessary for the activation of pig oocytes. In this respect, the NO-dependent oocyte activation differs from activating protocols based on the elevation of intracellular levels of free calcium ions, which is successful even after a short-term treatment (several minutes) with calcium-enhancing drugs (e.g. Machatý et al., 1997; Jílek et al., 2000, 2001). On the other hand, several hours of treatment are needed when the activation of pig oocytes is induced using cyclopiazonic acid, which elevates the intracellular calcium levels through the inhibition of calcium-dependent ATPases acting as intracellular sarco(endo)plasmic Ca^{2+} (Petr et al., 2000).

One of the factors involved in the slow effect of NO on oocyte activation could be the penetration of NO-donors to the oocytes. It is also possible that a continual exposure of oocytes to the NO-donor did not enable optimal intracellular signalling. This possibility is supported by our unpublished experiments during which a repeated short-term exposure of pig oocytes to the NO-donor resulted in a significant activation rate even when the total time of exposure to the NO-donor did not exceed 2 hours.

The inadequacy of an activating stimulus after NO-donor treatment is also indicated by poor cleavage which does not proceed beyond the 4-cell stage after activation by the NO-donor (Petr et al., 2005a). In addition to the inadequacy of an activating stimulus exhibited by the NO-donor, the very low cleavage rate observed in our experiment could also be due to other reasons. We cannot exclude the blockage of the embryonic cell cycle through NO-dependent events because NO is known as a regulator of the mitotic cell cycle (Tanner et al., 2000; Gibbs, 2003; Valenti et al., 2003), and it is able to suppress proliferation in somatic cells (Mooradian et al., 1995; Janssens et al., 1998).

We can conclude that NO is able to induce parthenogenetic activation in mammalian oocytes. This stimulus is not adequate and current protocols for the treatment of oocytes with NO-donors are not suitable for reproductive biotechnologies. It is necessary to look for other types of activating stimuli. There is a possibility of exposing oocytes to an NO-donor repeatedly for a short time and of imitating the natural pulsatile pattern which is obvious when NO-levels are followed in fertilized oocytes. Another possibility is to combine the NO-donor treatment with the effect of other molecules acting downstream on the NO-dependent signalling cascade or on other signalling cascades involved in the activation of mammalian oocytes.

We intended, for example, to combine the treatment of oocytes with the NO-donor and inhibitors of protein synthesis, kinase inhibitors of kinase activators. Such a combined treatment improved the results after oocyte activation with ionophore (Jílek et al., 2000, 2001; Ito et al., 2003) or ethanol (Petr et al., 1996).

What role is played by calcium in NO-dependent oocyte activation?

The observed interconnection between the calcium-dependent and NO-dependent signalling cascade (Hyslop et al., 2001; Leckie et al., 2003) attracts one's attention to the role of calcium during the NO-induced activation of mammalian oocytes. Petr et al. (2005b) showed that the NO-induced activation of pig oocytes was prevented after intracellular calcium was bound using the chelator BAPTA-AM. This clearly showed that the oocyte activation using the NO-donor SNAP could not occur without an adequate intracellular calcium store.

The NO-dependent signalling cascade is regulated by calcium through several mechanisms. Production of NO in the cell depends on the activation of NOS by calmodulin, which is itself activated by calcium ions (Lamas et al., 1992; Nathan, 1992). On the other hand, NO influences the levels of intracellular calcium through the regulation of calcium ion channels and pumps which modulate the influx and efflux of calcium between the cell and extracellular spaces (Berridge et al., 2000).

The important role of NO interaction with calcium channels was also confirmed in the study which demonstrated the lack of NO-induced parthenogenetic activation in oocytes treated with verapamil, the blocker of calcium channels (Petr et al., 2005b). Verapamil is known to block L-type calcium channels (Atlas and Adler, 1981). It is not able to completely block the influx of calcium ions into the oocyte, because mammalian oocytes express wide spectra of another type of calcium channels (Lee et al., 2004b). A certain amount of calcium ions can enter the oocyte from the extracellular milieu even after treatment with verapamil. However, L-type calcium channels evidently play an important role in pig oocytes, because their blockage using verapamil induces a block of meiotic maturation (Kaufman and Homa, 1993) and results in an abnormal distribution of intracellular calcium stores (Rozinek et al., 2003). Based on data from the experiment performed by Petr et al. (2005b), it can be concluded that the function of the L-type calcium channel also plays a key role in the parthenogenetic activation of pig oocytes using the NO-donor treatment.

NO could also regulate the mobilization of calcium ions from their intracellular stores. Calcium from this source is released through either ryanodine or inositol triphosphate receptors (Clapham, 1995). The blockage of IP_3R did not prevent parthenogenetic activation in the NO-donor treated pig oocytes (Petr et al., 2005b). This occurred despite the fact that IP_3R-inhibitors – heparin or xestospongin C – were used in concentrations which were able to prevent the parthenogenetic activation of the pig oocytes using inositol triphosphate (Petr et al., 2002). The dose of xestospongin C also seems to be sufficient to block IP_3R, because we used xestospongin C at concentrations which were effective for the blockage of calcium release in mouse oocytes (Kang et al., 2003). On the other hand, the blockage of RyRs prevented the parthenogenetic activation of pig oocytes using the NO-donor. This indicates that the NO-dependent activation of pig oocytes depends on the mobilization of intracellular calcium stores from RyR but is independent of the mobilization of calcium stores from IP_3R. The important role of RyR could be due to the activation of these receptors after their S-nitrosylation by NO (Xu et al., 1998).

The importance of RyR for oocyte activation is not clear. RyR were not found in oocytes of some species (hamster – Miyazaki et al., 1992) or are thought to be of negligible importance in others (cattle – Yue et al., 1995). However, RyR can be very important in the activation of pig oocytes, because the stimulation of these receptors resulted in significantly increased intracellular calcium levels (Machatý et al., 1997). Moreover, the stimulation of RyRs is clearly able to induce the parthenogenetic activation of matured pig oocytes, although the development of eggs activated according to this protocol is very low (Petr et al., 2002).

Contrary to the situation in RyRs, IP_3Rs are thought to be very important in the proper activation of mammalian oocytes. Petr et al. (2002) demonstrated that during parthenogenetic activation in pig oocytes, the inositol triphosphate-sensitive calcium stores were mobilized first and their stimulation triggered the mobilization of calcium from ryanodine-sensitive stores. This arrangement

was described as a two-store model by Tesarik and Sousa (1996) in human oocytes.

The parthenogenetic activation of pig oocytes using a NO-donor seems to be triggered by the mobilization of calcium from RyRs. This indicates that the signalling cascade relevant to the two-store model is activated only partially at the level of RyRs and the higher level of IP_3R is bypassed. The developmental competence of pig parthenogenetic embryos is much higher in embryos developing after the stimulation of IP_3Rs than in embryos developing after the stimulation of RyRs (Petr et al., 2002). With this fact in mind it is interesting to note that the development of parthenogenetic embryos generated using an NO-donor treatment is very poor and is comparable to the development of embryos generated using the stimulation of RyRs bypassing the stimulation of IP_3Rs (Petr et al., 2005b).

Based on the above-mentioned data, we can state that the activation of mammalian oocytes using NO depends on the calcium ions which are released to the oocyte cytoplasm through RyR. This at least partially explains the inadequacy of the activating stimuli given by NO-donors, because calcium release through RyR seems to be inadequate for the full oocyte activation. This finding opens up new vistas for the development of new activating protocols which would combine NO-donor treatment with the action of molecules normalizing the signalling of calcium ions, e.g. through the stimulation of IP_3R. At the same time, the new hypothesis is open to testing. This hypothesis states that the stimulation of the NO-dependent signalling cascade could improve the efficiency of treatments based on the stimulation of calcium-dependent signalling cascades, e.g. after combined treatment with the calcium ionophore and NO-donor.

Which processes are specific to NO-induced oocyte activation?

In many types of somatic cells (e.g. smooth muscle cells, platelets or endothelial cells), numerous NO-effects are mediated by soluble guanylate cyclase (sGC) and by the synthesis of cyclic guanosine monophosphate (cGMP) (Murad, 1994; Friebe and Koesling, 2003). sGC and cGMP also play an important role in the ovary (Grasselli et al., 2001; LaPolt et al., 2003; Shi et al., 2004) and it was proved that they are also involved in the regulation of meiosis (Tornell et al., 1984, 1990).

Petr et al. (2006) demonstrated that the activation of *in vitro* matured pig oocytes via the NO-dependent signalling pathway was mediated through cGMP. This suggestion is supported by an observation that sGC inhibitors effectively suppressed oocyte activation after the treatment of oocyte with an NO-donor and this is further supported by an observation that 8-Br-cGMP – an analogue of cGMP more resistant to phosphodiesterase – enhances the effects of a suboptimal dose of the NO-donor SNAP on oocyte activation. With respect to the fact that the cGMP-dependent protein kinase G is one of the main target systems of the signalling molecule of cGMP (Scott, 1991), the role of cGMP in the activation of pig oocytes induced by an NO-donor was further emphasized by an observation that the inhibition of cGMP-dependent kinase inhibits oocyte activation (Petr et al., 2006).

The key role of the cGMP-dependent signalling pathway for parthenogenetic activation seems to be specific to oocyte activation using the NO-donor, because the inhibition of cGC or PKG in oocytes treated with calcium ionophore did not prevent parthenogenetic activation. However, NO-dependent intracellular signalling could be triggered even after oocyte treatment with calcium ionophore, because Hattori et al. (2004) described the increased NO-production in pig oocytes treated with calcium ionophore. Despite this fact, NO is not necessary for ionophore-induced activation because, contrary to NO-induced activation, the inhibition of NOS did not prevent the parthenogenetic activation of oocytes treated with calcium ionophore (Petr et al., 2007).

The molecular mechanism of NO-dependent activation of pig oocytes remains unclear. In mammalian oocytes, the blockage of meiosis at metaphase II is induced by the activation of a complex formed by cyclin B and cyclin-dependent kinase Cdc2 and by stabilization of this complex. These events are dependent on the Mos-MAP kinase signalling pathway, which induces the inhibition of the anaphase-promoting complex/cyclosome (APC/C) (Kishimoto, 2003). The maintenance of this blockage is dependent on the action of Emi1 proteins, which protect APC/C against binding to its substrate (Reimann and Jackson, 2002; Tung et al., 2005). Exit from the stage of metaphase II is dependent on the destabilization of the Cdc2-cyclin B complex and cyclin destruction (Kishimoto, 2003).

As we already mentioned, these processes are generally considered to be calcium-dependent and NO-dependent signalling is interconnected with these processes. This interconnection of NO- and Ca-dependent signalling pathways can be mediated via cGMP and cGMP-dependent protein kinase through several processes.

Protein kinase G phosphorylates and activates the ADP-ribosyl cyclase, which synthesizes cADP ribose, an agonist of ryanodine receptors (Lee, 1996a,b; Graeff et al., 1998) releasing free calcium ions from intracellular calcium stores (Clapper et al., 1987; Galione et al., 1991). This signalling pathway is functional in sea urchin eggs, because the increase in intracellular NO levels induces an increase in cGMP and subsequently induces the increase in intracellular levels of free calcium ions through de novo synthesis of cyclic ADP ribose (Sethi et al., 1996; Willmott et al., 1996). On the other hand, it was demonstrated by Lee and Yang (2004) that the cGMP-mediated calcium release was not required for the rise of calcium during fertilization. However, there are some data demonstrating the ability of cADP ribose to induce the parthenogenetic activation of oocytes. The microinjection of cADP ribose induced the elevation of intracellular levels of free calcium ions in pig oocytes (Machatý et al., 1997) and this treatment induces the activation of pig oocytes and their subsequent parthenogenetic development (Petr et al., 2002).

In addition to cADP ribose, an endogenous ryanodine agonist, protein kinase G can also regulate the levels of the endogenous agonist of the inositol triphosphate receptor. It inhibits the activity of phospholipase C, which hydrolyzes phosphatidyl inositol 4, 5 diphosphate to inositol 1, 4, 5-triphosphate (IP_3) and diacylgylcerols (DAG) (Rapoport, 1986; Ruth et al., 1993; Wang et al., 1998a). This process decreases the intracellular levels of the endogenous IP_3 receptor (IP_3R) agonist, inositol 1, 4, 5-triphosphate. The other product of hydrolysis of phosphatidyl inositol 4, 5 diphosphate – diacylglycerols are known as activators of protein kinase C (Gomez-Fernandez et al., 2004), which also plays an important role in the regulation of mammalian oocyte activation (Quan et al., 2003; Sedmíková et al., 2006). cGMP-dependent kinase is known even to directly regulate the functions of inositol triphosphate receptors through their phosphorylation (Haug et al., 1999). All these effects are worthy of interest, because pig oocytes release free calcium ions after stimulation of their inositol triphosphate receptors (Machatý et al., 1997) and this treatment induced the activation of pig oocytes and their parthenogenetic development (Petr et al., 2002).

The cGMP-dependent signalling pathway can also influence the release of free calcium ions, acting directly on the calcium ion channels. This effect has been described in various types of somatic cells (Cataldi et al., 1999; Jiang et al., 2000; Centonze et al., 2001; Carabelli et al., 2002; D'Ascenzo et al., 2002) and also in oocytes (VanCoppenolle et al., 1997). The effects of protein kinase G close the ion channels through which free calcium ions entered the cell from extracellular spaces (Alioua et al., 1998; Fukao et al., 1999). Various types of these channels are expressed in mammalian oocytes (Lee et al., 2004a) and it is clear that their function is essential for meiosis especially in pig oocytes (Kaufman and Homa, 1993; Rozinek et al., 2003). We cannot exclude a possibility that nitric oxide also regulates the activation of oocytes through processes which does not depend on cGMP.

Based on these data we can conclude that the cGMP-dependent signalling cascade plays an important role in mammalian oocyte activation, but it is not involved in oocyte activation which is induced by the calcium ionophore treatment. This fact opens up a possibility of testing new protocols for an activation treatment based on the traditional stimulation of calcium-dependent signalling cascades combined with a treatment using chemicals stimulating cGMP-dependent kinase or using chemicals imitating the effects of cGMP. The stimulation of the cGMP-dependent signalling cascade could imitate the final effects of stimulation of the NO-dependent signalling cascade and it could improve the results of oocyte activation similarly like a treatment based on the stimulation of both the calcium ionophore and NO-donor.

Which signalling cascades remained silent in NO-induced oocyte activation?

It is obvious that the NO-donor does not trigger all processes necessary for the successful development of a pig oocyte into a viable embryo. One of these missing processes could be the signalling cascade of protein kinase C (PKC), which is known as one of the target systems of the intracellular calcium ion signalling. PKC is therefore considered to be one of the enzymes playing an important role in

oocyte activation (Bement, 1992; Fan et al., 2003; Sedmíková et al., 2006).

Protein kinase C belongs to serine/threonine kinase and its numerous isotypes can be classified into three groups. Conventional PKCs (cPKCs), represented by PKC-α, -β_I, -β_{II} and -γ isotypes, can be activated by free calcium ions and diacylglycerols (DAG). Novel PKCs (nPKCs), represented by PKC -δ, -ε, -η, -μ, and -θ isotypes, are independent of calcium ions, but they can be activated by DAG. Atypical PKCs (aPKCs), represented by PKC -ζ, -λ, and -τ isotypes, are independent of both the calcium ion and DAG (Liu and Heckman, 1998).

The expression of individual PKC isotypes depends on the type of cells and their stage of development (Aderem, 1995). Several authors reported the presence of isotypes PKC-α, -β_I, -β_{II}, -γ, -δ, -ε, -μ, -λ, and -ζ in rodent oocytes at the stage of metaphase II (Gangeswaran and Jones, 1997; Raz et al., 1998; Luria et al., 2000; Pauken and Capco, 2000; Downs et al., 2001; Eliyahu et al., 2001; Eliyahu and Shalgi, 2002; Viveiros et al., 2003) and mRNA for PKC-α, -δ, and -λ (Gangeswaran and Jones, 1997; Raz et al., 1998) although the data on the PKC isotype spectrum and their mRNA are different. Fan et al. (2002a,b) demonstrated PKC-α, -β_I, -γ isotypes in both immature and mature porcine oocytes.

The role of PKC in the activation of oocytes matured to MII was confirmed in experiments with *Xenopus* and mouse oocytes treated with PKC. There occurred processes that were typical of this activation (Bement and Capco, 1991; Colonna et al., 1997; Gallicano et al., 1997). According to other authors, however, the PKC activation does not result in the complete spectrum of processes typical of oocyte activation (Moore et al., 1995; Ducibella and Lefevre, 1997). The effects of individual PKC isotypes were not studied in these experiments, DAG activating all isotypes of cPKC and nPKC are usually used for PKC activation.

In porcine oocytes, PKC was observed to participate in the regulation of the resumption of meiotic maturation (Jung et al., 1992; Coskun and Lin, 1995; Kim and Menino, 1995; Su et al., 1999; Shimada et al., 2001). Fan et al. (2002b) demonstrated a change in the localization of PKC -α, -β_I, -γ in maturing porcine oocytes and after their activation. On the other hand, Sun et al. (1997) did not observe metaphase II transition and meiosis resumption in mature porcine oocytes after PKC stimulation, which indicates that PKC does not trigger these processes. Green et al. (1999) reported that PKC inhibition together with protein kinase A and myosin light chain kinase inhibition resulted in the activation of the porcine oocyte.

A significant role of PKC in the activation of pig oocytes after the calcium ionophore treatment was proved by Sedmíková et al. (2006). Based on the experiment with inhibitors specific to certain isotypes of PKC, they proved that PKC-δ played a key role. This is evident from the fact that bisindolylmaleimide I, which is an inhibitor of both calcium-dependent cPKC and calcium-independent nPKC (isotypes PKC-α, -β_I, -β_{II}, -γ, -δ, -ε), effectively blocks the activation induced in porcine oocytes by the treatment with calcium ionophore A23187. The fact that Go6976 (a specific inhibitor of PKC-α and -β_I) is unable to block the activation of oocytes by ionophore suggested that these cPKCs do not play a significant role in the activation of porcine oocytes induced in this way. The importance of the PKC-β isotype was not confirmed in the experiments in which hispidin (a specific inhibitor of PKC-β) did not affect the oocyte activation by ionophore. On the other hand, the marked effect of rottlerin (a specific inhibitor of PKC-δ) indicates that calcium-independent nPKC may play a significant role in the activation of porcine oocytes. However, it should be considered that all effects of rottlerin need not be due to the specific inhibition of PKC-δ. The PKC-δ-independent effect of rottlerin was demonstrated e.g. in astrocytes (Susarla and Robinson, 2003). Based on *in vitro* studies, it is concluded that rottlerin also inhibits other enzymes, e.g. calmodulin-dependent kinase III (Gschwendt et al., 1994) or p38-regulated/activated protein kinase (Davies et al., 2000).

The role of PKC indicates that signalling pathways in both modes of parthenogenetic activation are markedly different. No significant effect of the PKC inhibitors bisindolylmaleimide I, hispidin, Go6976 on the activation of pig oocytes after NO-donor treatment was observed (Petr et al., 2007). This indicates that the oocyte activation induced by the nitric oxide donor depends on PKC to a very limited extent or is completely independent of PKC. This is also supported by our previous observation that contrary to activation using the calcium ionophore, activation using nitric oxide did not induce the exocytosis of cortical granules (Petr et al., 2005a). There is an agreement that PKC plays a key role in cortical granule exocytosis (Fan

et al., 2002b). The inadequacy of oocyte activation after treatment with the nitric oxide donor is also indicated by the very limited cleavage of resulting parthenogenetic embryos (Petr et al., 2005a). There is no data on the role of PKC in these events in oocytes treated with the nitric oxide donor.

Based on these data, it is clear that the oocyte activation using calcium ionophore depends on PKC, especially on the activity of PKC-δ. However, PKCs are not stimulated after oocyte activation using the NO-donor. So there is an emerging possibility of enhancing the effects of oocyte activation using the NO-donor with concomitant PKC stimulation or with stimulation of selected PKC isotypes. A similar treatment was shown to be effective after pig oocyte activation using calcium ionophore (Ito et al., 2003).

What are other possible mechanisms involved in NO-induced oocyte activation?

The molecular mechanism of the NO-dependent activation of pig oocytes remains unclear. In mammalian oocytes, the blockage of meiosis at metaphase II is induced by the activation of a complex formed by cyclin B and cyclin-dependent kinase Cdc2 and by the stabilization of this complex. These events are dependent on the Mos-MAP kinase signalling pathway, which induces the inhibition of the anaphase-promoting complex/cyclosome (APC/C) (Kishimoto, 2003). The maintenance of this blockage is dependent on the action of Emi1 protein, which protects APC/C against binding to its substrate (Reimann and Jackson, 2002). Exit from the stage of metaphase II is dependent on the destabilization of the Cdc2-cyclin B complex and cyclin destruction (Murray and Johnson, 2000). This process is generally considered to be calcium-dependent, and the primary role of calcium ions in oocyte activation was confirmed even in studies comparing the role of the Ca-dependent and NO-dependent signalling cascade in oocyte activation (Hyslop et al., 2001; Leckie et al., 2003). On the other hand, it is clear from these studies that the NO-dependent signalling cascade is involved in the regulation of calcium signalling in oocyte activation.

During its activation, the oocyte exploits calcium both from extracellular and intracellular sources. Intracellular calcium is mobilized through ryanodine or inositoltriphosphate receptors (Williams, 2002). In sea urchin eggs, the increase in intracellular NO levels induces an increase in intracellular levels of free calcium ions through *de novo* synthesis of cyclic ADP ribose (Sethi et al., 1996; Willmott et al., 1996), which is known as an agonist of ryanodine receptors (Sitsapesan and Williams, 1995). Cyclic ADP ribose alone is able to induce parthenogenetic activation in pig oocytes (Petr et al., 2002).

There are many other mechanisms which could be involved in the NO-dependent activation of pig oocytes. NO activates many molecules through their nitrosylation. It induces the nitrosylation of cysteine on the protein molecule of ryanodine receptors and this nitrosylation activates ryanodine receptors (Xu et al., 1998). The importance of ryanodine receptors for parthenogenetic activation of pig oocytes has already been mentioned (Machatý et al., 1997; Petr et al., 2002).

NO also nitrosylates the Ras protein (Lander et al., 1995), which is involved in the MAP kinase signalling cascade in oocytes (Schmitt and Nebreda, 2002). The possibility of the action of NO on Ras seems plausible with regard to our unpublished data indicating that the inhibition of Ras is able to inhibit the NO-induced activation of pig oocytes.

Another mode of the action of NO is tyrosine nitration (Davis et al., 2001). This can influence the activity of calcium-dependent ATPases, which act as calcium pumps and recycle free calcium ions from the cytoplasm to their intracellular deposits (especially to the endoplasmic reticulum) and regulate the mobilization of intracellular sources. Doutheil et al. (2000) described the inhibition of calcium-dependent ATPases and subsequent exhaustion of endogenous calcium deposits under the effects of NO donors on somatic cells.

The intracellular calcium stores in the endoplasmic reticulum of mouse oocytes play an important role during oocyte activation (Swann and Lai, 1997; Mohri et al., 2001). In pig oocytes, the inhibition of calcium dependent ATPases is able to induce parthenogenetic activation (Petr et al., 2000). On the other hand, it is not clear whether the endoplasmic reticulum is a target for this treatment of pig oocytes, because the endoplasmic reticulum is not abundant in pig oocytes and other organelles are an important source of intracellular calcium (e.g. mitochondria, vacuole or yolk granule) (Petr et al., 2000, 2001).

Based on these data it is clear that many different signalling cascades could be stimulated when the mammalian oocyte is activated using NO-donors.

It is necessary to further investigate these events to characterize them and use them for optimization of the protocol for oocyte activation using NO-donors.

CONCLUSIONS

The NO-induced activation of mammalian oocytes is at least partly stimulated by alternative signalling cascades which are not involved in oocyte activation after treatment by traditional activating protocols (e.g. treatment with calcium ionophore). These alternative signalling cascades could not be involved in oocyte activation after its fertilization with the sperm. On the other hand, NO stimulates signalling cascades which are not necessary for oocyte activation through calcium-dependent processes (e.g. calcium ionophore, electric stimulation or fertilization by the sperm). For these reasons, nitric oxide does not provide an adequate activation stimulus and does not induce full embryonic development. This substantially limits the usage of NO in reproductive biotechnologies, e.g. for oocyte activation for cloning using nuclear transfer or for oocyte activation for the establishment of uniparental embryonic stem cells.

Further research into intracellular signalling in calcium- or NO-induced oocyte activation is needed. Based on data from this research, the NO-donor treatment could be modified to give a good performance. Additional treatments could either block redundant signalling pathways or stimulate the signalling cascades which remained inactive after NO treatment. For example we can suggest the inhibition of the cGMP-signalling cascade or activation of selected isotypes of PKC. Similar modifications of protocols for oocyte activation could enhance the efficiency of NO-donor treatment and introduce it to reproductive biotechnologies.

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