Zygote **14** (February), pp. 9–16. © 2006 Cambridge University Press
doi:10.1017/S0967199406003546 Printed in the United Kingdom

Nitric-oxide-dependent activation of pig oocytes: the role of the cGMP-signalling pathway

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Date submitted: 07.06.05. Date accepted 01.10.05

Summary

Pig oocytes matured *in vitro* were parthenogenetically activated (78%) after treatment with 2 mM nitric oxide-donor (±)-*S*-nitroso-*N*-acetylpenicillamine (SNAP) for 24 h. Inhibition of soluble guanylyl cyclase with the specific inhibitors 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ) or 6-anilino-5,8-quinolinequinone (LY83583) suppressed the SNAP-induced activation in a dose-dependent manner (23% of activated oocytes after treatment with 400 μM ODQ; 12% of activated oocytes after treatment with 40 μM LY83583). 8-Bromo-cyclic guanosine monophosphate (8-Br-cGMP), a phosphodiesterase-resistant analogue of cGMP, enhances the effect of suboptimal doses (0.1 or 0.5 mM) of the NO donor SNAP. DT3, a specific inhibitor of cGMP-dependent protein kinase (PKG, PKG), is also able to inhibit the activation of pig oocytes after NO donor treatment. Involvement of the cGMP-dependent signalling pathway is specific for NO-induced oocyte activation, because both the guanylyl cyclase inhibitor ODQ and the PKG inhibitor DT3 are unable to inhibit activation in oocytes treated with the calcium ionophore A23187. These data indicate that the activation of pig oocytes with an NO donor is cGMP-dependent and that PKG plays an important role in this mode of oocyte activation.

Keywords: Activation, cGMP, Nitric oxide, Pig oocyte

Introduction

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 an activating stimulus. Under natural conditions, this stimulus is brought into the oocyte by the sperm (Yanagimachi, 1988). The precise nature of this stimulus is not known. Nevertheless, the activation is important for many areas of research, including the cloning of mammals using nuclear transfer or intracytoplasmic sperm injection, because the successful development of embryos generated using these techniques depends on an appropriate activating stimulus.

Sperm induces oscillation of the intracellular levels of free calcium ions in the fertilized oocyte and that is why the activation of mammalian oocytes is considered to be a calcium-dependent process (Swann & Ozil, 1994). On the other hand, nitric oxide (NO) has also recently been suggested as an intracellular signal triggering activation of the oocyte (Kuo *et al.*, 2000).

NO represents an important signalling molecule and is synthesized in cells by nitric oxide synthase (NOS) from molecular oxygen and L-arginine in a process which also generates L-citrulline (Kwon *et al.*, 1990; Lamas *et al.*, 1992; Herrero & Gagnon, 2001).

However, the NO-dependent signalling cascade does not represent the primary stimulus for oocyte activation. This has been shown in studies which demonstrated a sharp increase in intracellular levels of free calcium ions preceding the increase in intracellular levels of NO in activated sea urchin eggs (Leckie *et al.*, 2003), and in activated tunicate and mouse

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https://doi.org/10.1017/S0967199406003546 Published online by Cambridge University Press

oocytes (Hyslop *et al.*, 2001). Nevertheless, we can conclude from the above-mentioned studies that an NO-dependent signalling cascade is functional in oocytes and is activated during oocyte activation after fertilization or parthenogenesis.

In a previous study we showed that stimulation of the NO-dependent signalling cascade is able to activate pig oocytes (Petr *et al.*, 2005*a*). We also proved that the activation of pig oocytes with NO donor is calcium-dependent (Petr *et al.*, 2005*b*).

However, NO is a potent intracellular regulator in many target systems and the role of these target systems in NO-induced oocyte activation remains unclear. Besides other effects, NO is known to bind the haem group of soluble guanylyl cyclase (sGC) and hence increase cyclic guanosine monophosphate (cGMP) production (Bellamy *et al.*, 2002). This type of action of NO has been well established in smooth muscle cells, platelets, endothelial cells (Murad, 1994), the kidney (Sayago & Beierwaltes, 2001), sperm (Revelli *et al.*, 2002), parotid acinar cells (Looms *et al.*, 2001) and porcine granulosa cells (Grasselli *et al.*, 2001). sGC is active in ovarian tissue (Ishimaru *et al.*, 2001; Shi *et al.*, 2004) and a large body of evidence indicates the potential roles of cGMP as an important cyclic nucleotide in the regulation of the ovarian functions (LaPolt *et al.*, 2003) including the regulation of oocyte meiosis (Tornell *et al.*, 1984, 1990). On the other hand, there is compelling evidence that NO can induce its biological effects via non-cGMP-dependent pathways (e.g. binding to haem-containing proteins other than sGC or *S*-nitrosylation of a variety of proteins, such as the ryanodine receptor or Ras protein) (Davis *et al.*, 2001).

To test the hypothesis that NO-induced parthenogenetic activation of pig oocytes is mediated by sGC through the cGMP-dependent pathway, we activated pig oocytes with the NO donor (±)-*S*-nitroso-*N*-acetylpenicillamine (SNAP) and we interfered in the cGMP-signalling pathway. The generation of cGMP was inhibited by 1H-[1,2,4]oxadiazolo [4,3-a]quinoxalin-1-one (ODQ) or 6-anilino-5,8-quinolinequinone (LY83583). ODQ is known as a selective inhibitor of NO-sensitive guanylyl cyclase. It does not chemically inactivate NO or NOS; nevertheless, it does inhibit cGMP generation in response to NO donors. LY 83583 is known to be a competitive inhibitor of soluble guanylyl cyclase, which lowers the production of cGMP levels in a wide range of tissues. Cell-permeable 8-bromo-cGMP, a cGMP analogue more resistant to phosphodiesterase, was used to mimic the cGMP effects in an oocyte treated with the NO donor. cGMP as a second messenger has many target systems. One of the most important is cGMP-dependent kinase (or PKG, PKG). To investigate the role of PKG in oocytes activated using the NO donor we used DT3, a cGMP-dependent protein kinase inhibitor.

Materials and methods

Isolation and culture of oocytes

Pig ovaries were obtained from a local slaughterhouse from gilts at an unknown stage of the estrous cycle and transported to the laboratory within 1 h in a saline solution (0.9% sodium chloride) at 39 °C. Fully grown oocytes were collected from follicles by aspirating those that were 2–5 mm in diameter with a 20-gauge needle. Only oocytes with compact cumuli were chosen for further study. Before culture, the oocytes were washed three times in a maturation culture medium.

The oocytes were cultured in a modified M199 medium (GibcoBRL, Life Technologies, Paisley, Scotland) containing sodium bicarbonate (0.039 ml of a 7.0% solution per millilitre of the medium), calcium lactate (0.6 mg/ml), gentamicin (0.025 mg/ml), HEPES (1.5 mg/ml), 13.5 IU eCG :6.6 IU hCG/ml (P.G.600 Intervet, Boxmeer, The Netherlands) and 10% of fetal calf serum (GibcoBRL, Life Technologies, Germany, lot no. 40F2190F).

The oocytes were cultured for 48 h in 3.5 cm diameter Petri dishes (Nunc, Roskilde, Denmark) containing 3.0 ml of the culture medium at 39 °C in a mixture of 5.0% CO_2 in air.

Evaluation of oocyte activation

At the end of the culture, the oocytes were mounted on slides, fixed with acetic alcohol (1:3, v/v) for at least 24 h, and stained with 1.0% orcein. The oocytes were examined under a phase-contrast microscope. Activation was considered to have occurred if the oocytes were in the pronuclear stage. Oocytes remaining at metaphase II or arrested at anaphase II or at telophase II were not considered as activated.

Experimental design

Experiment 1 was performed to demonstrate the ability of the NO donor SNAP to activate *in vitro* matured pig oocytes. The oocytes were matured for 48 h *in vitro*. They were then denuded of cumulus cells by repeated pipetting through a narrow glass pipette and cultured in a culture medium supplemented with 0, 0.1, 0.5, 1.0, 2.0 or 5.0 mm SNAP. We determined the ratio of activated oocytes at the end of the culture.

Experiment 2 was performed to investigate the role of guanylyl cyclase in the activation of *in vitro* matured pig oocytes using an NO donor. The matured and denuded oocytes were cultured in culture medium supplemented with 2.0 mm SNAP with the guanylyl

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cyclase inhibitor ODQ in concentrations of 0, 50, 100, 200 or 400 μM or with the guanylyl cyclase inhibitor LY83583 in concentrations of 0, 5, 10, 20 or 40 μM. We determined the ratio of activated oocytes at the end of the culture. The control experiments during which *in vitro* matured oocytes were cultured with LY83583 (40 μM) or ODQ (400 μM) did not reveal any side-effects of these compounds on pig oocytes, since all oocytes remained at the stage of metaphase II.

To investigate the specificity of the effects of guanylyl cyclase for NO-induced oocyte activation, the oocytes activated using the calcium ionophore A23187 were cultured with the specific guanylyl cyclase inhibitor ODQ. For this purpose, the *in vitro* matured oocytes (after 48 h of *in vitro* culture) were denuded of cumulus cells and then cultured in a medium supplemented with ODQ (400 μM) for 30 min to allow the inhibitor to bind to guanylyl cyclase. Subsequently oocytes were treated for 10 min in protein-free medium supplemented with A23187 (25 μM) and ODQ (400 μM). After this treatment, the oocytes were carefully washed in an ionophore-free medium supplemented with ODQ (400 μM) and cultured for 23 h in an ODQ-supplemented medium (400 μM). We determined the ratio of activated oocytes at the end of the culture.

Experiment 3 was performed to investigate the role of cGMP in the NO-induced activation of *in vitro* matured pig oocytes. The matured and denuded oocytes were cultured in culture medium supplemented either with 0, 0.1, 0.5, 1 or 2.0 mm SNAP alone or with 0, 0.1, 0.5, 1 or 2.0 mm SNAP and 1 mm of the cGMP analogue 8-bromo-cGMP. We determined the ratio of activated oocytes at the end of the culture.

Experiment 4 was performed to investigate the role of cGMP-dependent kinase (PKG) in NO-induced activation of *in vitro* matured pig oocytes. The matured and denuded oocytes were cultured for 48 h *in vitro* in culture medium supplemented with 2.0 mm SNAP with the PKG inhibitor DT-3 in concentrations of 0, 50, 100 or 200 μM. We determined the ratio of activated oocytes at the end of the culture. The control experiments, during which the *in vitro* matured oocytes were cultured with DT3 (200 μM), did not reveal any side-effects of these compounds on the pig oocytes, since all oocytes remained at the stage of metaphase II.

To investigate the specificity of the effects of PKG for NO-induced activation, the oocytes activated using the calcium ionophore A23187 were cultured with the specific PKG inhibitor DT3. For this purpose, the *in vitro* matured oocytes were denuded of cumulus cells and then cultured in a medium supplemented with DT3 (200 μM) for 30 min to allow the inhibitor to bind to PKG. Subsequently, the oocytes were treated for 10 min in a protein-free medium supplemented with A23187 (25 μM) and DT3 (200 μM). After this treatment, the oocytes were carefully washed in ionophore-free medium supplemented with DT3 (200 μM) and cultured for 23 h in a DT3-supplemented medium (200 μM). The ratio of activated oocytes was determined at the end of the culture.

Table 1 Activation of pig oocytes using the NO donor SNAP

Concentration of SNAP (mM)	Rate of activated oocytes (%)	No. of oocytes
0	2[a]	120
0.1	25[b]	120
0.5	36[b]	120
1.0	70[c]	120
2.0	78[c]	120
5.0	82[c]	120

Oocytes were cultured for 48 h *in vitro* and were then treated with the NO donor SNAP for 24 h.
[a,b,c]Statistically significant differences ($p < 0.05$) in the ratio of activated oocytes are indicated by different superscripts.

Statistical analysis

Data from all experiments were subjected to statistical analysis. Each experiment was performed four times. The results were pooled for presentation and evaluated by chi-square analysis (Snedecor & Cochran, 1980). The mean percentage of oocytes or embryos reaching the given stage of maturation or development in all trials did not vary from the pooled percentage by more than 2.5%. A p value of less than 0.05 was considered significant.

Results

In experiment 1 we demonstrated that the NO donor SNAP is able to activate pig oocytes in a dose-dependent manner (Table 1). When used in a concentration of 2.0 mM, SNAP activated 78% of the oocytes. A further increase in SNAP concentration did not result in any significant increase in the activation rate. We therefore chose treatment with 2.0 mm SNAP for further experiments. After activation of the oocytes with this concentration of SNAP for 24 h, we observed several types of chromatin arrangements, i.e. one extruded polar body and one pronucleus (12% of all activated eggs), one polar body and two pronuclei (45% of all activated eggs), two polar bodies and one pronucleus (36% of all activated oocytes), or one polar body and more than two pronuclei (7% of all activated oocytes).

In experiment 2 we demonstrated that guanylyl cyclase inhibitors blocked NO-induced oocyte activation in a dose-dependent manner. Data for ODQ are

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Table 2 Effect of the inhibitor of guanylyl cyclase ODQ on the activation of pig oocytes with the NO donor SNAP

Concentration of ODQ (μM)	Rate of activated oocytes (%)	No. of oocytes
0	68^{a}	120
50	58^{a}	120
100	44^{b}	120
200	34^{bc}	120
400	23^{c}	120

Oocytes were matured for 48 h *in vitro* and then treated with 2 mm SNAP and various doses of ODQ for another 24 h.
^{a,b,c}Statistically significant differences ($p < 0.05$) in the ratio of activated oocytes are indicated by different superscripts.

Table 3 Effect of the inhibitor of guanylyl cyclase LY83583 on the activation of pig oocytes with NO donor SNAP

Concentration of LY83583 (μM)	Rate of activated oocytes (%)	No. of oocytes
0	79^{a}	120
5	64^{b}	120
10	51^{c}	120
20	42^{c}	120
40	12^{d}	120

Oocytes were matured for 48 h *in vitro* and were then treated with 2 mm SNAP and various dose of LY83583 for another 24 h.
^{a,b,c,d}Statistically significant differences ($p < 0.05$) in the ratio of activated oocytes are indicated by different superscripts.

shown in Table 2 and data for LY83583 are given in Table 3. An effective dose of 400 μM ODQ was chosen for the experiment, which investigated the role of guanylyl cyclase in oocytes activated using the calcium ionophore A23187. Seventy-eight per cent of the oocytes ($n = 120$) treated with 25 μM of calcium ionophore for 10 min were activated. ODQ did not prevent ionophore-induced activation. Eighty-two per cent of the oocytes ($n = 120$) were activated when treated with ionophore and ODQ. This is a non-significant difference when compared with the oocytes treated with ionophore alone ($p > 0.05$) but it is a significantly higher activation rate than in oocytes treated with SNAP and 400 μM ODQ (82% vs 23%, $p < 0.05$).

In experiment 3 we observed that the cGMP analogue 8-bromo-cGMP enhances the effect of the NO donor SNAP. The addition of 1 mm 8-Br-cGMP significantly increased the effect of suboptimal doses of SNAP (0.1 or 0.5 mM) (Table 4).

In experiment 4 we demonstrated that DT3, the inhibitor of cGMP-dependent protein kinase, inhibits activation of pig oocytes in a dose-dependent manner (Table 5). An effective dose of 200 μM DT3 was chosen for the experiment, which investigated the role of cGMP-dependent kinase in oocytes activated using the calcium ionophore A23187. After treatment with 25 μM calcium ionophore for 10 min, 76% of the *in vitro* matured oocytes ($n = 120$) were activated. DT3 did not prevent ionophore-induced activation. Seventy-five per cent of the oocytes ($n = 120$) were activated when treated with ionophore and then cultured with

Table 4 Effect of 8-bromo-cGMP on the activation of pig oocytes using the NO donor SNAP

Concentration of SNAP (mM)	Rate of activated oocytes (%)		No. of oocytes: 8-Br-cGMP supplemented/8-Br-cGMP-free
	8-Br-cGMP supplemented	8-Br-cGMP-free	
0	$7^{a\text{-}1}$	$4^{a\text{-}1}$	120/120
0.1	$40^{b\text{-}1}$	$25^{b\text{-}2}$	120/120
0.5	$53^{b\text{-}1}$	$36^{b\text{-}2}$	120/120
1.0	$63^{bc\text{-}1}$	$70^{c\text{-}1}$	120/120
2.0	$71^{c\text{-}1}$	$78^{c\text{-}1}$	120/120

Oocytes were matured for 48 h *in vitro* and were then treated either with various doses of SNAP alone (8-Br-cGMP-free) or with various doses of SNAP with 1 mm of 8-bromo-cGMP (8-Br-cGMP-supplemented). Oocytes were treated with 8-Br-cGMP and/or SNAP for another 24 h.
^{a,b,c}Statistically significant differences ($p < 0.05$) in the ratio of activated oocytes for each type of treatment (8-Br-cGMP-free or -supplemented), i.e. differences within each column, are indicated by different alphabetical superscripts.
^{1,2}Statistically significant differences ($p < 0.05$) in the ratio of activated oocytes between treatment (8-Br-cGMP-free or -supplemented) for each respective SNAP concentration (i.e. differences between columns) are indicated by different numerical superscripts.

Table 5 Effect of the PKG inhibitor DT3 on the activation of pig oocytes using the NO donor SNAP

Concentration of DT3 (μM)	Rate of activated oocytes (%)	No. of oocytes
0	74^{a}	120
50	52^{b}	120
100	36^{c}	120
200	17^{d}	120

Oocytes were matured for 48 h *in vitro* and then were treated with 2 mm SNAP and various dose of DT3 for another 24 h.
^{a,b,c,d}Statistically significant differences ($p < 0.05$) in the ratio of activated oocytes are indicated by different superscripts.

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DT3. This is a non-significant difference ($p > 0.05$) when compared with oocytes treated with calcium ionophore and then cultured in a DT3-free medium. The activation rate in oocytes treated with ionophore and then cultured with 200 μM DT3 is significantly higher than in oocytes cultured with SNAP and 200 μM DT3 (75% vs. 17%, $p < 0.05$).

Discussion

In the present study we demonstrated that the activation of *in vitro* matured pig oocytes via the NO-dependent signalling pathway is mediated through cGMP. It is known that NO activates sGC and induces cGMP synthesis (Hofmann *et al.*, 2000). The cGMP-mediated effects of NO have been described in many types of somatic cells (Murad, 1994; Sayago & Beierwaltes, 2001; Looms *et al.*, 2001) as well as in sperm (Revelli *et al.*, 2002) and ovarian tissue (Grasselli *et al.*, 2001; Ishimaru *et al.*, 2001; Shi *et al.*, 2004; LaPolt *et al.*, 2003). Based on our data, we suggest that in addition to somatic body cells and somatic compartments of the ovary, the NO-induced cGMP-dependent pathway is also active in the pig oocyte.

This suggestion is supported by the observation that both the sGC inhibitors used in our study, ODQ and LY83583, effectively suppressed oocyte activation after treatment of the oocyte with an NO donor. Although LY83583 is widely used for the inhibition of sGC, its action on the cGMP-signalling pathway is not clear. Besides its effects on sGC, LY83583 has also been described as inhibiting NOS, eliciting NO auto-oxidation and generating the superoxide anion, which could scavenge NO (Li *et al.*, 1998; Revelli *et al.*, 2001). We cannot exclude the possibility that these side-effects of LY83583 were active under our conditions and that they could have blocked NO-induced oocyte activation, since the inhibition of NOS with its specific inhibitor is able to block activation of pig oocytes induced by an NO donor (Petr *et al.*, 2005*a*). On the other hand, ODQ is known as a specific inhibitor of sGC (Garthwaite *et al.*, 1995; Brunner *et al.*, 1996; AbiGerges *et al.*, 1997) and exhibits the same effects as LY83583. This indicates that NO donors induce activation of pig oocytes at least partially through activation of sGC.

This notion is further supported by our 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. 8-Br-cGMP is able to simulate elevated cGMP levels and to substitute the cGMP lacking after the weaker stimulation of sGC by a lower dose of NO donor.

The cGMP-dependent PKG is one of the main target systems of the signalling molecule of cGMP (Scott, 1991). For this reason, the role of cGMP in the activation of pig oocytes induced by NO donor was further emphasized by the observation that the inhibition of cGMP-dependent kinase inhibits oocyte activation.

The key role of the cGMP-dependent signalling pathway for parthe-nogenetic activation seems to be specific for oocyte activation using an NO donor, because inhibition of cGC or PKG in oocytes treated with calcium ionophore did not prevent parthenogenetic activation. NO-dependent intracellular signalling could be triggered even after oocyte treatment with calcium ionophore, because Hattori *et al.* (2004) described increased NO production in pig oocytes treated with calcium ionophore. However, based on our unpublished data, we suggest that 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.

Moreover, based on the results of the present study, we can conclude that even if NO is released in the ionophore-treated oocyte, activation of the NO-triggered cGMP-signalling pathway is not necessary for pig oocyte activation using a calcium ionophore.

From data published by Kuo *et al.* (2000), Hyslop *et al.* (2001) and Leckie *et al.* (2003) it is clear that calcium- and NO-dependent signalling pathways are interconnected in oocytes, with calcium playing the primary role. In our previous study (Petr *et al.*, 2005*b*) we also demonstrated that the activation of pig oocytes with an NO donor strongly depends on calcium. Our data showed that calcium for NO-induced activation of the pig oocyte is mobilized from both extracellular and intracellular stores and that intracellular calcium is mobilized from the ryanodine receptors but not at all from inositol triphosphate receptors (Petr *et al.*, 2005*b*).

This interconnection of NO- and Ca-dependent signalling pathways can be mediated via cGMP and cGMP-dependent protein kinase through several processes. PKG phosphorylates and activates the ADP-ribosyl cyclase that synthesizes cADP ribose, an agonist of ryanodine receptors (Lee, 1996; 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 (1996) that cGMP-mediated calcium release is not required for the rise in calcium during fertilization. However, there are some data demonstrating the ability of cADP ribose to induce parthenogenetic activation of oocytes. The microinjection of cADP ribose induced the elevation

https://doi.org/10.1017/S0967199406003546 Published online by Cambridge University Press

of intracellular levels of free calcium ions in pig oocytes (Macháty *et al.*, 1997) and this treatment induces activation of pig oocytes and their subsequent parthenogenetic development (Petr *et al.*, 2002).

In addition to cADP ribose, an endogenous ryanodine agonist, PKG can also regulate the levels of endogenous agonist of the inositol triphosphate receptor. It inhibits activity of phospholipase C, which hydrolyses phosphatidyl inositol 4,5-diphosphate to inositol 1,4,5-triphosphate (IP_3) and diacylgylcerols (DAG) (Rappaport, 1989; Ruth *et al.*, 1993; Wang *et al.*, 1998). This process decreases the intracellular levels of endogenous IP_3 receptor (IP_3R) agonist, inositol 1,4,5-triphosphate. The other product of hydrolysis of phosphatidyl inositol 4,5-diphosphate – diacyglycerols – are known as activators of the protein kinase C (Gómez-Fernandez *et al.*, 2004) that also play an important role in the regulation of mammalian oocyte activation (Quan *et al.*, 2003). cGMP-dependent kinase is known even to directly regulate the functions of inositol triphosphate receptors through their phosphorylation (Huang *et al.*, 1999). All these effects are worthy of interest, because pig oocytes release free calcium ions after stimulation of their inositol triphosphate receptors (Macháty *et al.*, 1997) and this treatment induced activation of pig oocytes and their parthenogenetic development (Petr *et al.*, 2002).

The cGMP-dependent signalling pathway can also influences the release of free calcium ions, acting directly on calcium ion channels. This effect has been described in various types of somatic cells (D'Ascenzo *et al.*, 2002; Carabelli *et al.*, 2002; Centonze *et al.*, 2001; Jiang *et al.*, 2000; Cataldi *et al.*, 1999) and also in oocytes (Van Coppenolle *et al.*, 1997). The effects of the PKG closed 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.*, 2004) and it is clear that their function is essential for meiosis especially in pig oocytes (Kaufman & Homa, 1993; Rozinek *et al.*, 2003).

We cannot exclude the possibility that NO also regulates the activation of oocytes through processes that do not depend on cGMP. NO activates many molecules through their nitrosylation. It induces 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 (Macháty *et al.*, 1997; Petr *et al.*, 2002).

NO also nitrosylates the Ras protein (Lander *et al.*, 1996), which is involved in the MAP kinase signalling cascade in oocytes (Schmitt & Nebreda, 2002). The possibility of the action of NO on Ras seems plausible, given our unpublished data indicating that inhibition of Ras is able to inhibit the NO-induced activation of pig oocytes.

Another mode of cGMP-independent 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 & 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 endoplasmic reticulum is the 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, vacuoles or yolk granules) (Petr *et al.*, 2001).

Based on data presented in this study we can conclude that NO-induced activation of pig oocytes is at least partially mediated via the cGMP-dependent signalling cascade, with PKG playing an important role. However, further studies are needed for more precise elucidation of cGMP-dependent signalling in pig oocytes activated using an NO donor, especially with respect to the fact that cGMP-dependent signalling could be unique to the NO-induced activation and is not necessary for other modes of activation, such as calcium ionophore.

Acknowledgements

We thank Mrs Lucy Westcott for editorial assistance with this manuscript. This work was supported by grant no. 523/03/0029 from GACR, grant no. MZE0002701401 from MZe CR, grant no. 523/03/H076 from GACR and grant no. MSM 6046070901.

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