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Possible Intracellular Regulators of Female Sexual Maturation

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Summary

Protein kinases, transcription factors and other apoptosis- and proliferation-related proteins can regulate reproduction, but their involvement in sexual maturation remains to be elucidated. The general aim of the *in vivo* and *in vitro* experiments with porcine ovarian granulosa cells was to identify possible intracellular regulators of female sexual maturation. For this purpose, proliferation (expression of proliferating cell nuclear antigen – PCNA, mitogen-activated protein kinases – ERK 1,2 related MAPK and cyclin B1), apoptosis (expression of the apoptotic protein Bax and apoptosis regulator Bcl-2 protein), expression of some protein kinases (cAMP dependent protein kinase – PKA, cGMP-dependent protein kinase – PKG, tyrosine kinase – TK) and cAMP responsive element binding protein 1 (CREB-1) was examined in granulosa cells isolated from ovaries of immature and mature gilts. Expression of PCNA, ERK1,2 related MAPK, cyclin B1, Bcl-2, Bax, PKA, CREB-1, TK and PKG in porcine granulosa cells were detected by immunocytochemistry. Sexual maturation was associated with significant increase in the expression of Bcl-2, Bax, PKA, CREB-1 and TK and with decrease in the expression of ERK1,2 related MAPK, cyclin B1 and PKG in granulosa cells. No significant difference in PCNA expression was noted. The present data obtained from *in vitro* study indicate that sexual maturation in females is influenced by puberty-related changes in porcine ovarian signaling substances: increase in Bcl-2, Bax, PKA, CREB-1, TK and decrease in ERK1,2 related MAPK, cyclin B1 and PKG. It suggests that these signaling molecules could be potential regulators of porcine sexual maturation.

Key words

Ovary • Kinases • Proliferation • Apoptosis • Transcription factor

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Introduction

Sexual maturation is associated with ovarian follicular growth and differentiation (Onagbesan *et al.* 2009, Palma *et al.* 2012). These processes are governed by hormones, growth factors (Kolesarova *et al.* 2009a,b, 2010a,b, Roychoudhury *et al.* 2009, 2014), which through protein kinases and transcription factors, affect ovarian cell proliferation, apoptosis and secretion activity (Onagbesan *et al.* 2009, Sirotkin 2013). There is indirect evidence for involvement of several candidate signaling substances in control of sexual maturation and/or related ovarian follicle development.

Action of hormones and growth factors on ovarian folliculogenesis and functions is mediated *via* protein kinases and related proliferation- and apoptosis-related peptides (Sirotkin *et al.* 2000, 2008). The involvement of cAMP/protein kinase A (PKA)-dependent intracellular mechanisms (Makarevich *et al.* 2000, Sirotkin and Grossmann 2003, 2006) in the regulation of proliferation- and apoptosis-related

substances (Sirotkin and Grossmann 2003, 2006) has already been reported. Furthermore, cAMP/PKA can regulate the secretion activity of mammalian ovarian cells as noted in cases of porcine (Sirotkin *et al.* 2004), chicken (Sirotkin and Grossmann 2006) and human (Sirotkin *et al.* 2008) ovarian cells and also mediate the action of hormones and growth factors on ovarian functions (Makarevich *et al.* 2004a,b). Apoptosis-related substances are crucial in follicular selection, atresia and corpus luteum regression (Parborell *et al.* 2001, 2008, Greenfeld *et al.* 2007, Maeda *et al.* 2007). Mitochondrial apoptotic protein Bax is considered as the key pro-apoptotic substance (Elmore 2007), whilst apoptosis regulator Bcl-2 protein, which binds and inactivates Bax, has an opposite, anti-apoptotic action (Greenfeld *et al.* 2007, Lomonosova and Chinnadurai 2008). The mitogen-activated protein kinases (MAPK) signaling cascade including intracellular regulated kinases (ERK) also act as promoters of cell cycle progression as well as mediators of mitogenic action of hormones and growth factors (Lapthorn *et al.* 1994, Cameron *et al.* 1996, Sirotkin and Grossmann 2003), stimulators of ovarian cell proliferation, differentiation and secretion activity (Sirotkin and Grossmann 2003) and suppressors of apoptosis (Xia *et al.* 1995, Dent *et al.* 1998, Kyriakis 1999, Gunter *et al.* 2013). Tyrosine kinase (TK) localized in growth factor receptors and cytoplasm plays an important role in promoting cell proliferation, differentiation and mediation effects of some hormones and growth factors in signal transduction (Okamura *et al.* 2001, Sirotkin and Grossmann 2003, Arora and Scholar 2005). TK may be involved in activation of ovarian porcine follicle growth and maturation (Okamura *et al.* 2001) and in control of chicken ovarian cell proliferation and hormone release (Sirotkin and Grossmann 2003).

The involvement of cGMP dependent protein kinase (PKG) along with cGMP in control of the production of steroid, nonapeptide hormone, growth factor, cAMP and cAMP-dependent PKA, as well as the induction of apoptosis in porcine ovarian cells has been reported, too (Sirotkin *et al.* 2000). Protein kinases (PKA, MAPK) can also target cAMP responsive element binding protein 1 (CREB-1). It is required for mediating stimulatory influence of FSH on granulosa and luteal cells differentiation and steroidogenesis during the follicular recruitment estrous cycle and pregnancy of mouse (Mendelson and Kamat 2007). There exist indirect evidences for involvement of CREB in control of sexual maturation (Sirotkin *et al.* 2004, He *et al.* 2006). Cell cycle peptides especially proliferating cell nuclear antigen PCNA (Naryzhny and Lee 2001) and cyclin B1 (Wyllie *et al.* 1998) are also involved in ovarian cell proliferation, growth and development (Tomanek and Chronowska 2006). Proliferation-related peptide PCNA is a known promoter of the cell cycle transition through G1 and G2 phases. Furthermore, it activates the cyclin/cyclin dependent kinase complex (McHugh and Sarkar 2006, Moldovan *et al.* 2007), which promotes the G_2-M transition of the cell cycle (Hassan *et al.* 2001). Expression of PCNA and cyclin B1 in ovarian cells has been reported from different mammalian species (Hutt *et al.* 2006, Sirotkin *et al.* 2008).

The general aim of the *in vivo* and *in vitro* experiments with porcine ovarian granulosa cells was to identify possible intracellular regulators of female sexual maturation. For this purpose, expression of these signaling molecules were evaluated in granulosa cells collected from sexually mature and immature gilts of the same age.

Materials and Methods

Animals

Healthy gilts of Slovakian White breed (100-120 days of age) were reared under standard conditions at the Experimental Station of the Slovak University of Agriculture in Nitra, Slovakia. Conditions of their care and handling corresponded to the instructions of the European Commission (EC) no. 178/2002 and related EC documents and as approved by local ethics committee. Animals (n=35) were assigned at slaughter into two groups: sexually immature (n=18) and animals of the same age having reached sexual maturity (n=17) according to visual characteristics of ovaries (presence of follicles larger than 5 mm).

Preparation, culture and processing of granulosa cells

Ovaries were transported to the laboratory at 4 °C and washed in sterile physiological solution. Ovaries from immature and mature gilts were processed separately. Follicular fluid was aspirated from 3-5 mm follicles, granulosa cells were isolated by centrifugation for 10 min at 200xg followed by washing in sterile DMEM/F12 1:1 medium (BioWhittaker™, Verviers, Belgium) and resuspended in the same medium supplemented with 10 % fetal calf serum

(BioWhittaker™) and 1 % antibotic-antimycotic solution (Sigma, St. Louis, MO, USA) at a final concentration of 10^6 cells/ml of medium. Portions of the cell suspension were dispensed to Lab-Tek 16-welled chamber slides (Nunc Inc., International, Naperville, USA, 100 µl/well; for immunocytochemistry). Chamber slides were incubated at 37.5 °C and 5 % CO_2 in humidified air until a 75 % confluent monolayer was formed (5-7 days), at which point the medium was replaced with fresh medium. Further culture was performed in 300 µl medium in 16-welled chamber slide cells. After 2 days of culture the media from wells were removed, wells from chamber slides were washed in ice-cold PBS (pH 7.5). Cells were fixed for 1 h at room temperature in 4 % paraformaldehyde, dehydrated in alcohols (70, 80, 96 %; 10 min each) and stored in 96 % alcohol at –4 °C to await immunocytochemical analysis.

Immunocytochemistry

Immunocytochemistry was used to detect PKA, PKG, TK, ERK1,2 related MAP kinase, CREB-1, PCNA, cyclin B1, Bax, Bcl-2 in granulosa cells plated on chamber slides (Osborn and Isenberg 1994). Primary mouse monoclonal antibodies to each petide PKA, PKG, TK, CREB-1, PCNA, cyclin B1, Bax, Bcl-2 (cross-reacting with corresponding rat, human, porcine and chicken substances; all from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) were used as directed by the manufacturer at a dilution of 1:100 and ERK1,2 at a dilution of 1:50. Visualization of the primary antibody binding sites was done with a secondary rabbit polyclonal antibody against mouse IGs, labelled with horseradish peroxidase (Sevac, Prague, Czech Republic; dilution 1:500) and diaminobenzidine (DAB) reagent (Roche Diagnostics Corporation, IN, USA, 10 %). The presence of each peptide was determined by light microscopy. To verify these data, in some selected cases primary antibodies were visualized by secondary rabbit or goat monoclonal antibodies against mouse IGs labeled with FITC (Sevac, Prague, Czech Republic) and fluorescent microscopy. Negative control was presented by stained cells omitting primary antibody. During microscopic inspection, the percentage of cells containing visible antigen was determined.

Statistics

Each experimental group was represented by four chamber slide wells with granulosa cells. The data presented concerning the effects of each substance are means of values obtained in three separate experiments performed on separate days using separate ovaries obtained from 10-12 animals. The proportion of cells containing each analyzed substance was calculated following immunocytochemical analysis by counting at least 1000 cells per chamber slide well. Firstly, the data obtained in each experiment were processed by ANOVA. Thereafter, significant differences between the immature groups and mature gilts were evaluated by paired t-test or chi-square (χ^2) test by using statistical software Sigma Plot 9.0 (Jandel, Corte Madera, USA). Differences from controls ($p<0.05$) were considered as significant.

Results

Percentage of ovarian granulosa cells containing PCNA did not differ between sexually mature (22.4±4.1 %) and immature gilts (17.5±1.2 %) (Fig. 1). On the contrary, the expression of ERK1,2 related MAPK and cyclin B1 was significantly ($p<0.05$) lower in granulosa cells of sexually mature gilts (ERK1,2 35.3±1.6 %, cyclin B1 21.8±0.6 %) in comparison to their immature counterparts (ERK1,2 46.2±1.8 %, cyclin B1 38.2±1.6 %) (Fig. 1).

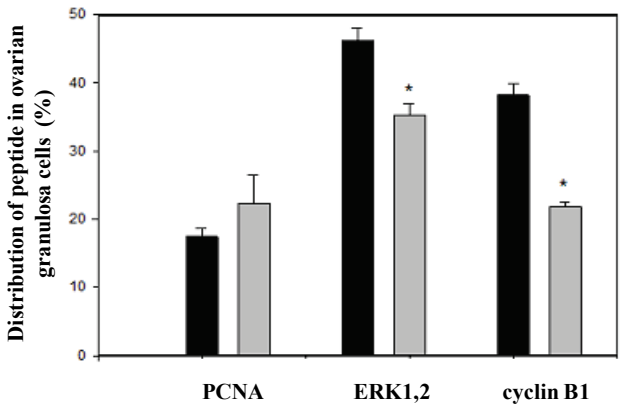

Fig. 1. Distribution of PCNA, ERK1,2 and cyclin B1 in ovarian granulosa cells of sexually immature and mature gilts. * significant difference ($p<0.05$) between corresponding groups of sexually immature (n=18, black column) and mature (n=17, grey column) gilts evaluated by t-test and chi-square (χ^2) test. Immunocytochemistry.

The expression of Bcl-2 and Bax by ovarian granulosa cells was significantly ($p<0.05$) higher in sexually mature gilts in comparison to immature animals (37.7±1.8 % vs. 28.7±1.0 % for Bcl-2 and 48.7±2.6 % vs. 31.9±2.3 % for Bax, respectively) (Fig. 2).

Proportion of cells containing PKA was also

significantly ($p<0.05$) higher in sexually mature gilts than in sexually immature animals (PKA 54.4±1.2 % vs. 32.7±1.7 %) (Fig. 3). Similarly, expression of TK was significantly ($p<0.05$) higher in sexually mature gilts in comparison to sexually immature ones (TK 41.4±1.1 % vs. 32.4±3.3 %) (Fig. 3). Also, proportion of cells containing CREB-1 was significantly ($p<0.05$) higher in sexually mature gilts than the immature animals (CREB 46.3±1.0 % vs. CREB 38.6±0.7 %). However, the expression of PKG was significantly lower ($p<0.05$) in granulosa cells of sexually mature gilts than the immature animals (PKG 16.7±2.2 % vs. PKG 61.3±2.1 %) (Fig. 3).

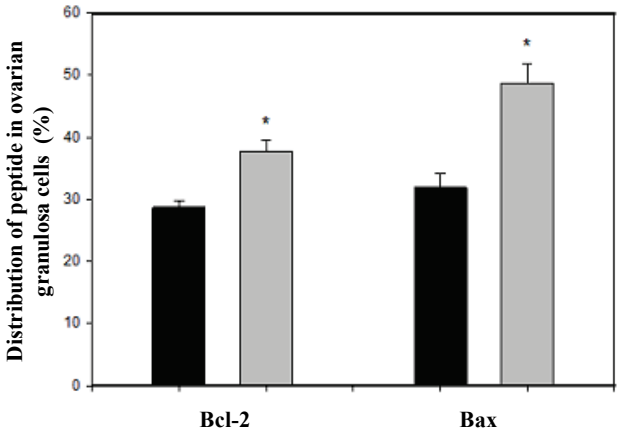

Fig. 2. Distribution of Bcl-2 and Bax in ovarian granulosa cells of sexually immature and mature gilts. * significant difference ($p<0.05$) between corresponding groups of sexually immature (n=18, black column) and mature (n=17, grey column) gilts evaluated by t-test and chi-square (χ^2) test. Immunocytochemistry.

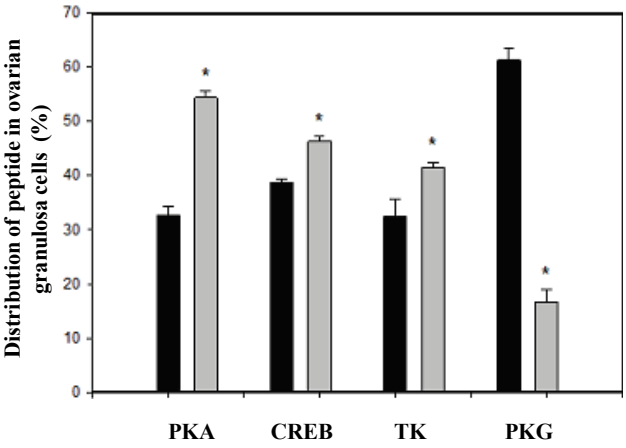

Fig. 3. Distribution of PKA, CREB, TK and PKG in ovarian granulosa cells of sexually immature and mature gilts. * significant difference ($p<0.05$) between corresponding groups of sexually immature (n=18, black column) and mature (n=17, grey column) gilts evaluated by t-test and chi-square (χ^2) test. Immunocytochemistry.

Discussion and Conclusions

Do peptides of cell proliferation (PCNA, ERK1,2 related MAPK and cyclin B1) relate to sexual maturation?

Follicular growth and development in porcine ovary was associated with increased expression of PCNA in granulosa cell (Tomanek and Chronowska 2006). Peng *et al.* (1998) reported decreased expression of PCNA in granulosa cells during apoptosis. Our investigation did not reveal any differences in PCNA in granulosa cells between immature and mature gilts (Peng *et al.* 1998). In contrast to PCNA, the expression of cyclin B1 was associated with sexual maturation. Since cyclin B1 is a promoter and marker of G-phase of cell cycle (Wyllie *et al.* 1998), it might be suggested that sexual maturation is associated with suppression of cell cycle at this phase. Since ERK1,2 related MAPK is an important marker and promoter of cell cycle (Grossmann 2009), its role in stimulation of ovarian cell proliferation and related follicle growth during puberty may be suggested. During establishment of ovarian cyclicity the expression and probably the importance of ERK1,2 related MAPK declines. Besides cell proliferation ERK1,2 related MAPK can control apoptosis. In mammalian cells, the MAPK pathway can prevent (Allen *et al.* 1999, Anderson and Tolkovsky 1999, Nishio *et al.* 1999) or induce (Goillot *et al.* 1997, Bhat and Zhang 1999) apoptosis depending on the type of cell and the extracellular stimuli that initiate the pathway. Therefore, the changes in ERK1,2 related MAPK as observed in the present study could be due to its involvement in control of apoptosis during sexual maturation. Evidence also persists (Sirotkin and Grossmann 2003) that MAPK could be involved not only in control of apoptosis, but also in control of ovarian secretion activity and in mediating the effect of hormonal regulators of reproduction.

Results of the present study indicate that sexual maturation is associated with a reduction in the expression of ERK1,2 related MAPK and cyclin B1, but not of PCNA, which could result in reduction of ovarian cell proliferation, increase in their apoptosis and might even change their secretion activity and response to hormonal regulators during establishment of ovarian cycle.

Do anti-apoptotic peptide Bcl-2 and pro-apoptotic peptide Bax relate to sexual maturation?

In this study, sexual maturation in gilts was found to be associated with increased expression of both Bcl-2 and Bax by ovarian granulosa cells. Through their effect on

apoptosis, these peptides could be involved in control of ovarian follicular growth, development and fertility. This is probably the first indication of involvement of ovarian Bax and Bcl-2 in control of porcine sexual maturation. The puberty-related increase in expression of both Bax and its antagonist Bcl-2 as observed in the present study suggest the involvement of these apoptosis-related peptides in regulation of porcine sexual maturation, although understanding their exact role in control of porcine reproduction requires further investigation.

Do PKA, CREB-1, TK and PKG relate to sexual maturation?

Stimulatory influence of PKA on ovarian secretory activity and in mediating the action of hormones and growth factors has been demonstrated previously (Makarevich *et al.* 2004a,b). The present study for the first time reports involvement of PKA in regulation of not only basal ovarian functions, but also of sexual maturation. It is possible that sexual maturity-related increases in the expression of PKA as observed in this study are important for sexual maturity-associated increases in hormone and growth factor release and action.

It was previously mentioned that TK can be involved in activation of porcine ovarian follicle growth and maturation (Okamura *et al.* 2001) and in control of chicken ovarian cell proliferation and hormone release (Sirotkin and Grossmann 2003). Our observations present further involvement of TK in control of sexual maturity-related changes in ovarian functions. Our observations provide the first indications of involvement of PKG in regulation of porcine sexual maturation. Involvement of cGMP/PKG system in control of release of porcine ovarian hormones has been reported previously (Sirotkin *et al.* 2000).

Also, our data provide the first indications of the role of CREB-1 in sexual maturation and related processes in gilts. Although involvement of CREB-1 in control of sexual maturation (Sirotkin *et al.* 2004, He *et al.* 2006) and in mediating the effect of growth factor on these processes (Sirotkin and Grossmann 2003) in non-porcine species has been documented, details of CREB-1 targets and action remain to be studied.

Possible interrelationships between studied substances

Effect of hormones and growth factors on the ovary can be mediated by protein kinases and protein kinases-dependent transcription factors. PKA can mediate the action of hormones and growth factors (Makarevich *et al.* 2004a,b). Furthermore, hormones and growth factors can affect ovarian function during and after puberty through MAPK-dependent intracellular mechanisms. At least, growth factors can activate MAPK in a variety of cell types (Lapthorn *et al.* 1994), and inhibitors of MAPK cascade can block the mitogenic action of the growth factors (Alessi *et al.* 1995). Furthermore, ability of PKA and TK to affect MAPK and MAPK-activated CREB-1 in non-ovarian cells has been reported (Gao *et al.* 2009, McAlees and Sanders 2009, Sun *et al.* 2009, Zu *et al.* 2009). Therefore, the functional interrelationships between these substances within the ovary in regulating porcine sexual maturation cannot be excluded. Fine interrelationships between analyzed processes occurring in porcine ovary during sexual maturation require further elucidation. Nevertheless, the present observations expand the existing knowledge concerning changes during sexual maturation in porcine ovarian hormones, growth factors and growth factors binding proteins. Furthermore, this is the first indication of involvement of some intracellular signaling substances in control of this process. Obtained results suggest that sexual maturation is associated with increase in expression of apoptosis-related substances (Bcl-2, Bax), PKA, TK, PKG, CREB-1, with decreases in the expression proliferation-related substances of ERK1,2 related MAPK and cyclin B1, but not PCNA. Analyzed data indicate puberty-related changes in porcine ovarian signaling substances: Bcl-2, Bax, PKA, CREB-1, TK, ERK1,2 related MAPK, cyclin B1 and PKG. Results obtained from both *in vivo* and *in vitro* studies indicate the involvement of some apoptosis- and proliferation-related substances, protein kinases and transcription factor CREB-1 in porcine sexual maturation. The results of present study indicate that sexual maturation is associated with decrease in ovarian cells proliferation and increase in their secretory activity, apoptosis and expression of some protein kinases and transcription factor. Although the puberty-related changes do not provide direct evidence of the involvement and physiological role of these signaling molecules in control of sexual maturation, our study enables to identify several extra-and intracellular signaling substances, which could be potential candidates for induction of porcine puberty and sexual maturation.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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