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Effect of proteasome inhibitor MG132 on *in vitro* maturation of pig oocytes

Eva Chmelíková[1], *Markéta Sedmíková*[1], *Radko Rajmon*[1], *Jaroslav Petr*[2], *Dana Švestková*[1] *and František Jílek*[1]

Czech University of Agriculture in Prague and Research Institute of Animal Production, Prague, Czech Republic

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Summary

The present study aimed to demonstrate the dependence of meiotic maturation in pig oocytes on the activity of the protease complex proteasome. The proteasome inhibitor MG132 blocked the exit of maturing pig oocytes from metaphase I stage. Seventy-five per cent of the oocytes were blocked at metaphase I when they were cultured with 10 μM MG132. The blocking effect of MG132 was expressed only when the oocytes were exposed to an inhibitor before the 18th hour of *in vitro* culture. The effects of MG132 are fully reversible. However, a significant proportion of oocytes (46%) cultured for 48 h in MG132-supplemented medium and then for 24 h in MG132-free medium did not block meiosis at the stage of metaphase II and underwent spontaneous parthenogenetic activation. On the basis of our data we can conclude that exit from the metaphase I stage of meiosis is proteasome-dependent in pig oocytes matured *in vitro*. On the other hand, our data also indicate that other proteasome-independent events are involved in regulating the exit from metaphase I.

Keywords: Maturation, MG132, Oocyte, Pig, Proteasome

Introduction

Meiosis in mammalian oocytes is triggered during the fetal life of the female but is interrupted in the late diplotene stage. The resumption of meiosis occurs after puberty, when the selected population of oocytes completes its meiotic maturation during each oestrous cycle. After resumption of meiosis, the oocyte undergoes germinal vesicle breakdown (GVBD), enters the stage of metaphase I, goes through anaphase I and telophase I and reaches metaphase II, when meiosis is again blocked (Wassarman, 1988). The further progress of meiosis and its completion is dependent on the stimulus provided to the oocyte by the sperm (Yanagimachi, 1988).

Meiosis is regulated by a wide network of events. Phosphorylation and dephosphorylation play a key role among these events (Motlík & Kubelka, 1990). Besides phosphorylation and dephosphorylation, meiosis is also regulated through the synthesis and degradation of specific proteins (Murray *et al*., 1989; Glotzer *et al*., 1991). From this point of view, a very important role is performed by proteasome (Tokumoto *et al*., 1997) – a multicatalytic protease able to hydrolyse C-terminal peptide bonds to acidic, basic and hydrophobic amino acid residues. Proteasome represents about 1% of all proteins in the mammalian cell and it is the main pathway for protein degradation (Goldberg, 1995; Coux *et al*., 1996).

A role for proteasome during meiosis was first demonstrated in invertebrates (Kawahara *et al*., 1992; Sawada *et al*., 1992). Proteasome was also shown to regulate meiosis in mammalian oocytes (Josefsberg *et al*., 2000). The inhibition of proteasome blocked the maturation of rat oocytes at the stage of metaphase I. This effect of proteasome is attributed to the proteasome-dependent destruction of cyclin B (Josefsberg *et al*.,

All correspondence to: E. Chmelíková, Department of Veterinary Science, Czech University of Agriculture in Prague, 165 21, Prague 6, Czech Republic. Tel.: +420 224382931. Fax: +420 234381841. e-mail: chmelikova@af.czu.cz
[1] Czech University of Agriculture in Prague, Faculty of Agronomy, Department of Veterinary Science, Kamýcká 129, Prague 6, Czech Republic.
[2] Research Institute of Animal Production, Přátelství 815, Prague 10, Czech Republic.

2000). Cyclin B forms a complex with cyclin-dependent kinase $p34^{cdc2}$ and this complex is responsible, in its active state, for the induction of metaphase of the cell cycle. The destruction of cyclin results in the inactivation of the complex and exit from metaphase (Murray *et al*., 1989).

The results obtained during the study of proteasome in rat oocytes are not relevant in all respect to the oocytes of large domestic animals, because the regulation of meiosis differs in many aspects between rodents and ungulates (Thibault *et al*., 1987). Exit from metaphase occurred in pig oocytes even after the inactivation of the cyclin B-cyclin dependent kinase $p34^{cdc2}$ complex without the destruction of cyclin through proteasome-dependent proteolysis. In this case the active complex is inactivated through its phosphorylation (Kikuchi *et al*., 1999, 2000). The role of proteasome in the maturation of pig oocytes is not clear and the aim of the present study was to investigate the role of proteasome through its inhibition using the specific cell-permeable inhibitor MG132 (Lee & Goldberg, 1996).

Materials and methods

Isolation of oocytes

Pig ovaries were obtained from a local slaughterhouse from gilts at an unknown stage of the oestrous 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 by aspirating follicles that were 2–5 mm in diameter with a 20-gauge needle. Only oocytes with compact cumuli were chosen for further studies.

Oocyte culture

Before culture the oocytes were washed three times in the 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% solution per millilitre of medium), calcium lactate (0.6 mg/ml), sodium pyruvate (0.25 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% fetal calf serum (GibcoBRL, Life Technologies, Germany, lot no. 40F2190F).

The oocytes were cultured in 3.5 cm diameter Petri dishes (Nunc, Roskilde, Denmark) containing 3 ml of the culture medium at 39 °C in a mixture of 5% CO_2 in air.

Evaluation of the oocytes

At the end of the culture, the oocytes were relieved of cumulus cells by repeated pipetting through a narrow glass pipette and their morphology was evaluated under an inverted microscope. The oocytes were then mounted on slides, fixed with acetic alcohol (1:3, v/v) for at least 24 h and stained with 1% orcein. The oocytes were examined under a phase-contrast microscope. The stages of maturation were determined according to the criteria of Motlík & Fulka (1976). Activation was considered to have occurred if the oocytes were in the pronuclear stage and a polar body was visible.

Design of experiments

Experiment 1 was carried out to investigate the effects of the proteasome inhibitor MG132 on the *in vitro* maturation of pig oocytes. Oocytes were cultured in a culture medium supplemented with 0.5, 1, 2, 5, 7.5, 10 or 20 μM MG132 for 48 h. The MG132 was diluted in dimethyl sulphoxide (DMSO) and added to the culture medium. The final concentration of DMSO did not exceed 0.1%. The control oocytes were cultured with medium supplemented with 0.1% DMSO for 48 h.

The aim of experiment 2 was to investigate the time interval during which oocytes cultured *in vitro* maintained sensitivity to MG132. Oocytes were cultured for 6, 12, 18 or 24 h in MG132-free medium and then cultured in medium supplemented with 10 μM MG132 to complete a total culture time of 48 h (i.e. they were cultured for another 42, 36, 30 or 24 h with MG132). The control oocytes were cultured in MG132-free medium for 48 h or in MG132-supplemented medium for 48 h.

Experiment 3 was carried out to determine the reversibility of the effects of MG132 on the *in vitro* maturation of pig oocytes. Oocytes were cultured with 10 μM MG132 for 48 h and were then cultured in MG132-free medium for 8 or 24 h. The control oocytes were cultured in medium supplemented with MG132 for 48, 56 or 72 h or in MG132-free medium for 48 or 72 h.

Statistical analysis

Data from all experiments were subjected to statistical analysis. Each experiment was carried out four times. The results were pooled for presentation and evaluated by chi-square analysis (Snedecor & Cochran, 1957). The mean percentage of oocytes or embryos reaching a given stage of maturation or development in all experiments did not vary from the pooled percentage by more than 2.5%. A p value of less than 0.05 was considered significant.

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Table 1 Effects of the proteasome inhibitor MG132 on *in vitro* maturation of pig oocytes

	MG132 (μM)							
	0	0.5	1	2	5	7.5	10	20
% of metaphase II oocytes[a]	92^{A}	87^{AB}	78^{B}	80^{B}	75^{B}	43^{C}	25^{D}	22^{D}
No of oocytes	120	120	120	120	120	120	120	120

Oocytes were cultured for 48 hours with different concentrations of MG132.[A,B,C,D]Significant differences ($p < 0.05$) between the treatments are indicated by different superscripts.
[a] The remaining oocytes were observed at the stage of metaphase I.

Results

Under our culture conditions, about 95% of the oocytes matured *in vitro* to metaphase II stage within 48 h. The remaining oocytes (4–8%) were at metaphase I. In control experiments, the oocytes were cultured in medium supplemented with 0.1% DMSO. The percentage of matured oocytes (92–96% of oocytes at metaphase II after 48 h culture) did not differ from that obtained in DMSO-free medium.

In experiment 1 we demonstrated that the proteasome inhibitor MG132, at concentrations above 1 μM, significantly inhibits the maturation of pig oocytes to metaphase II and blocks the maturation of a significant proportion of oocytes at metaphase I. Maximal inhibition was observed after treatment with 10 μM MG132 (Table 1).

In experiment 2 we demonstrated that the effect of MG132 is dependent on the addition of the inhibitor before the 18th hour of culture. We did not observe any significant effect of MG132 when this proteasome inhibitor was added to the pig oocytes at the 24th hour of culture or later (Table 2).

Interesting results were obtained in experiment 3 during which we demonstrated full reversibility of the inhibition of maturation of pig oocytes cultured with 10 μM MG132. After 48 h of culture with MG132, an 8 h culture in MG132-free medium allowed completion of maturation to metaphase II in 75% of the oocytes. When the 48 h culture with MG132 was followed by a longer culture in MG132-free medium, a significant proportion of oocytes (46%) underwent parthenogenetic activation. All activated oocytes had one extruded polar body and developed one pronucleus. A non-significant portion of oocytes (2%) was activated already after an 8 h reversal of the inhibitory block (Table 3).

Discussion

In our study we proved that exit from metaphase I of meiosis in pig oocytes is dependent on proteasome-dependent proteolysis. The addition of the proteasome inhibitor MG132 did not influence germinal vesicle breakdown and entry to the metaphase I stage of meiosis, but significantly reduced the proportion of oocytes that were able to continue maturation to stages beyond metaphase I. This inhibitory effect occurred in a dose-dependent manner, with the maximum effect at a concentration of 10 μM. Even at 20 μM MG132 we observed a significant proportion of the oocytes (22%) that were able to escape the inhibitory effect and to complete their maturation at the stage of metaphase II. On the basis of these data, pig oocytes seem to be more resistant to the effects of MG132 than rat oocytes (Josefsberg *et al.*, 2000), in which a concentration above 2 μM induced a complete block of meiosis at

Table 2 Effect of pre-culture time in MG132-free medium on the maturation of pig oocytes in MG132-supplemented medium (10 μM)

Pre-culture in MG132-free medium (h)	Culture in MG132-supplemented medium (h)	Stage of oocyte maturation (%)[a]				Total no. of oocytes
		M I	A I	T I	M II	
0	48	77^{A}	0^{A}	0^{A}	23^{A}	120
6	42	68^{A}	0^{A}	0^{A}	32^{A}	120
12	36	54^{B}	0^{A}	4^{A}	32^{A}	120
18	30	50^{B}	2^{A}	3^{A}	45^{B}	120
24	24	8^{C}	0^{A}	0^{A}	92^{C}	120
48	0	3^{C}	1^{A}	1^{A}	95^{C}	120

Oocytes were cultured in MG132-free medium for 6, 12, 18 or 24 h and then in MG132-supplemented medium (10 μM) to complete a total culture time of 48 h (i.e. 42, 36 or 30 h culture in MG132-supplemented medium). The control oocytes were cultured in MG132-supplemented medium (10 μM) or in MG132-free medium for 48 h.
[A,B,C] Significant differences ($p < 0.05$) between the percentage of the respective stage of oocyte maturation between various treatments (i.e. differences within a column) are indicated by different superscripts.
[a] M I, metaphase I; A I, anaphase I; T I, telophase I; M II, metaphase II.

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Table 3 The effect of the reversal of the inhibitory effect of MG132 on the maturation of pig oocytes *in vitro*

Type of culture		Stage of maturation (%)[a]					No. of oocytes
MG132-supplemented medium (h)	MG132-free medium (h)	M I	A I	T I	M II	Activated	
48	0	76^{A}	0^{A}	0^{A}	24^{A}	0^{A}	120
56	0	79^{A}	0^{A}	0^{A}	21^{A}	0^{A}	120
72	0	80^{A}	0^{A}	1^{A}	17^{A}	2^{A}	120
48	8	3^{B}	7^{A}	3^{A}	75^{C}	2^{A}	120
48	24	0^{B}	0^{A}	2^{A}	52^{B}	46^{B}	120
0	48	4^{B}	0^{A}	0^{A}	96^{D}	0^{A}	120
0	72	0^{B}	0^{A}	0^{A}	100^{D}	0^{A}	120

Oocytes were cultured in MG132 (10 μM), then carefully washed and subsequently cultured for 8 or 24 h in MG132-free medium. Control oocytes were cultured in MG132-supplemeted medium for 48, 56 or 72 h or in MG132-free medium for 48 or 72 h.

[A,B,C,D] Significant differences ($p < 0.05$) between the percentage of the respective stage of oocyte maturation between various treatments (i.e. differences within a column) are indicated by different superscripts.

[a] M I, metaphase I; A I, anaphase I; T I, telophase I; M II, metaphase II.

metaphase I. We can speculate that other proteasome-independent events also play an important role in the regulation of exit from metaphase I in pig oocytes.

Exit from metaphase I and entry in to anaphase I is generally attributed to a drop in the activity of maturation promoting factor (MPF) induced by inactivation of kinase activity of the complex formed by cyclin B and cyclin-dependent kinase $p34^{cdc2}$ (Motlík & Kubelka, 1990; Murray *et al*., 1989). The inactivation of this complex occurred after degradation of cyclin through ubiquitine-dependent proteolysis by proteasome (Glotzer *et al*., 1991; Motlík & Kubelka, 1995). From this point of view we can easily explain the block of meiosis at the stage of metaphase I in MG132-treated pig oocytes.

However, the complex of cyclin B and cyclin-dependent kinase $p34^{cdc2}$ can also be inactivated without the destruction of cyclin. After phosphorylation at specific sites, the complex loses its kinase activity. This mode of inactivation of MPF occurred, for example, during the *in vitro* aging of pig oocytes matured to the stage of metaphase II (Kikuchi *et al*., 1999). The exit of pig oocytes from metaphase I shares many regulatory events with exit from metaphase II (e.g. calcium-dependent events; Petr *et al*., 1997, 1999). If the proteasome-independent mechanism for inactivation of the complex of cyclin B and cyclin-dependent kinase $p34^{cdc2}$ functions also at the stage of metaphase I, it could induce a drop in MPF activity necessary for exit from metaphase I and progress of meiosis to metaphase II.

We demonstrated that pig oocytes matured for 24 h *in vitro* are insensitive to the proteasome inhibitor MG132. A significant inhibitory effect was observed only when MG132 was added to the cultured oocytes before the 18th hour of *in vitro* culture. On the basis of our data on the time sequence of maturation in pig oocytes (Petr *et al*., 1997), we can conclude that the sensitivity of pig oocytes to proteasome inhibition is limited to the stages preceding entry to metaphase I. This is different from the situation in rat oocytes described by Josefsberg *et al*. (2000). Rat oocytes are sensitive to proteasome inhibition by MG132 even during the transition from metaphase I to anaphase I. Under our culture conditions, transition from metaphase I to anaphase I occurred in pig oocytes cultured *in vitro* for 26–28 h (Petr *et al*., 1997), when sensitivity to MG132 had already disappeared.

Our further experiments revealed the full reversibility of the inhibitory action of MG132 on meiosis at the stage of metaphase I. After wash-out the inhibitor, a significant proportion of the oocytes (75%) had already completed their maturation to the stage of metaphase II after 8 h. This is a significantly higher reversal rate than that observed in rat oocytes (about 40%) by Josefsberg *et al*. (2000) and it confirms that the concentrations used are harmless to pig oocytes.

Under our culture conditions, the transition from metaphase I to metaphase II takes about 12 h (Petr *et al*., 1997). Completion of maturation in the oocytes reversed from the MG132 block within 8 h indicates acceleration of meiosis. This again indicates that the transition from metaphase I to metaphase II is not regulated by proteasome-dependent proteolysis alone. These proteasome-independent events were able to continue even under the effects of the inhibitor MG132 and, after the restoration of proteasome function following wash-out of the inhibitor, the transition from the metaphase I to the metaphase II was faster. Similar acceleration of meiosis was observed during reversal of

the effects of other inhibitors of meiosis, such as cycloheximide (Ye *et al*., 2002), 6-dimethylaminopurine (Avery *et al*., 1998) or dibutyrylcyclic AMP with testosterone (Petr *et al*., 1996).

Interesting data were obtained after reversal of the effects of the inhibitor MG132 for a longer culture interval (24 h) in MG132-free medium. A significant proportion of oocytes (46%) did not undergo a meiotic block at metaphase II and these oocytes underwent parthenogenetic activation. All parthenogenetically activated oocytes exhibited extrusion of the first polar body and one pronucleus. The presence of a polar body excludes the possibility that the observed pronuclear structure was formed after the decondensation of chromatin at metaphase I as occurred e.g. after the treatment of metaphase I pig oocytes with cycloheximide (Ding *et al*., 1992; Rozinek *et al*., 1996).

The reason for the absence of the meiotic block at the stage of metaphase II after reversal of MG132 is not clear. During meiotic maturation of the oocyte, MPF decreased at exit from metaphase I, then increased again and was stabilized. The second meiotic block at the stage of metaphase II is due to the stabilized high activity of MPF. We can only speculate that accelerated maturation after reversal of the MG132-block caused asynchrony of the increase in MPF relative to other regulatory events. As a result of this asynchrony, MPF is not stabilized, it is vulnerable to inactivation and spontaneous parthenogenetic activation can occur. The inactivation of non-stabilized MPF could be influenced by proteasome-independent events, which are clearly able to allow transition of a certain proportion of the oocytes from metaphase I to metaphase II. On the other hand, the proportion of oocytes activated after reversal of MG132 is significantly higher than the proportion that are insensitive to the MG132-induced block at the stage of metaphase I. This indicates that the cohort of oocytes resistant to the effect of MG132 on the transition from metaphase I to metaphase II is not identical to the cohort which undergo spontaneous parthenogenetic activation during the reversal of MG132.

From our data we can conclude that exit from metaphase I of meiosis is proteasome-dependent in pig oocytes matured *in vitro*. On the other hand, our data also indicate that other proteasome-independent events are involved in the regulation of exit from metaphase I.

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