Inhibition of cathepsin B activity reduces apoptosis by preventing cytochrome c release from mitochondria in porcine parthenotes

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Abstract. Cathepsin B, a lysosomal cysteine protease of the papain family, has recently been implicated in the quality and developmental competence of bovine preimplantation embryos. In this study, to determine whether inhibition of cathepsin B activity can improve porcine oocyte maturation and early embryo developmental competence, we supplemented in vitro maturation or embryo culture media with E-64, a cathepsin B inhibitor. Cathepsin B activity was high in poor quality germinal vesicle stage oocytes, but no differences in mRNA expression or protein localization were observed between good and poor quality oocytes, which were categorized based on morphology. Following treatment with 1 μM E-64, cathepsin B activity sharply decreased in 4-cell and blastocyst stage embryos. E-64 had no effect on cell number but significantly (P < 0.05) increased blastocyst formation and decreased the number of apoptotic cells in blastocysts. It also significantly (P < 0.05) enhanced mitochondrial membrane potential in blastocysts, reducing the release of cytochrome c and resulting in decreased expression of Caspase-3 and Caspase-9. In conclusion, inhibition of cathepsin B activity in porcine parthenotes using 1 μM E-64 resulted in attenuation of apoptosis via a reduction in the release of cytochrome c from mitochondria.

Key words: Cathepsin B, Cytochrome c, Embryo development, Oocyte maturation

Animal reproductive technology has proved to be very important in the last three decades, and the development of efficient procedures for assisted reproduction in livestock has generated multiple opportunities for commercialization. Oocytes subjected to in vitro maturation (IVM) are used in the vast majority of laboratories producing embryos by somatic cell nuclear transfer, in vitro fertilization and parthenogenetic activation (PA); however, the efficiency of development is lower than that of oocytes matured in vivo [1, 2]. In assisted reproductive technology, the capacity of development is determined by the quality of the oocytes and blastocysts produced following a long period of in vitro maturation and development, with high quality oocytes and blastocysts showing the capacity for successful development [3]. Thus, it is necessary to improve IVM and culture systems to produce embryos of good quality and high developmental competence [4].

In the early stages of embryonic development, apoptosis is closely related to embryo quality. Apoptosis, or programmed cell death, is a widespread biological phenomenon and is typically characterized by membrane blebbing, chromatin condensation, and DNA fragmentation [5]. Apoptosis involves a number of membrane receptors and a signal transduction cascade, resulting in the activation of several cysteine proteases known as caspases [6, 7]. In mammalian cells, the release of caspase activators from mitochondria regulates apoptosis [8]. In vivo, apoptosis first appears in 32- to 64-cell embryos and is subsequently observed throughout embryogenesis [5]. Although apoptosis occurs in mammalian blastocysts and is in fact crucial for further development, alterations to this process in the blastocyst compromise development and may lead to early embryonic death or fetal anomalies [9]. Apoptosis has been shown to compromise the developmental competence of mammalian oocytes [10, 11] and has been related to embryonic arrest and fragmentation in early stage embryos [12].

In recent years, cathepsins, and in particular cathepsin B, have been found to influence bovine oocyte developmental competence by regulating apoptosis [10, 13, 14]. Cathepsin B is a lysosomal cysteine protease of the papain enzyme family involved in inducing apoptosis, degrading the extracellular matrix, catabolizing intracellular proteins; and processing prohormones into active forms [15]. Cathepsin B can activate caspases indirectly through induction of mitochondrial membrane degradation, leading to translocation of apoptosis-inducing components from the mitochondria to the cytosol [16].

The function of cathepsin B has been well established in bovine cumulus-oocyte complexes (COCs), and an increase in its activity has been observed in poor quality and heat-shocked oocytes [10, 13]. Artificially inhibiting cathepsin B with the specific inhibitor E-64 greatly improves the developmental competence of preimplantation embryos and increases the number of good quality embryos by attenuating apoptosis [14].
Until now, success rates for in vitro development of porcine embryos have been very low, partly due to poor culture conditions and apoptosis during embryonic development [17, 18]. Although the role of cathepsin B has been elucidated in bovine oocytes, very little information exists regarding its function in porcine oocytes and early stage embryos. In the present study, we investigated the activity of cathepsin B in both porcine GV stage oocytes and PA embryos; and evaluated the consequences of its inhibition using E-64. Furthermore, mitochondrial membrane potential, apoptosis and cytochrome c release were analyzed.

Materials and Methods

Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Oocyte collection and sorting

Prepubertal porcine ovaries were obtained from a local slaughterhouse. Oocytes of good and poor quality were separated based on a previously published method [19]. In brief, COCs with more than three layers of cumulus cells were collected and defined as the good quality group, while denuded oocytes or COCs with dark cumulus cells were separated and considered the poor quality group. For evaluation of cathepsin B activity, all COCs were denuded by repeated pipetting in 0.1% hyaluronidase. The denuded oocytes were then washed three times in IVM medium prior to use in a cathepsin B activity assay.

IVM, PA; and culture of embryos

After collection, oocytes were cultured for 44 h in IVM media; consisting of tissue culture medium 199 (Medium 199, Gibco, Grand Island, NY, USA) supplemented with 0.57 mM cysteine, 10 ng/ml epidermal growth factor, 0.5 IU/ml luteinizing hormone; and 0.5 IU/ml follicle stimulating hormone. To evaluate the influence of E-64 on embryo development, oocytes matured in the presence of 0, 1, 10, or 100 μM E-64 were parthenogenetically activated with calcium ionophore A23187 (50 μM) for 5 min. Following this, oocytes and embryos were incubated with blocking solution of PVA-PBS containing 3.0% BSA at 37 C for at least 1 h. Next, oocytes and embryos were permeabilized using PVA-PBS containing 1% Triton X-100 at 37 C for 5 min. Following this, oocytes and embryos were incubated with a blocking solution of PVA-PBS containing 3.0% BSA at 37 C for at least 1.5 h and subsequently incubated overnight at 4 C with an anti-cathepsin B antibody (diluted 1:100; ab58802, Abcam, Cambridge, MA, USA). To investigate the colocalization of mitochondria and cytochrome c, blastocysts were incubated with 5 μM MitoTracker Red at 37 C for 45 min, and after three washes with IVC medium, they were fixed in 4% polyvinyl alcohol (PV A-PBS). Then the cells were fixed in 3.7% paraformaldehyde for 30 min at room temperature. Next, the blastocysts were permeabilized

| Table 1. Primer sequences and product sizes used for RT-qPCR |

| Gene     | GenBank accession number | Primer sequence (5’–3’) | Length (bp) |
|----------|--------------------------|-------------------------|-------------|
| Cathepsin B | NM_001097458             | F:GGCCCTCTATGACTCGACATGT R:TTTGAGACGGGGTGAGGC | 150         |
| Caspase 3 | NM_214131                | F:GGATTAGACGAGCACTGTTGGG R:CCGCTTCTGAATTCGCTCA | 124         |
| Caspase 9 | XM_003127618             | F:GTCTGCCAACCACCTAGTGAC R:AGGGTGCCCAGCCTCATTAT | 264         |
| Gapdh    | NM_001206359             | F:CGTGTCCGTTGTGATCGTTGA R:TGACGAGGGTGTTGAGG | 209         |
as described above and incubated with blocking solution at 37 C for 1 h. The samples were then incubated with an anti-cytochrome c antibody (diluted 1:100; ab110325, Abcam) at 37 C for 2 h. After incubation with primary antibodies, all samples were exposed to a fluorescein isothiocyanate-labeled secondary antibody for 1 h at 37 C. Thereafter, they were washed twice with PVA-PBS, treated with 5 μg/ml Hoechst 33342 for 10 min, washed twice more, and then mounted on glass slides. Microscopy was performed using a Zeiss LSM 710 inverted confocal laser scanning microscope with a 63× oil immersion objective (Zeiss Microimaging, Jena, Germany). Images were processed using the ZEN software (Zeiss). The colocalization of mitochondria and cytochrome c was analyzed using Image-Pro Plus (version 6.0; Media Cybernetics, Rockville, MD, USA).

Detection of intracellular cathepsin B activity

Detection of cathepsin B activity in oocytes and embryos was carried out using a Magic Red Cathepsin B Assay Kit (ImmunoChemistry Technologies, Bloomington, MN, USA) according to a previously published method [14]. In brief, germinal vesicle (GV) stage oocytes of good or poor quality, and 4-cell and blastocyst stage embryos were incubated in 500 ml PVA-PBS containing 2 ml of reaction mix in a humidified atmosphere of 5% CO₂ for 30 min. After rinsing in PBS with 3 mg/ml polyvinylpyrrolidone, the freshly stained embryos were mounted onto a glass slide and observed under a fluorescence microscope (Eclipse Ti, Nikon, Tokyo, Japan).

Detection of intracellular cathepsin B in oocytes classified by quality

To determine the relationship between cathepsin B and COC quality, the mRNA expression, localization; and activity of cathepsin B were measured in denuded oocytes classified as being of good and poor quality. There were no obvious differences in mRNA level (Fig. 1A) and protein distribution (Fig. 1B) in GV stage oocytes. Cathepsin B was clearly observed to be localized in the cytoplasm, and its activity was detected in the cytosol of both good and poor quality oocytes. The activity assay revealed that the red fluorescence of hydrolyzed cresyl violet (Arginine-Arginine₂) appeared stronger (P < 0.05) in poor quality oocytes than in those of good quality (Fig. 1C).

Expression and localization of cathepsin B in porcine PA embryos

To investigate the expression and localization of cathepsin B, porcine embryos of various stages, from the 1-cell to blastocyst stage, were used for RT-qPCR and immunostaining. As shown in Fig. 2A, compared with the 1-cell stage, similar cathepsin B mRNA levels (P > 0.05) were observed in 2- and 4-cell stage embryos. However, expression was dramatically reduced (P < 0.05) in the morula and blastocyst stages compared with the 1-cell stage. During earlier embryonic stages, cathepsin B protein displayed a uniform distribution in the cytoplasm, but was detected as strong dots in the cytoplasm of morulae and blastocysts (Fig. 2B).

Effect of E-64 on oocyte maturation

To assess the inhibitory effect of E-64 on oocyte maturation, COCs were matured with 0, 1, 10, or 100 μM E-64. There were no significant differences in maturation rate using concentrations of 0, 1, or 10 μM, but 100 μM of E-64 significantly reduced this rate (42.51 ± 6.19%) compared with the control group (74.71 ± 6.60%, P < 0.05, Fig. 3).

Effect of E-64 on embryo development and apoptosis

To investigate the inhibitory effect of cathepsin B can increase blastocyst formation and reduce apoptosis, activated oocytes were cultured in medium containing 0, 1, 10, or 100 μM E-64. After treatment, the activity of cathepsin B was significantly reduced in both 4-cell and blastocyst stage embryos (Figs. 4A and 4B).
Embryos treated with 1 μM (40.3 ± 4.06%) or 10 μM E-64 (39.2 ± 3.33%) displayed increased blastocyst formation compared with the control group (31.6 ± 3.91%). However, formation of blastocysts at a concentration of 100 μM (3.7 ± 3.04%) was lower than that of the control group (P < 0.01, Fig. 5A). Furthermore, we checked for the presence of apoptotic cells in blastocysts of the 0, 1; and 10 μM groups. Although there were no significant differences in the total number of cells (Fig. 5B), the number of apoptotic cells was notably lower in the 1 μM (6.8 ± 0.67, P < 0.05) and 10 μM groups (4.5 ± 0.58, P < 0.01) compared with the control group (9.8 ± 0.99, Fig. 5C).
Effect of E-64 on the expression of apoptosis-related genes

To explore the effect of cathepsin B inhibition on the expression of apoptotic genes, blastocysts cultured in the presence or absence of 1 μM E-64 were analyzed by RT-qPCR. The results demonstrated that cathepsin B inhibition markedly reduced the expression of caspase-9 and caspase-3 in blastocysts (P < 0.05, Fig. 6).

Effect of E-64 on colocalization of mitochondria and cytochrome c

To determine the manner in which inhibition of cathepsin B reduces apoptosis, the colocalization of mitochondria and cytochrome c was analyzed, as shown in Fig. 7. In untreated blastocysts, cytochrome c was dispersed throughout the cytoplasm, while it displayed a strong blot-like distribution in the E-64 treatment group, and was distinctly colocalized with mitochondria. Pearson’s correlation showed that E-64 treatment resulted in obvious colocalization compared with the control group, and that cytochrome c was weakly present in the cytoplasm in the treated group.

Effect of E-64 on mitochondrial membrane potential in blastocysts

To explore the mechanism by which E-64 affects the development of porcine parthenotes, mitochondrial membrane potential in blastocysts was measured. The results are shown in Fig. 8. E-64 treatment resulted in significantly higher mitochondrial membrane...
potential (P < 0.05) compared with the control group.

Discussion

In the present study, we investigated the expression, subcellular distribution, and activity of cathepsin B in porcine oocytes of poor and good quality. Inhibition of cathepsin B dramatically enhanced the development of PA embryos by protecting mitochondrial membranes and thus preventing cytochrome c release and apoptosis in blastocysts.

Follicular atresia is a key component of oogenesis and consisting of the removal of follicles not selected for ovulation. More than 99% of follicles undergo atresia in mammalian ovaries [24]; thus only very few oocytes of the highest quality are available for IVM following aspiration from the ovaries. Cathepsin-induced apoptosis is one mechanism by which follicular atresia is induced in mammalians [25, 26]. We therefore hypothesized that cathepsin B activity reduces the quality of both oocytes and early stage embryos in pigs.

To test this hypothesis, we first investigated the mRNA expression,
protein distribution, and activity of cathepsin B in GV stage oocytes. Although no difference in expression and distribution were observed, higher activity was seen in oocytes of poor quality. These results agree with those of similar studies using bovine cells [13]. In addition, it is generally accepted that a low oocyte quality compromises development following activation [19]. These findings demonstrate that cathepsin B activity is negatively correlated with oocyte quality, and that such activity can be used as an indicator of oocyte quality.

We subsequently investigated the distribution of cathepsin B in embryos. A very weak signal was seen in embryos of 1 to 4 cells, while a clustered localization pattern was present in morulae and blastocysts. This result corresponds well with the observation that apoptosis first appears at the 32- to 64-cell stages [5] and strongly indicates that cathepsin B is closely related to this process in embryos.

To test whether cathepsin B induces apoptosis in blastocysts, E-64 was employed to inhibit its activity. When treated with E-64, 4-cell and blastocyst stage embryos showed reduced cathepsin B activity, and the blastocyst formation rate increased as a result of cathepsin B inhibition. Furthermore, the results of our TUNEL assay clearly and that such activity can be used as an indicator of oocyte quality.

In conclusion, we found that cathepsin B induces apoptosis during the early stages of embryo development by degrading mitochondrial membranes, causing the release of cytochrome c into the cytosol; and resulting in the activation of caspases-9 and caspase-3. In addition, we established that cathepsin B activity can be used as a marker to evaluate the quality of porcine oocytes.

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References

1. Rath D, Niemann H, Torres CR. In vitro development to blastocysts of early porcine embryos produced in vivo or in vitro. Theriogenology 1995; 43: 913–926. [Medline] [CrossRef]
2. Macháty Z, Day BN, Prather RS. Development of early porcine embryos in vitro and in vivo. Biol Reprod 1999; 59: 451–455. [Medline] [CrossRef]
3. Lindner GM, Wright RW Jr. Bovine embryo morphology and evaluation. Theriogenology 1983; 20: 407–416. [Medline] [CrossRef]
4. Pawlik P, Renksa N, Pers-Kamcozy E, Warzych E, Lechtiak D. The quality of porcine oocytes is affected by sexual maturity of the donor gilt. Reprod Biol 2011; 11: 1–18. [Medline] [CrossRef]
5. Brill A, Torchinsky A, Carp H, Toder V. The role of apoptosis in normal and abnormal embryonic development. J Assist Reprod Genet 1999; 16: 512–519. [Medline] [CrossRef]
6. Thornberry NA. The caspase family of cysteine proteases. Br Med Bull 1997; 53: 478–496. [Medline] [CrossRef]
7. Turk B, Stoka V. Protease signalling in cell death: caspases versus cysteine cathepsins. FEBS Lett 2007; 581: 2761–2767. [Medline] [CrossRef]
8. Estañouer J, Valtelle F, Yassesir JL, Mignotte B. The mitochondrial pathways of cathepsin B. Adv Exp Med Biol 2004; 557: 157–183. [Medline] [CrossRef]
9. Brison DR, Schultz RM. Apoptosis during mouse blastocyst formation: evidence for a role of survival factors including transforming growth factor alpha. Biol Reprod 1997; 56: 1088–1096. [Medline] [CrossRef]
10. Baldouza AZ, Yamanaka K, Sakatani M, Kawahara M, Hegag AO, Zaabel SM, Takahashi M. Cathepsin B activity has a crucial role in the developmental competence of bovine cumulus-oocyte complexes exposed to heat shock during in vitro maturation. Reproduction 2013; 146: 407–417. [Medline] [CrossRef]
11. Cui XS, Jeong YJ, Lee HY, Cheon SH, Kim NH. Fetal bovine serum influences apoptosis and apoptosis-related gene expression in porcine parthenotes developing in vitro. Reproduction 2004; 127: 125–130. [Medline] [CrossRef]
12. Yu M, Qiu ZL, Li H, Zeng WS, Chen LN, Li QH, Quan S. Association between cell apoptosis and the quality of early mouse embryos. Nan Fang Yi Xue Da Xue Xue Bao 2011; 31: 409–413 (in Chinese). [Medline]
13. Balbouza AZ, Yamanaka K, Sakatani M, Hegag AO, Zaabel SM, Takahashi M. Cathepsin B activity is related to the quality of bovine cumulus oocyte complexes and its inhibition can improve their developmental competence. Mol Reprod Dev 2010; 77: 439–448. [Medline] [CrossRef]
14. Balbouza AZ, Yamanaka K, Sakatani M, Hegag AO, Zaabel SM, Takahashi M. Intracellular cathepsin B activity is inversely correlated with the quality and developmental competence of bovine preimplantation embryos. Mol Reprod Dev 2010; 77: 1031–1039. [Medline] [CrossRef]
15. Stoka V, Turk B, Turk V. Lysosomal cysteine proteases: structural features and their role in apoptosis. IURBM Life 2005; 57: 347–353. [Medline] [CrossRef]
16. Bossy-Wetzel E, Newmeyer DD, Green DR. Mitochondrial cytochrome c release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J 1998; 17: 37–49. [Medline] [CrossRef]
17. Mateusen B, Van Soom A, Maes DG, Donnay I, Ducaeteau L, Lequarre AS. Porcine embryo development and fragmentation and their relation to apoptotic markers: a cinematographic and confocal laser scanning microscopic study. Reproduction 2005; 129: 443–452. [Medline] [CrossRef]
18. Peters JK, Milliken G, Davis DL. Development of porcine embryos in vitro: effects of culture medium and donor age. J Anim Sci 2001; 79: 1578–1583. [Medline] [CrossRef]
19. Alvarez GM, Dalvit GC, Achi MV, Miguez MS, Cetica PD. Immature oocyte quality and maturational competence of porcine cumulus-oocyte complexes subpopulations. Biol Reprod 2009; 33: 167–177. [Medline] [CrossRef]
20. Nakatsuka M, Iwamura S, Yoshida S. Birth of piglets through the non-surgical transfer of blastocysts produced in vitro. J Reprod Dev 2004; 50: 487–491. [Medline] [CrossRef]
21. Cui XS, Li XY, Jeong YJ, Jan HH, Kim NH. Gene expression of coxSa, 5b, or 6b1 and their roles in preimplantation mouse embryos. Biol Reprod 2006; 74: 601–610. [Medline] [CrossRef]
22. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402–408. [Medline] [CrossRef]
23. Ge H, Tollner TL, Hu Z, Dai M, Li X, Guan H, Shan D, Zhang X, Lv J, Huang C, Dong Q. The importance of mitochondrial metabolic activity and mitochondrial DNA replication during oocyte maturation in vitro on oocyte quality and subsequent embryo developmental competence. Mol Reprod Dev 2012; 79: 392–401. [Medline] [CrossRef]
24. Borchfeld AN. Development of follicles in the mammalian ovary. Int Rev Cytol 1991; 124: 43–101. [Medline] [CrossRef]
25. Seriram V, Richards JS. Cathepsin L gene expression and promoter activation in rodent
granulosa cells. *Endocrinology* 2004; 145: 582–591. [Medline] [CrossRef]

26. Dhanasekaran N, Sheela Rani CS, Moudgal NR. Studies on follicular atresia: lysosomal enzyme activity and gonadotropin receptors of granulosa cells following administration or withdrawal of gonadotropins in the rat. *Mol Cell Endocrinol* 1983; 33: 97–112. [Medline] [CrossRef]

27. Barrett AJ, Kirschke H. Cathepsin B, Cathepsin H, and cathepsin L. *Methods Enzymol* 1981; 80: 535–561. [Medline] [CrossRef]

28. Klose A, Zigrino P, Dennhöfer R, Mauch C, Hunzelmann N. Identification and discrimination of extracellularly active cathepsins B and L in high-invasive melanoma cells. *Anal Biochem* 2006; 353: 57–62. [Medline] [CrossRef]

29. Joy B, Sivadasan R, Abraham T E, John M, Sobhan PK, Seervi M, T R S. Lysosomal destabilization and cathepsin B contributes for cytochrome c release and caspase activation in embelin-induced apoptosis. *Mol Cancer* 2010; 49: 324–336. [Medline] [CrossRef]

30. Guicciardi ME, Deussing J, Miyoshi H, Bronk SF, Svingen PA, Peters C, Kaufmann SH, Gores GJ. Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. *J Clin Invest* 2000; 106: 1127–1137. [Medline] [CrossRef]

31. Jiang X, Wang X. Cytochrome C-mediated apoptosis. *Annu Rev Biochem* 2004; 73: 87–106. [Medline] [CrossRef]