Relationship between *In Vitro* Maturation and Plasminogen Activator Activity on Porcine Cumulus-Oocytes Complexes Exposed to Oxidative Stress

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ABSTRACT

This study was undertaken to evaluate the relationship between *in vitro* maturation and plasminogen activators (PAs) activity on porcine cumulus-oocytes complexes (COCs) exposed to oxidative stress. When COCs were cultured in maturation medium with hydrogen peroxide (H_2O_2), the proportion of the germinal vesicle breakdown (GVBD) and oocytes maturation were decreased with addition of H_2O_2, and were significantly ($p<0.05$) lower in medium with 0.1 mM H_2O_2 than control group. Also, the rate of degenerated oocytes was increased in as H_2O_2 concentration increased. When COCs were cultured for 48 h, three plasminogen-dependent lytic bands were observed: tissue-type PA (tPA); urokinase-type PA (uPA); and tPA-PA inhibitor (tPA-PAI). PA activity was quantified using SDS-PAGE and zymography. When H_2O_2 concentration was increased, tPA and tPA-PAI activities also increased in porcine oocytes cultured for 48 h, but not uPA. In other experiment, embryos were divided into three groups and cultured in (1) control medium, (2) control medium with 1.0 mM H_2O_2 and (3) control medium with 1.0 mM H_2O_2 along with catalase in concentrations of 0.01, 0.1, and 1.0 mg/ml, respectively. H_2O_2 decreased the rate of GVBD and maturation in porcine COCs but catalase revealed protective activity against oxidative stress caused by H_2O_2. In this experiment, tPA and tPA-PAI activities were higher in media with 1.0 mM H_2O_2 alone. Increasing concentration of catalase decreased tPA and tPA-PAI activities in porcine oocytes. These results indicate that the exposure of porcine follicular oocytes to ROS inhibits oocytes maturation to metaphase-II stage and increase the oocytes degeneration. Also, we speculated that increased ROS level may trigger tPA and tPA-PAI activities in porcine oocytes matured *in vitro*.

(Key words : Oxidative stress, Porcine oocyte, *In vitro* maturation, Plasminogen activator)

INTRODUCTION

In mammal, immature oocytes were commonly matured under higher (20%) concentrations of O_2 than those of matured *in vivo*, resulted in increased accumulation of reactive oxygen species (ROS) in the cytoplasm of developing embryos (Luvoni *et al.*, 1996). The ROS, toxic metabolites of oxygen, including the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxidase (H_2O_2), and hydroxyl radical ($OH^{\cdot}$) are important mediators of inflammatory tissue injury (Weiss, 1986) and can damage cell membranes (Aitken *et al.*, 1989) and DNA (Halliwell and Aruoma, 1991) and may play a role in apoptosis (Yang *et al.*, 1998). There are therefore striking similarities between many known action of oxygen-derived free radicals and the events leading to oocyte maturation. Gutteridge and Halliwell (1988) reported that low levels of ROS may act as "trigger" molecules. In bovine, the ROS produced with the hypoxanthine-xanthine oxidase system plays a role in induction of oocyte nuclear and/or cytoplasmic maturation (Patrick *et al.*, 1997). Also, COCs have less sensitivity to apoptotic signals triggered by oxidative stress during *in vitro* maturation (Hedeki *et al.*, 2000).

PAs are specific enzymes that cleave plasminogen to form the active protease plasmin. Mammalian have two forms of plasminogen activator, tissue-type PA (tPA) and urokinase-type PA (uPA) (Dano *et al.*, 1985). Although these PAs are believed to be important in extracellular tissue remodeling in many physiological process, including fibrinolysis, ovulation, mammary involution, implantation (Dano *et al.*, 1985), fertilization (Huarte *et al.*, 1993). Irigoyen *et al.* (1999) reported that the unrestrained generation of plasmin from plasminogen by the action of PA is potentially hazardous to cells.

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The pericellular activation of plasminogen is a powerful proteolytic pathway that able to trigger apoptosis (Rossignol *et al.*, 2004). Also, Kwaan *et al.* (2000) reported that addition of plasminogen activator inhibitor-1 (PAI-1), a known inhibitor of PAs, to culture media inhibits spontaneous and induced apoptosis of multiple cell lines. Although correlations have been reported between oocyte maturation and PA production, the relationship between oxidative stress and PA activity during *in vitro* maturation has not been elucidated in porcine oocytes.

Therefore, this study was conducted to evaluate the relationship between *in vitro* maturation and PAs activity on porcine cumulus-oocytes complexes (COCs) exposed to oxidative stress.

MATERIALS AND METHODS

Culture Media

All chemicals used in this study were purchased from Sigma-Aldrich Corporation (St. Louis, Mo, USA) unless otherwise stated. The medium used for oocyte maturation was BSA-free North Carolina State University-23 (NCSU-23; Petters and Wells, 1993) supplemented with 10% (v/v) porcine follicular fluid (pFF), 0.6 mM cysteine, 10 IU/ml human chorionic gonadotropin (hCG) and 10 IU/ml pregnant mare's serum gonadotropin (PMSG).

Preparation of Oocytes

Porcine ovaries were collected at a local slaughterhouse and kept in saline (NaCl, 0.9% w/v ; penicillin 100,000 IU/l ; streptomycin 100 mg/l and amphotericin B 250 μg/l) at 30 to 32℃. COCs were aspirated from 2 to 6 mm follicles with a 10 ml syringe with 18-gauge needle. The collected oocytes were washed three times with a specified maturation medium and each group of 10 COCs were matured in 50 μl drop of BSA-free NCSU-23 under mineral oil at 39℃, 5% CO_2, in air. After culture for 24 h, oocytes were washed three times and then cultured in maturation medium without hormones for another 24 h at 39℃, 5% CO_2, in air.

Experimental Design

To evaluate the relationship between *in vitro* maturation and PAs activity on porcine COCs exposed to oxidative stress, the COCs were matured for 48 h in maturation medium (NCSU-23) containing 4 different concentrations of hydrogen peroxide (None, 0.01, 0.10, 1.00 mM) and/or catalase (None, 0.01, 0.10, 1.00 mg/ml). After maturation culture, the changes of PA activity were determined in COCs by SDS-PAGE and zymography.

Assessment of Meiotic Maturation

After maturation, COCs were freed from cumulus cells by washing three times of Hepes-buffered TALP medium containing 0.1 (v/v) polyvinylalcohol (H-TL-PVA) with a small-bore pipette. The maturation stages of oocytes were examined under a phase-contrast microscope at a magnification of × 200 or × 400. The oocytes were classified as in the GVBD phase, metaphase II (MII), or as degenerated.

SDS-PAGE and Zymography

SDS-PAGE and zymography were performed using procedures described by Dyk and Menino (1991) with a slight modification. All experiments measuring PA activity were repeated at least three times. Protease activity was quantified by densitometric scanning of the zymograph using NIH Image 1.62 (Center for information Technology National Institutes of Health, Maryland, USA). PA activities were expressed relatively to the activity in a fixed sample, which was different in each experiment.

Statistics

Data were analyzed by ANOVA using the General Linear Models procedure of the Statistical Analysis System (SAS Institute Inc., Cary, NC). When *F*-test results were significant in ANOVA, individual data were further tested by Duncan's multiple-range test. Differences with values of $p<0.05$ were considered to be statistically significant. Data are presented as mean±standard error of the mean (SEM).

RESULTS

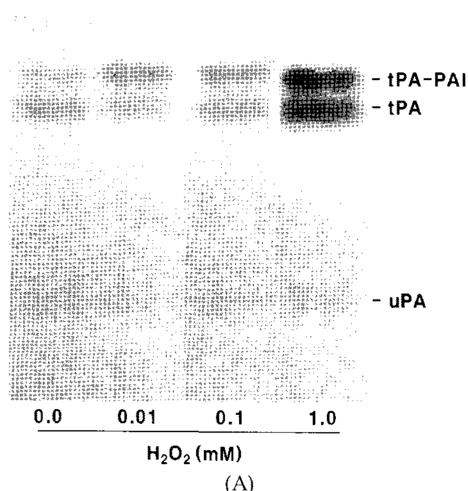
This study was shown that the effect of H_2O_2 and/or catalase on PAs activity and meiotic progression during maturation *in vitro* of porcine cumulus-oocytes complexes (COCs). The proportion of oocytes undergoing GVBD stage was significantly lower ($p<0.05$) in oocytes treated with 1.00 mM H_2O_2 and the maturation rate of oocytes was also significantly reduced ($p<0.05$) by addition of H_2O_2 to maturation medium. The incidence of degenerated oocytes was increased in higher H_2O_2 concentration (Table 1). The activities of tPA-PAI, tPA and uPA were evaluated in COCs at 48 h of maturation culture. When H_2O_2 concentration was increased, tPA and tPA-PAI activities were higher in porcine COCs cultured for 48 h, but not uPA (Fig. 1). On the other hand, H_2O_2 decreased the rate of GVBD and maturation in porcine COCs but catalase revealed protective activity against oxidative stress caused by H_2O_2 (Table 2). The activities of tPA and tPA-PAI were higher in media with 1.00 mM H_2O_2 alone. Increased catalase

Table 1. Effect of H_2O_2 on meiotic progression of porcine COCs

H_2O_2 (mM)	No. of oocytes	Percentage of nuclear stage (mean±sem)		
		GVBD	M-II	Degenerated oocytes
0.00	93	91.4±3.4[a]	82.8±7.6[a]	2.2±1.6[a]
0.01	93	83.9±8.7[a]	61.3±7.4[b]	8.6±3.1[a]
0.10	98	84.7±11.0[a]	60.2±8.1[b]	23.5±7.5[b]
1.00	107	44.9±12.3[b]	31.8±13.5[c]	66.4±8.4[c]

GVBD, germinal vesicle breakdown; M-II, metaphase-II. The results were expressed as mean±SEM (n=3). [a~c] Value with different superscripts in same columm is differ (p<0.05).

level has decreased tPA and tPA-PAI activity in porcine COCs (Fig. 2).



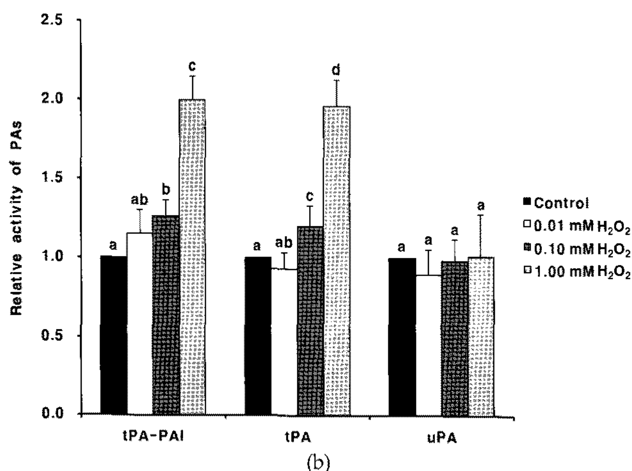
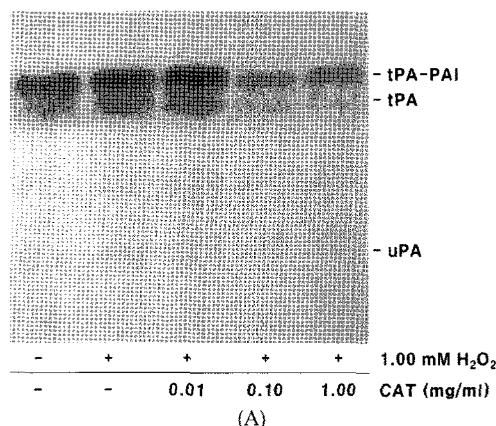


Fig. 1. Effects of H_2O_2 on PAs activity in *in vitro* matured porcine COCs. (A) Zymographic analysis of porcine COCs matured in medium with H_2O_2. (B) Intensity of PAs activity was quantified by densitometric scanning of zymography and expressed relative to the activity for tPA-PAI, tPA and uPA in the control group (None). Results are shown as mean±SEM (n=3). [a~d] Value with different letters are significantly different (p<0.05).



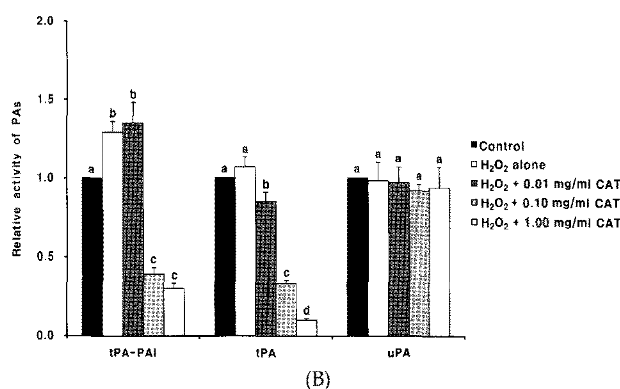


Fig. 2. Effects of catalase on PAs activity of porcine COCs treated with 1.00 mM H_2O_2. (A) Zymographic analysis of porcine COCs matured in medium with H_2O_2 and/or catalase. (B) Intensity of PAs activity was quantified by densitometric scanning of zymography and expressed relative to the activity for tPA-PAI, tPA and uPA in the control group (None). Results are shown as mean ±SEM (n=3). [a~d] Value with different letters are significantly different (p<0.05).

DISCUSSION

Although type of PAs detected in COCs seemed to be different according to the different species, the increase of PAs activity during maturation *in vitro* or *in vivo* has been reported in pig (Kim and Menino, 1995) and rat (Liu and Hsueh, 1987). Huarte *et al.* (1985) reported that tPA, but not tPA-PAI and uPA, activity was increased in cumulus-free mouse oocytes during maturation *in vitro*. In rats and mice, oocyte accumulates tPA mRNA during the maturation process and translation of this mRNA is triggered upon resumption of meiotic maturation (Huarte *et al.*, 1985; 1987). In our previous study, we reported that activities of tPA-PAI, tPA and uPA were observed in porcine oocytes that were attached with cumulus cells during maturation *in vitro* (Ann *et al., 2006).* Also, the addition of xanthine and xanthine oxidase to maturation medium increased the proportion of oocytes remaining GV stage and de-

Table 2. Effect of catalase on meiotic progression of porcine COCs treated with 1.00 mM H_2O_2

H_2O_2 (1.00 mM)	Catalase (mg/ml)	No. of oocytes	Percentage of nuclear stage (mean±sem)		
			GVBD	M-II	Degenerated oocytes
-	-	95	84.4±4.2[a]	83.2±6.3[a]	3.2±2.9[a]
+	-	96	35.4±6.8[b]	20.8±7.1[b]	71.9±5.7[b]
+	0.01	99	38.4±11.0[b]	27.3±8.3[b]	62.6±10.6[bc]
+	0.10	98	59.2±4.8[c]	45.9±5.0[c]	55.1±3.5[cd]
+	1.00	101	68.3±7.3[c]	52.5±8.4[c]	48.5±6.4[d]

GVBD, germinal vesicle breakdown; M-II, metaphase-II. The results were expressed as mean±SEM (n=3). [a~d] Value with different superscripts in same columm is differ (p<0.05).

generated-oocytes. In COCs exposed to ROS, the increased activity of tPA-PAI as well as tPA was observed (Sa *et al.*, 2010). In present study, the higher activity of tPA and tPA-PAI were observed in porcine COCs underwent oxidative stress by H_2O_2.

PAs are serine protease that cleaves plasminogen to form the active protease plasmin. Although these enzymes are believed to be important in extracellular tissue remodeling in many physiological and pathologic process (Dano *et al.*, 1985; Sappino *et al.*, 1989), the unrestrained generation of plasmin by plasminogen activation is potentially hazardous to cells. Thus, the process of plasminogen activation is strictly controlled through the availability of PAs, localized activation, and interaction with specific inhibitor (PA inhibitors) (Irigoyen *et al.*, 1999). Results of this study showed that addition of H_2O_2 to maturation medium increased the proportion of oocytes remaining GVBD stage and degenerated oocytes. In COCs exposed to H_2O_2, the increased activity of tPA as well as tPA-PAI was observed. However, the addition of catalase improved the maturation rate of porcine COCs and reduced the increase of tPA and tPA-PAI activity induced by H_2O_2. These results indicate that the enhanced activity of tPA and tPA-PAI may be associated with oxidative stress during porcine oocytes maturation periods. Rossignol *et al.* (2004) have demonstrated that pericellular activation of plasminogen is a powerful proteolytic pathway that able to trigger apoptosis and that protease nexin-1, a potent serine proteinase inhibitor, inhibited the activity of plasmin and tPA via the formation of inhibitory complexes and prevented cell detachment and apoptosis. PA inhibitor type-1 (PAI-1) may play a significant role in regulating plasmin formation at the cell surface (Lee *et al.*, 1996) and increased expression of PAI-1 is associated with decreased apoptosis of neoplastic cells (Chen *et al.*, 2004). ROS may induce the PA activity in porcine oocytes during *in vitro* maturation and PA activity may be relevant to apoptotic cell death in porcine oocytes by oxidative stress (Sa *et al.*, 2010).

In summary, the present study provides information concerning on the effect of H_2O_2 and/or catalase on PAs activity and meiotic progression during maturation *in vitro* of porcine COCs. The proportion of oocytes remaining GV stage and oocytes-degenerated were increased in the COCs exposed to oxidative stress by H_2O_2. The higher activity of tPA and tPA-PAI were observed in porcine COCs underwent oxidative stress by H_2O_2. However, the addition of catalase to oocytes maturation medium revealed protective activity against oxidative stress caused by H_2O_2 and decreased tPA and tPA-PAI activity in porcine COCs.

These results suggest that oxidative stress may induce the PAs activity in porcine COCs during maturation *in vitro* and antioxidant may be relevant to the reduction of increasing activity of PAs caused by oxidative stress.

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