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Changes of Plasminogen Activator Activity under Heat Stress Condition in Porcine Endometrium

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ABSTRACT The aim of this study was to investigate effect of heat stress on expression levels of plasminogen activators (PAs) related mRNAs and proteins, and changes of PAs activity in porcine endometrial explants. The endometrial explants (200 ± 50 mg) were isolated from middle part of uterine horn at follicular phase (Day 19-21) and were pre-incubated in serum-free culture medium at 38.5°C in 5% CO_2 for 18 h. Then, the tissues were transferred into fresh medium and were cultured at different temperature (38.5, 39.5, 40.5 or 41.5°C) for 24 h. The expression level of urokinase-type PA (*uPA*), type-1 PA inhibitor (*PAI-1*), type-2 PAI (*PAI-2*), and heat shock protein-90 (*HSP-90*) mRNA were analysis by reverse-transcription PCR and proteins were measured by western blotting. The supernatant were used for measurement of PAs activity. In results, mRNA and protein levels of HSP-90 was higher in 41.5°C treatment groups than other treatment groups ($p < 0.05$). The expression of *uPA*, *PAI-1*, and *PAI-2* mRNA were slightly increased by heat stress, however, there were no significant difference. Heat stress condition suppressed expression of active uPA and PAI-2 proteins ($p < 0.05$), whereas PAI-1 protein was increased ($p < 0.01$). Although PAI-1 protein was increased and active uPA was decreased, PAs activity was greatly enhanced by exposure of heat stress ($p < 0.05$). These results suggest that heat stress condition could change intrauterine microenvironment through regulation of PAs activity and other factors regarding with activation of PAs might be regulate by heat stress. Therefore, more studies regarding with regulatory mechanism of PAs activation are needed.

Keywords: endometrium, heat shock protein, heat stress, pigs, plasminogen activators activity

INTRODUCTION

In the pig industry, reproductive performance is one of important factors that are directly influenced to economic benefit, and it is affect by environmental and genetic factors. Furthermore, another factors such as feed, breed, temperature, light and nutrient condition, and water consumption influenced to reproductive performance (Peltoniemi et al., 1999). Pigs have a thick subcutaneous fat and small number of sweat glands, and these physiological features led to higher sensitivity of pig than other species and seasonal infertility (Love et al., 1993). Continuous exposure into high temperature is caused to disrupt various physiological and cellular phenomenon including redistribution of blood flow, function of germ cells, embryo development, delayed puberty of gilts and a reduced

litter size during summer (Claus and Weiler, 1985; Prunier et al., 1996; Li et al., 2015).

A increasing of temperature in the testis led to reduced sperm motility and concentration, and increase population of abnormal spermatozoa in ejaculate (Hansen, 2009). Also, heat stress induced hypoxia and oxidative stress in cells through generation of reactive oxygen species (ROS), and it lead to apoptosis and DNA fragmentation in spermatogenic cells (Paul et al., 2009). Paul et al. (2009) reported that fertilized oocytes with heat stress-exposed sperm had lower developmental competence than fertilized oocytes with normal sperm in mice. Female reproductive events are disrupted by heat stress as well as male reproduction. Heat stress exposure in heifers and lactating cows in summer was caused reduced fertilization and early embryonic development (Sartori et al., 2002). Moreover, heat stress could alter steroid secretion, gene expression, and follicle growth that are caused to disrupt oocyte maturation (Roth et al., 2001). Maternal heat stress could induced generation of ROS in female reproductive tracts and these might be involved in regulation of uterine environments.

As one of serine protease, plasminogen activators (PAs) convert inactive plasminogen to active plasmin, and this proteases are divided two form as urokinase-type (uPA) and tissue-type (tPA). Activation of PAs was regulated by their specific inhibitors (type-1 PA inhibitor, PAI-1; type-2 PA inhibitor, PAI-2). These PAs and their inhibitors are secreted by variety of cells, and they play an important role in tissue remodeling via degradation of fibrin and activation of matrix metalloproteinase. These physiological roles of PAs system are closely involved in various reproductive phenomenon including angiogenesis, implantation, placentation, ovulation and fertilization (Bazer, 2013). In our previous study (Hwangbo et al., 2016), heat stress on porcine uterine epithelial cells slightly decreased two-type of PAs and PAIs and significantly increased expression of tPA protein. However, monolayer cultured cells in *in vitro* environment lose their physiological function and this model is not suitable for investigation of uterine biology. Therefore, we used explant culture model for more precise reaction of uterus under heat stress condition, and the aim of this stud was to investigate effect of heat stress on expression levels of plasminogen activators (PAs) related mRNAs and proteins, and changes of PAs activity in porcine endometrial explants.

MATERIALS AND METHODS

Endometrial explant culture and induction of heat stress

All procedures that involved the use of animals were approved by the Kangwon National University Institutional Animal Care and Use Committee (KIACUC-09-0139). The uterus was collected from a local slaughterhouse and was transported to the laboratory within 2 h on ice. Estrous cycle of uterus was identified by morphology of follicles and uterus at follicular phase (Day 19-21), which has up to 6 mm in a diameter of antral follicles, was used. Porcine uterus was washed twice using Hank's Balanced Salt

Table 1. Primer sequences used for RT-PCR

Gene	Primer sequence (5′→3′)	Product size (bp)	Annealing temp.	Accession number
uPA	F: CCTACAAGTACTTCTC R: GCAAACCAAGGCTGGTTTCTC	460	55°C	NM_213945
tPA	F: AGGAGGCCTCTATGCTGACA R: GGCACACAGCATATTGTTGG	544	59°C	NM_214054
PAI-1	F: GCCGATGCCATCTTCGTGCA R: TCCAGGATGTCGTAGTAACGGC	400	60°C	NM_213910
PAI-2	F: CGACTCAGCGCAAATCAGTA R: GTCATTCTTTCCCGACATGC	360	55°C	XM_003121697
HSP-90	F: CGGAAATCGCCCAGTTGATG R: ATGAACGCCTTGGTCCCAGA	286	59°C	NM_213973.2
ACTB	F: AAATGGGCACGTTGTGGGTG R: AGGCCAACCGGGAGAAGATG	159	60°C	XM_003124280

uPA, urokinase-type plasminogen activator; *tPA*, tissue-type plasminogen activator; *PAI-1*, type-1 plasminogen activator inhibitor; *PAI-2*, type-2 plasminogen activator; *HSP-90*, heat shock protein-90; *ACTB*, actin-beta.

Solution (HBSS) containing 0.1% (w/v) BSA and uterine horn was separated from connective tissues. Endometrial tissues (200 ± 50 mg) in middle part of uterine horn were isolated from perimetrium and pre-incubated in serum-free Dulbecco's modified eagle's medium/Ham's F-12 nutrient mixture (DMEM/F-12) containing 0.1% (v/v) antibiotic-antimycotic and 20 μg/mL amphotericin B. To induce heat stress, tissues were incubated at 38.5 (control group), 39.5 40.5 and 41.5°C for 24 h after pre-incubation.

Reverse transcription-PCR

To extract total RNA from cultured tissues, trizol reagen (Takara, Japan) was used and cDNA was synthesized using Maxime TR Premix (iNtRON Biotechnology, Korea) according to manufacturer's protocol. Concentration of total RNA and cDNA was quantified by Nanodrop 2000 (Thermo Scientific Nanodrop, Wilmington, DE, USA). All of primers for specific sequence in *uPA, tPA, PAI-1, PAI-2,* heat shock protein-90 (*HSP-90*) mRNA were designed using Primer 3 plus (Table 1) and PCR was conducted. The PCR productions were confirmed by 2% agarose gel electrophoresis containing ethidium bromide (EtBr, Bioneer, Korea) and band images were analyzed by Image J software (Version 1.46; National Institutes of Health, Bethesda, MD, USA).

Western blot

Cultured tissues were homogenized in 200 μL mammalian protein extract reagent (M-PER) buffer and rotated for protein extraction at room temperature within 1 h. The resulting homogenate was centrifuged at 12,000 rpm and 4°C for 20 min and the supernatants were collected in a fresh tube. Total protein concentrations were determined according to the method of Bradford. Protein (50 μg) was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for 90 min at 150 V and transferred to polyvinylidene fluoride (PVDF) membranes for 60 min at 190 V. Membranes were blocked in 5% skim milk for 45 min at room temperature and incubated with the following primary antibodies at 4°C overnight: rabbit anti-HSP90 (1:1000, Calbiochem, USA), mouse anti-β-actin (1:500, Novus, USA) and anti-PAI-2 (1:500, Santa Cruz Biotechnology, Santa Cruz, Ca, USA), and goat anti-uPA and anti-PAI-1 (1:500, Santa Cruz Biotechnology). Membranes were washed three times in Tris-buffered saline/tween-20 (TBS-T) and incubated at room temperature for 60 min using the HRP-conjugated secondary antibodies, donkey anti-mouse IgG (1:1000, Santa cruz, USA), donkey anti-goat IgG (1:1000, Santa cruz, USA), and goat anti-rabbit IgG (1:1000, Santa cruz, USA). All proteins were visualized using the West Save ECL kit (Ab frontier) and detected using a gel documentation system and image

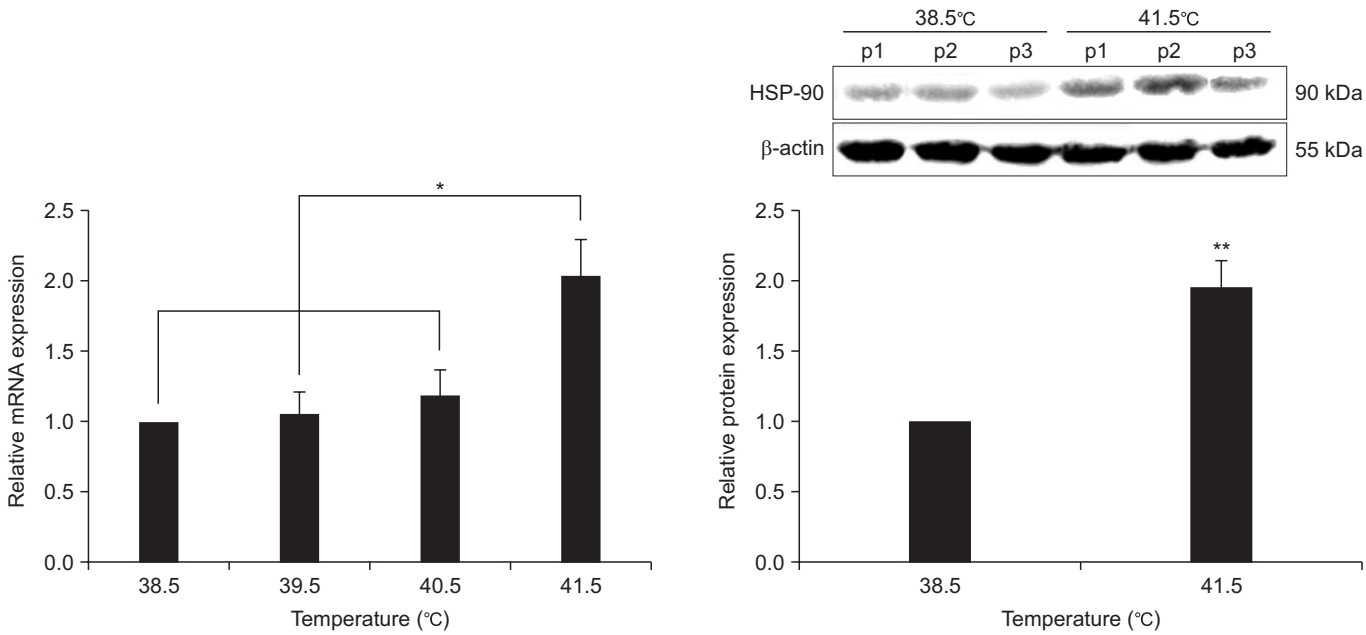

Fig. 1. The expression of heat shock protein-90 (HSP-90) mRNA and protein in porcine endometrial tissues exposed to different temperature (38.5, 39.5, 40.5 and 41.5°C). Asterisk indicates significant differences (*$p < 0.05$, **$p < 0.01$).

J was used for image analysis.

PAs activity assay

Samples of the collected culture medium (20 μL) were dispensed into a 96-well microplate and mixed with 30 μL of a plasminogen working solution (2.5 μg/well plasminogen; Sigma-Aldrich). The solution was incubated at 38°C for 1 h. After incubation, 200 μL of substrate buffer [0.18 mM Z-L-Lys-SBzl hydrochloride, 0.22 mM 5,5′-dithiobis-(2-nitrobenzoic acid), and 0.01% Triton X-100] was added and was further incubated at 38°C for 1 h. PA activity was determined by absorbance at the wavelength of 405 nm using a microplate reader.

Statistical analysis

All numerical data representing each parameter were analyzed using the Statistical Analysis System Software (SAS version 9.4). Data are presented means ± SEM, and comparisons among treatment groups were conducted by t-test using a generalized linear model (GLM) in the SAS package. A value of $p < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

Expression of HSP-90 mRNA and protein in heat stress-exposed explants

To confirm temperature that heat stress is induced in porcine endometrial tissue, mRNA and protein levels of HSP-90 were measured (Fig. 1). The expression of *HSP-90* mRNA was higher in 41.5°C-exposed tissue than other treatment groups (38.5, 39.5 and 40.5°C) ($p < 0.05$). Heat stress in 41.5°C culture condition increased HSP-90 protein compared with control group as well as mRNA expression ($p < 0.05$).

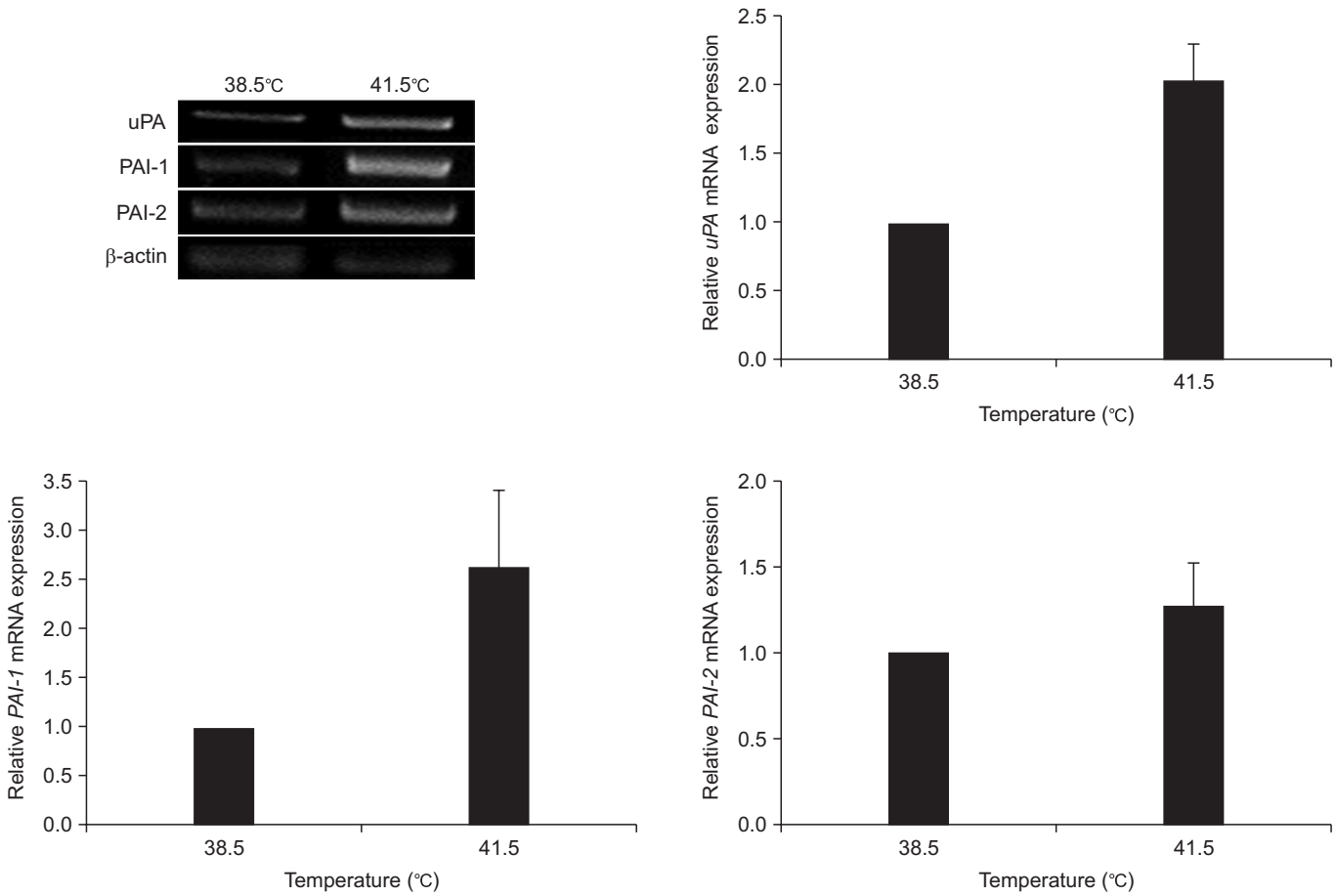

Fig. 2. Effect of heat stress on urokinase-type plasminogen activator (*uPA*), type-1 PA inhibitor (*PAI-1*) and type-2 PA inhibitor (*PAI-2*) mRNA in porcine endometrial tissues exposed to different temperature (38.5 and 41.5°C).

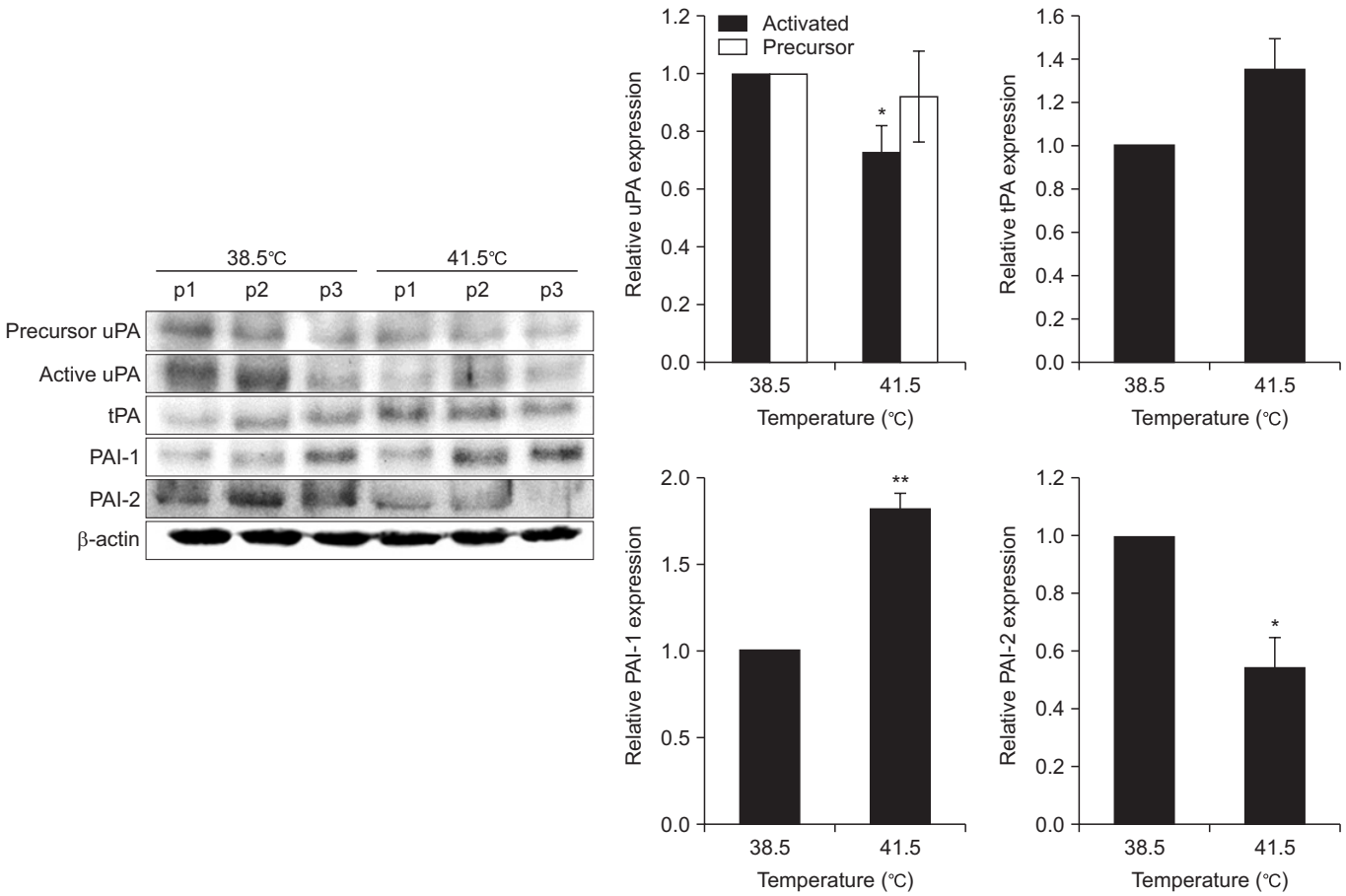

Fig. 3. Change of protein levels of urokinase-type plasminogen activator (uPA), tissue-type PA (tPA), type-1 PA inhibitor (PAI-1) and type-2 PA inhibitor (PAI-2) in porcine endometrial tissues by heat stress condition. Asterisk indicates significant differences (*$p < 0.05$, **$p < 0.01$).

Change of PAs activity and expression under heat stress condition

The expression level of mRNA and protein of PAs related factors under heat stress condition were showed in Fig. 2 and Fig. 3, respectively. Heat stress treatment slightly increased *uPA*, *PAI-1*, and *PAI-2* mRNA (*tPA* mRNA did not detected), but there were no significant difference. Although mRNA levels were not changed by heat stress, activated form of uPA and PAI-2 proteins were reduced in treatment group ($p < 0.05$), whereas PAI-1 protein was increased by heat stress (Fig. 4, $p < 0.01$). Interestingly, PAs activity in supernatants from heat stress-exposed explants was greatly higher than control group ($p < 0.05$) and this activity pattern was not corresponded with protein expression pattern.

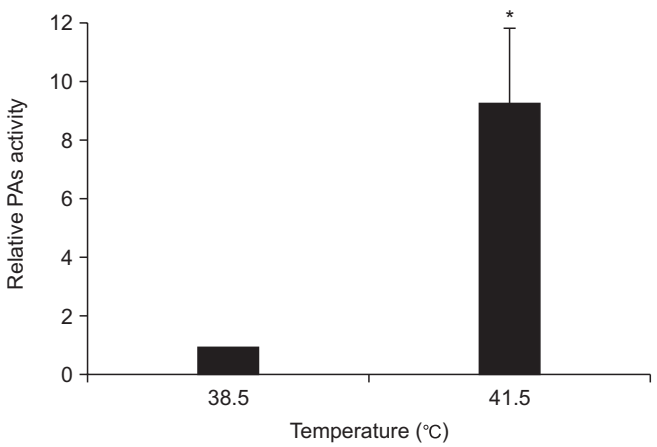

Fig. 4. Effect of heat stress condition on plasminogen activators (PAs) activity in porcine endometrial tissues. Asterisk indicates significant differences (*$p < 0.05$).

DISCUSSION

This present study was conducted to confirm effect of heat stress on expression of two-types of PAs and PAIs, and change of PAs activity in porcine endometrial tissues. The expression of HSP-90 mRNA and protein was significantly increased in cultured explants with 41.5°C condition. All of mRNA were did not affected by heat stress in tissues. Protein level of uPA and PAI-2 were suppressed in heat stress-exposed tissues, whereas PAI-1 was stimulated. Despite upregulation of PAI-1 and downregulation of uPA, PAs activity was increased by heat stress.

Ambient temperature and light duration that are increased in the summer are directly affect to reproductive performance in pigs. In the domestic animals, continuous exposure by high temperature led to reduce milk production, growth rate of litters and feed intake (Schoenherr et al., 1989). In particular, feed intake in pigs is closely related with various reproductive phenomenon including activity and steroid hormone concentration in ovary, activation of aromatase, delayed ovulation, and reduction of luteinizing hormone receptor (Dourmad et al., 1994). Also, heat stress is caused disruption of oocytes function, decreased embryo development and reduction of steroid hormone secretion in cows (Roth et al., 2001; Sartori et al., 2002). These reports demonstrated that reproduction of mammalians is directly or indirectly influenced by heat stress.

Heat stress is defined as one of stress that occurs as a result of increasing external and internal body temperature. In present study, both of mRNA and protein expression of HSP-90 were increased in 41.5°C-exposed explants, especially, mRNA expression in 41.5°C-exposed explants was higher than other temperature groups. Commonly, pigs maintain their body temperature around 37.8-40.7°C (Li et al., 2015) and HSPs are produced by cells in response to exposure to stressful conditions. Thus, this result indicated that heat stress in pigs might be induce by internal temperature up to 41°C.

As one of female reproductive tracts, uterus plays roles in transport of spermatozoa, embryo development and construction of microenvironment for implantation. These physiological roles of uterus are influenced by change of steroid hormones, cytokines, and gonadotropins. Although mRNA levels of *uPA*, *PAI-1* and *-2* were not changed by heat stress, protein levels were differently regulated under heat stress condition. Kobayashi et al. (2013) reported that expression of microsomal prostaglandin E synthase 1 mRNA was enhanced by summer heat stress in bovine oviductal epithelial cells, however, protein level of prostaglandin E2 was not altered. Therefore, heat stress in uterus and oviduct would selectively regulate transcription or translation process.

Rensis and Scaramuzzi (2003) reported that blood flow into uterus in cows was reduced by increasing of intrauterine temperature. As one of proteases for tissue remodeling, plasmin, which is converted by PAs, play an important role in angiogenesis. In this study, heat stress in 41.5°C condition reduced protein level of uPA and PAI-2, and increased PAI-1 protein. These expression pattern of PAs was seem to be correspond with reduced blood flow through suppression of angiogenesis. However, activation of PAs was greatly stimulated by heat stress. In regulatory mechanism of PAs activity, steroid hormones and various factors such as α2-anitiplasmin, α2-macroglobulin, Kalikrein, Factor XIα and XIIα participate in conversion of plasminogen and suppression of plasmin activity. Lee et al. (2014) reported that PAs activity in three-dimensional cultured porcine endometrial cells was suppressed by 17β-estradiol treatment. Therefore, increased PAs activity by heat stress might be regulated by other regulatory factors and altered steroid hormones, and it is expected that one of responses against reduced blood flow into uterus.

In conclusion, our findings showed that heat stress in porcine endometrium is occurred up to 41.5°C conditions and it up-regulated PAs activity in protein level. Increasing of PAs activity might be related with regulation of blood flow into reproductive tracts under heat stress condition, however, regulatory mechanism of PAs activation by heat stress is still unclear. Therefore, investigation of other factors regarding with plasmin activity under heat stress in porcine endometrium is required.

CONFLICTS OF INTEREST

No potential conflict of interest relevant to this article was reported.

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