Summer heat stress affects prostaglandin synthesis in the bovine oviduct

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Abstract
Summer heat stress (HS) negatively affects reproductive functions, including prostaglandin (PG) F2α secretion in the endometrium, and decreases fertility in cattle. In the present study, we examined the effects of elevated temperatures on PG synthesis in oviductal epithelial cells. The epithelial cells obtained from the ampulla and isthmus of the oviduct were incubated at various temperatures (38.5, 39.5, 40.0, and 40.5°C) for 24 h. In the ampulla, PGE2 concentration was higher at 40.5°C than at 38.5°C, while PGF2α production was not affected by the temperatures in this range. The expressions of microsomal PGE synthase 1 (PTGES1), cytosolic PGES (PTGES3), and heat shock protein 90 (HSP90AA1, HSP90) mRNAs and proteins were higher at 40.5°C than at 38.5°C in the ampullary epithelial cells. Seasonal changes in the expressions of PGES and HSP90AA1 mRNAs in oviductal tissues were also investigated. The expressions of PTGES3 and HSP90AA1 mRNAs were higher in the ampullary tissues in summer than in winter. In summary, elevated temperatures stimulated PGE2 production in the ampullary oviduct by increasing the expressions of PGESs and HSP90AA1, which can activate cPGES. The overall results suggest that HS upsets PG secretions and reduces oviductal smooth muscle motility, which in turn could decrease gamete/embryo transport through the oviduct.

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Introduction
In mammals, the oviduct plays roles in the transport of gametes and fertilized oocytes and the development of embryos (Menezo & Guerin 1997, Suarez 2008, Ulbrich et al. 2010). After fertilization has occurred in the ampulla of the oviduct, the embryo is transported to the uterus within a few days. The transport to the uterus is caused by waves of contraction and relaxation of the smooth muscle and the ciliation of the oviductal epithelial cells (Halbert et al. 1976, Hunter 2012). Prostaglandin (PG) E2 and PGF2α concentrations in the ovulatory phase (CL) or the dominant follicle are highest in the periovulatory phase (Wijayagunawardane et al. 1998). These PGs seem to control gamete/embryo transport by stimulating smooth muscle motility (Wijayagunawardane et al. 2001).

PG biosynthesis starts from the liberation of arachidonic acid from phospholipids (Okuda et al. 2002). Cyclooxygenases convert arachidonic acid to PGH2, which is the precursor for various PGs including PGE2 and PGF2α (Garavito et al. 2002, Gabler et al. 2008). The conversion of PGH2 to PGE2 or PGF2α is catalyzed by the specific downstream enzymes PGE synthase (PGES) and PGF synthase (PGFS) respectively (Arosh et al. 2002). The bovine oviduct expresses three PGES isozymes: microsomal PGES1 (mPGES1), mPGES2, and cytosolic PGES (cPGES) (Gauvreau et al. 2010).

Summer heat stress (HS) weakens estrous behavior, suppresses follicular development, and causes early embryo mortality, all of which decrease the fertility rate in cows (Gwazdauskas et al. 1975, Cavestany et al. 1985, De Rensis & Scaramuzzi 2003, Sakatani et al. 2004, Hansen 2009). HS also increases PGF2α production in the endometrium, which might result in the early regression of CL or the death of embryos (Putney et al. 1988, Malayer et al. 1990). HS is of special concern to the livestock industry because of recent global warming.

Heat shock proteins (HSPs) are highly expressed under hot conditions and protect cells from various stresses including heat and reactive oxidants (Kregel 2002). In particular, HSP90AA1 (HSP90) is known to activate cPGES, which increases the production of PGE2 in rat fibroblasts (Tanioka et al. 2003). Thus, HS may stimulate PGE2 production in the oviductal epithelium, which would reduce gamete/embryo transport.

We hypothesized that HS negatively affects PG synthesis in the bovine oviduct. In the present study, to prove the above hypothesis, we investigated the effects of elevated temperatures on the synthesis of PGE2.
and PGF$_{2\alpha}$ in cultured bovine oviductal epithelial cells. We also studied the expressions of PGESs and HSP90AA1 in bovine oviducts obtained in winter and summer.

Materials and Methods

Collection of bovine oviducts

Oviducts from Holstein cows were collected at a local abattoir within 10–20 min of exsanguination. The stages of the estrous cycle were determined based on a macroscopic observation of the ovaries and uteri (Okuda et al. 1988, Miyamoto et al. 2000). After trimming of the oviducts that were ipsilateral to the CL, the ampullary and isthmic sections were immediately frozen and stored at −80°C until mRNA extraction. For cell culture experiments, the oviducts were submerged in ice-cold saline and transported to the laboratory.

Isolation of oviductal cells

Oviductal tissues collected at days 0–3 after ovulation were utilized for cell culture. The oviduct was separated into infundibulum, ampulla, ampullary–isthmic junction, isthmus, and utero–tubal junction sections (Fig. 1). The epithelial cells were isolated from the ampullary and isthmic sections of the oviduct. A Teflon catheter (internal diameter: 0.5 mm) was inserted into the ampullary and isthmic sections of the oviduct, and the luminal wall was washed five times with 1 ml of sterile Hank’s balanced salt solution (HBSS; Sigma–Aldrich), 0.1% (wt/vol) BSA, 100 IU/ml penicillin, and 100 µg/ml streptomycin (at a flow rate of 10 ml/min, 38°C, for 30 min). After perfusion, the dissociated epithelial cells were filtered through metal meshes (150 and 77 µm) to remove undissociated tissue fragments. The filtrates were washed by centrifugation (180 g for 10 min at 4°C) with Tris-buffered ammonium chloride (pH 7.5) to remove hemocytes and with DMEM (Sigma–Aldrich) supplemented with 0.1% (wt/vol) BSA, 100 IU/ml penicillin, and 100 µg/ml streptomycin. After the washes, the cells were counted using a hemocytometer. Cell viability was >95% as assessed by 0.5% (wt/vol) trypan blue dye exclusion. The final pellets of the epithelial cells were resuspended in DF (DMEM/Ham’s F-12; 1:1 (vol/vol) Invitrogen supplemented with 10% (vol/vol) bovine serum (Invitrogen), 20 mg/ml gentamicin (Invitrogen), and 2 mg/ml amphotericin B (Sigma–Aldrich)). These cells were seeded at a density of 1.0×10⁵ viable cells/ml in 25 cm² culture flasks (Greiner Bio-One, Frickenhausen, Germany) and cultured at 38.5°C in a humidified atmosphere of 5% CO₂ in air. Because the epithelial cells attached 24–48 h after plating, the medium in the epithelial cell culture was replaced 48 h after plating. The medium was changed every 2 days until confluence was reached.

To purify the epithelial cells, the cells were trypsinized on reaching sub-confluence. Briefly, the cells in the culture flasks were washed with PBS (−) twice. After washing, 0.02% (wt/vol) porcine trypsin (1000–2000 BAEE units/mg solid; Sigma–Aldrich) with 0.008% (wt/vol) EDTA (Sigma–Aldrich) in PBS was added to the flasks, and the cells were incubated for 5 min at 38.5°C to detach stromal cells. Then, the solution containing the stromal cells was removed, and the remaining cells were incubated with 2 mmol/l EDTA in PBS for 2 min at 38.5°C. After incubation, the solution was removed and the cells were washed with PBS (−) and the cells were incubated for 10 min at 38.5°C with 0.02% (wt/vol) bovine trypsin in PBS. After incubation, the cells were washed by centrifugation (180 g for 10 min at 4°C) with the culture medium. Then, the cells were placed in fresh DF to adjust them to a density of 1.0×10⁵ viable cells/ml. These cells were seeded in 3–12 mm² well plates (Thermo Fisher Scientific, Waltham, MA, USA), 48-well plates (Greiner Bio-One), and 75 cm² culture flasks (Greiner Bio-One) and cultured at 38.5°C in a humidified atmosphere of 5% CO₂ in air. The medium was changed every 48 h until confluence was reached. When the cells reached confluence (10–11 days after starting the culture), they were used for experiments.

The homogeneity of the epithelial cells was evaluated using immunofluorescence staining for specific markers of epithelial (cytokeratin) and stromal (vimentin) cells as described previously (Malayer & Woods 1998, Tanikawa et al. 2008) with our modification. Briefly, oviductal epithelial and endometrial stromal (control) cells were seeded on collagen-coated sterile cover glasses in six-well plates at a density of 5.0×10⁴ cells/ml. The endometrial stromal cells were isolated as described previously (Tanikawa et al. 2008). In brief, the cells were fixed with 4% (wt/vol) paraformaldehyde (Nacalai Tesque, Inc., Kyoto, Japan) for 10 min 48 h after plating. Then, the cells were incubated with 5% (wt/vol) skim milk (Nacalai Tesque, Inc.) in PBS containing 0.1% (vol/vol) Tween-20 (PBS-T) for 60 min at room temperature. After blocking, the cells were

![Diagram](image)

Figure 1 Five sections into which the oviducts were separated (1, infundibulum; 2, ampulla; 3, ampullary–isthmic junction; 4, isthmus; and 5, utero–tubal junction). The ampulla and isthmus sections were utilized for mRNA determination and epithelial cell culture.
incubated with specific primary antibodies to cytokeratin (anti-cytokeratin-IgG-mouse; Sigma–Aldrich, 1:500 dilution) or vimentin (anti-vimentin-IgG-mouse; Sigma–Aldrich, 1:5000 dilution) in 5% (wt/vol) skim milk overnight at 4 °C. After incubation, the cells were incubated again with secondary antibodies (anti-mouse-IgG Alexa 594 conjugate-donkey; Sigma–Aldrich; 1:500 dilution for cytokeratin 18; anti-mouse-IgG FITC conjugate-donkey; Sigma–Aldrich; 1:500 dilution for vimentin, in PBS-T) for 60 min at room temperature and mounted with ProLong Gold Antifade Reagent with DAPI (Invitrogen) on slide glasses. Fluorescence was observed using a fluorescence microscope (Olympus, Tokyo, Japan). In the epithelial cell culture, contamination of the oviductal stromal cells was <1% (Fig. 2).

**Experiment 1: effects of various temperatures on PG production in cultured bovine oviductal epithelial cells**

Epithelial cells that reached confluence in the four-well plates were used for this experiment. The cells were incubated with serum-free DF (DMEM/Ham's F-12 1:1 (vol/vol) supplemented with 0.1% BSA, 5 μg/ml holo-Transferrin (Sigma–Aldrich), and 2 μg/ml insulin (Sigma–Aldrich)) at 38.5, 39.5, 40.0, and 40.5 °C for 24 h. After incubation, the medium was collected in a 1.5 ml tube containing a 1% stabilizer solution (0.3 mol/l EDTA and 1% (w/v) acetylsalicylic acid, pH 7.3), and the concentrations of PGE$_2$ and PGF$_{2\alpha}$ in the medium were measured by enzyme immunoassay. The cells were collected for the measurement of DNA amount by DNA assay.

**Experiment 2: effects of elevated temperatures on PGES mRNA expressions in cultured bovine oviductal epithelial cells**

Epithelial cells that reached confluence on the 48-well plates were used for this experiment. The cells were incubated with serum-free medium at 38.5 and 40.5 °C for 24 h. After incubation, the cells were collected and used for the investigation of the expressions of PTGES, PTGES2, and PTGES3 mRNAs.

**Experiment 3: comparison of mRNA expression of PGEs and HSP90AA1 in summer and winter in bovine oviductal tissues**

For the determination of the expressions of PGEs and HSP90AA1 mRNAs, oviductal tissues (days 0–6 after ovulation) were collected from 14 cows in winter (November–March, 2011–2012) and 14 cows in summer (July–September, 2012). The cows were slaughtered at a local abattoir in Okayama, Japan. Average temperatures in Okayama during the sampling periods were 5.3 °C in winter and 27.5 °C in summer. The range of temperatures during the summer sampling period was 22.1–36.8 °C. Although the average temperature is reported as 27.5 °C herein, there were 33 days (of 46 days) when the daily maximum temperature was >33 °C. This climate seems to be under an apparent HS condition for dairy cows. The temperature data were obtained from Japan Meteorological Agency.
total RNA as described previously (Sakamoto et al. 2006). All primers were designed to amplify a product as shown in Table 1, and the specificity of each primer set was confirmed by running the PCR products on a 2.0% agarose gel. Protocol conditions consisted of denaturation at 95 °C for 3 min, followed by 45 cycles at 94 °C for 15 s, 60 °C for 20 s, and 72 °C for 15 s with a final dissociation (melting) curve analysis.

To standardize the relative level of the expression of each mRNA, three potential housekeeping genes, β-actin (ACTB), 18S rRNA (RNA18S1), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), were initially tested. The expression of GAPDH mRNA was most stable among those of the three genes, so the transcripts were selected as internal controls in our experiments. To analyze the relative level of the expression of each mRNA, the 2−ΔΔCT method was used (Livak & Schmittgen 2001).

**Western blotting**

Each protein in the cultured bovine oviductal cells was detected by western blotting analysis as described previously (Nishimura et al. 2008). Briefly, the cultured cells were lysed in 200 μl lysis buffer, and the protein concentrations were determined by the BCA method (Osnes et al. 1993). The proteins were heated with a SDS gel-loading buffer containing 1% (v/v) β-mercaptoethanol (Wako Pure Chemical Industries Ltd., Osaka, Japan) at 95 °C for 10 min. The samples (50 μg protein) were loaded on 10% (v/v) SDS–PAGE (200 V, 80 min) gel and transblotted onto a 0.2 μm nitrocellulose membrane (GE Healthcare, Milwaukee, WI, USA; 250 mA, 180 min). The membrane was then incubated in PVDF Blocking Reagent for Can Get Signal (Toyobo, Osaka, Japan) for 60 min at room temperature. After blocking, the membrane was cut into two pieces, and each piece was incubated separately with specific primary antibodies to HSP90AA1 (anti-HSP90-IgG-mouse; Abcam, Cambridge, UK, 1:1000 dilution) and ACTB (anti-β-actin-IgG-mouse; Sigma–Aldrich, 1:20 000 dilution) in Can Get Signal Immunoreaction Enhancer Solution 1 (Toyobo) overnight at 4 °C. After incubation, the membrane pieces were incubated again with a secondary antibody (anti-mouse, HRP-linked whole antibody produced in sheep; GE Healthcare, 1:5000 dilution for HSP90AA1 and 1:40 000 dilution for ACTB) in Can Get Signal Immunoreaction Enhancer Solution 2 for 60 min at room temperature. The signal was detected using the ECL Western Blotting Detection System (GE Healthcare), and the intensity of the immunological reaction mixture was estimated by measuring the optical density in the defined area by computerized densitometry using Image J (National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis**

All the experimental data are reported as mean ± S.E.M. The data of PG production are reported as a percentage of control. The statistical significance of differences was assessed by ANOVA followed by Bonferroni/Dunn test for multiple comparisons using StatView (SAS Institute, Cary, NC, USA).

**Results**

**Experiment 1: effects of various temperatures on PG production in cultured bovine oviductal epithelial cells**

Temperature affected PGE_2 production in the cultured ampullary epithelial cells. PGE_2 concentration was significantly higher at 40.5 °C than at 38.5 °C (Fig. 3A).

![Figure 3](image-url)
Summer heat stress affects oviductal functions

On the other hand, elevated temperatures did not affect PGE₂ production in the isthmic epithelial cells (Fig. 3B). The culture temperature had no effect on PGF₂α production in either the ampullary cells or isthmic cells (Fig. 3C and D). In the experiments described below, the cells were incubated at 38.5 and 40.5 °C, because PGE₂ production at these temperatures is significantly different.

**Experiment 2: effects of elevated temperatures on PGES mRNA expressions in cultured bovine oviductal epithelial cells**

The expressions of PTGES (mPGES1) and PTGES3 (cPGES) mRNAs were higher at 40.5 °C than at 38.5 °C in the cultured ampullary epithelial cells (Fig. 4A and C; P<0.05). The expression of PTGES2 mRNA was not affected by elevated temperatures (Fig. 4B; P>0.05). The expression of PTGES mRNA was higher at 40.5 °C than at 38.5 °C in the isthmus (Fig. 4D; P<0.05). On the other hand, PTGES2 and PTGES3 transcripts were not affected by elevated temperatures (Fig. 4E and F; P>0.05).

**Experiment 3: comparison of mRNA expressions of PGESs and HSP90AA1 in summer and winter in bovine oviductal tissues**

The expression of PTGES3 mRNA in the bovine ampullary oviduct was higher in summer than in winter (Fig. 5C; P<0.05). In contrast, the mRNA expressions of PTGES and PTGES2 in the ampulla and all PGESs in the isthmus were not significantly different in summer and winter (Fig. 5; P>0.05). The expression of HSP90AA1 mRNA in the bovine ampullary oviduct was higher in summer than in winter (Fig. 6A; P<0.05). On the other hand, the expression of HSP90AA1 mRNA in the isthmic cells was not significantly different in summer and winter (Fig. 6B; P>0.05).

**Experiment 4: effect of elevated temperatures on HSP90AA1 mRNA and protein expressions in cultured bovine oviductal epithelial cells**

The expression of HSP90AA1 mRNA was higher at 40.5 °C than at 38.5 °C in the cultured ampullary (Fig. 7A; P<0.01) and isthmic (Fig. 7B; P<0.05) oviductal epithelial cells. The expression of HSP90AA1 protein was also higher at 40.5 °C than at 38.5 °C in the cultured cells obtained from both sections of the oviductal epithelium (Fig. 7C and D; P<0.05).

**Discussion**

In the present study, elevated temperatures promoted PGE₂ production, but did not affect PGF₂α production in the cultured ampullary oviductal epithelial cells (Fig. 3). The body temperature of cows is ~38.5 °C, but it

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**Figure 4** Effects of an elevated temperature (40.5 °C) on the expression of microsomal PGE synthase 1 (PTGES (mPGES1)) (A and D), PTGES2 (B and E), and cytosolic PGES (PTGES3 (cPGES)) (C and F) mRNAs in epithelial cells collected from the ampulla (white bars) and isthmus (black bars) of the bovine oviduct (mean ± S.E.M., n = 4 oviducts). Different superscript letters indicate a significant difference (P<0.05; ANOVA).

**Figure 5** Expressions of PTGES (A and D), PTGES2 (B and E), and PTGES3 (C and F) mRNAs in the bovine ampullary (white bars) and isthmic (black bars) oviducts (mean ± S.E.M.) in the postovulatory phase in winter (November 21 2011–March 1 2012; n=14) and summer (July 23–September 6, 2012, n=14). Different superscript letters indicate a significant difference (P<0.05; ANOVA).
increases under hot conditions. For example, the vaginal temperature of cows was found to increase to \( \sim 40.5 \, ^\circ C \) under high-temperature conditions (Nabenishi et al. 2011). Oviductal smooth muscle is relaxed by PGE2 and induced to contract by PGF\(_{2\alpha}\) (Al-Alem et al. 2007, Siemieniuch et al. 2009). Since gametes and embryos are transported by waves of contraction and relaxation, upsetting the balance between PGE2 and PGF\(_{2\alpha}\) secretions by HS may negatively affect the transport.

Elevated temperatures increased PGE2 production in the ampullary epithelial cells (Fig. 3). The increased PGE2 production could be due to either increases in the expressions of PGEs or increases in the PGES activity. Mammals have three PGEs isozymes: mPGES1, mPGES2, and cPGES (Murakami et al. 2002). In the present study, the expressions of PTGES and PTGES3 mRNAs were higher at 40.5 \( ^\circ C \) than at 38.5 \( ^\circ C \) in the ampullary epithelial cells (Fig. 4). However, only the expression of PTGES3 mRNA was higher in summer than in winter in the ampullary oviductal tissue (Fig. 5). Thus, cPGES may play an important role in the stimulation of PGE2 synthesis in the ampullary oviduct under HS. Since PGE2 is known to relax the oviductal smooth muscle (Al-Alem et al. 2007, Siemieniuch et al. 2009), HS may be a cause of summer infertility by decreasing the oviductal motility via the promotion of PGE2 production. The expression of PTGES mRNA in both the oviductal sections did not show any difference in summer and winter (Fig. 5) in contrast to that observed in the in vitro experiment (Fig. 4). This discrepancy may be due to the different in vivo and in vitro conditions. However, since the expression of PTGES3 was increased by HS both in vivo and in vitro, we believe that summer HS increases PGE2 production in bovine oviducts.

The expression of HSP90AA1 in the oviduct was higher in summer than in winter (Fig. 6) and was increased by elevated culture temperatures (Fig. 7). HSPs protect cells from various stresses including heat and reactive oxidants (Kregel 2002). HSPs are highly expressed at high temperatures and help the folding of proteins to prevent protein denaturation (Kregel 2002). In particular, HSP90AA1 plays crucial roles as a molecular chaperone in the activation of various client proteins including cPGES (Pearl & Prodromou 2006). In rat fibroblast cells, HSP90AA1 activates cPGES and consequently promotes PGE2 production (Tanioka et al. 2003). Therefore, it is possible that HS increases the expression of HSP90AA1 in the ampullary oviductal tissue (Fig. 5). Thus, it is possible that HS increases the expression of HSP90AA1 and the activity of cPGES, resulting in an increase in PGE2 production in bovine oviductal epithelial cells. Furthermore, the expression of HSP90AA1 in summer could be assumed to increase not only in the oviduct but also in other organs. Since HSP90AA1 is an important chaperone for the actions of various client proteins (Pearl & Prodromou 2006), HS may affect not only oviductal functions but also other body conditions. The expressions of HSP90AA1 and PTGES3 mRNAs in the ampullary oviduct were higher in summer than in winter, whereas there was no difference in them in the isthmic oviduct in summer and winter. It has been reported that the expression of HSPs is higher in porcine oocytes in summer than in winter, although there are no seasonal changes in porcine cumulus cell (Pennaressa et al. 2012). Therefore, it is possible that the expressions of HSP90AA1 and PTGES3 in the isthmic oviduct are not affected by different seasonal conditions in contrast to those in the ampullary oviduct.

Elevated temperatures did not affect PGE2 production in the isthmic epithelial cells (Fig. 3), although they stimulated the expression of PTGES mRNA (Fig. 4). In perioculatory cows, the expression of PTGES is lower in the isthmus of the oviduct than in the ampulla (Gauvreau et al. 2002).
et al. 2010). Thus, mPGES1 may contribute less to PGES activity in the isthmus than the other two PGESs. Additionally, since the culture temperature did not affect the expression of PTGES3 mRNA (Fig. 4), PGF2 production may be at a stable level in the isthmic oviductal epithelium. Mammals have two types of oviductal epithelial cells, ciliated and secretory, and the proportion of ciliated cells to secretory cells is different among the sections of the oviduct (Kolle et al. 2009). Thus, PGE2 production seems to be not affected by elevated temperatures in the isthmus due to the different cell type populations. In conclusion, HS is suggested to not affect the secretory function of PGs in the isthmic oviduct.

The overall findings suggest that HS upssets the balance between PGE2 and PGF2 secretion, which would be expected to decrease the motility of the oviductal smooth muscle, which is important for gamete/embryo transport. This could lead to the low fertility in cattle in summer.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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