Journal of Reproduction and Development, Vol. 61, No 5, 2015

—Original Article—

Effect of epidermal growth factor (EGF) on the phosphorylation of mitogen-activated protein kinase (MAPK) in the bovine oviduct *in vitro*: Alteration by heat stress

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Abstract. Epidermal growth factor (EGF) has been shown to be involved in control of the oviductal microenvironment. To elucidate the potential mechanisms responsible for the detrimental effect of heat stress and to identify the relation with the endocrine status, the effects of EGF on the level of phosphorylated mitogen-activated-protein kinase (MAPK) and proliferation of bovine oviductal epithelial cells (OECs) exposed to different cyclic ovarian steroidal environments (luteal phase (LP), follicular phase (FP) and postovulatory phase (PO)) and temperatures (mild heat stress (40 C) and severe heat stress (43 C)) were investigated. Western blot was performed to evaluate phosphorylated MAPK, while proliferation was analyzed by MTT assay. Stimulation of OECs with EGF alone or with EGF in the PO and FP environments significantly increased the amount of phosphorylated MAPK, with MAPK 44 phosphorylation being highest during exposure to PO conditions. These effects were not observed in the LP. Heat treatment completely blocked effects of EGF on phosphorylated MAPK. Additionally, severe heat stress led to a significantly lower basal level of phosphorylated MAPK. PD98059 (MAPK inhibitor) completely abolished EGF-stimulated MAPK phosphorylation and OECs proliferation. Overall the results indicate that EGF has the potential to increase the amount of phosphorylated MAPK in OECs and therefore could be involved in regulation of the bovine oviductal microenvironment. However, these regulatory mechanisms may be compromised in the presence of heat stress (high ambient temperature), leading to low fertility rates and impaired embryo survival.

Key words: Bovine oviduct, Cell culture, Epidermal growth factor, Heat stress, Mitogen-activated-protein kinase (MAPK)

(**J. Reprod. Dev. 61:** 383–389*,* 2015)

The environment of the mammalian oviduct is the site for ovum transport and maturation, capacitation of sperm, fertilization and early-embryo development and transport [1]. The oviductal contribution includes hormones, growth factors and their receptors, which have important roles in the physiology of the oviduct and embryo development [2]. A wide variety of growth factor systems (e.g. the insulin-like (IGF), fibroblast (FGF), transforming (TGF) and vascular endothelial (VEGF) growth factor systems) exists in the bovine oviduct [3–7], and their localization and estrous cycle-depended changes suggest a crucial role of growth factors in the regulation of oviductal contraction and overall oviductal secretions. They support the establishment of an optimal oviductal microenvironment, thus enabling fertilization and development of the embryo at the right time and in the right location [3–7].

After expression of the epidermal growth factor (EGF) and EGF receptor (EGFR) was demonstrated in the porcine oviduct, it was suggested that EGF has an autocrine/paracrine role in this tissue [8, 9]. For example, the activation of MAPK by EGF in the pig oviduct was enhanced by estradiol 17-β (E2) treatment, whereas progesterone (P4) treatment appeared to decrease the activity [10]. In addition it is known that MAPK activity is regulated by gonadotropin-releasing hormone (GnRH) and necessary for normal fertility [11]. The EGFR mRNA was found to be increased after E2 treatment, with concurrent increases in EGFR protein [12]. Furthermore, luteinizing hormone (LH) in combination with a basal level of P4 and a high concentration of E2 has a maximal stimulatory effect on the oviductal secretion of contraction-related substances in the bovine oviduct [4, 13–18]. Thus, the ovarian steroids act together with growth factors and other substances [3, 4] to achieve the optimal oviductal environment for fertilization and timely transportation of the embryo to the uterus. In addition, EGF was reported to increase bovine oocyte maturation and development [19] as well as blastocyst development [20].

However, the effect of EGF on bovine oviductal MAPK during the estrous cycle has not been well studied. Therefore, in the present study, the bovine oviductal epithelial cell (OEC) culture system was utilized to investigate the effect of EGF on the amount of phosphorylated MAPK42/44 and OECs proliferation. The influence of the steroidal environment in the oviduct, represented by simulated luteal, follicular and postovulatory phases in the cultured OECs, on the EGF-mediated effect was also evaluated.

Besides these internal factors, external factors that cause stress can

Received: May 13, 2014
Accepted: May 8, 2015
Published online in J-STAGE: June 8, 2015

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influence the physiological functions of the body [21], including the reproductive system. The compromising effects of environmental heat stress (HS) on various aspects of reproduction have been discussed before [21–23]. Among them are changes in estrus, follicular growth and luteolytic mechanisms, early embryonic development, the oviductal and uterine environments and conception rates. It has recently been reported that HS reduces oviductal smooth muscle motility, which in turn could decrease gamete/embryo transport through the oviduct. [24]. Thus, the cells were also subjected to different temperatures during the experiments to assess the possible effect of mild/severe heat stress on the effect of EGF action on the OECs. Even though the role of EGF in the heat-stressed oviduct is not fully understood, some studies have demonstrated a conducive effect of EGF on heat-stressed cells. In cultured mouse mammary epithelial cells, severe heat stress (42 C) elevated EGF protein as well as EGF mRNA expression, while inhibition of EGF production during severe heat stress dramatically reduced the ability of cells to survive [25]. Moreover, heat-stress-induced damage to the porcine small intestinal epithelium is associated with downregulation of epithelial growth factor signaling [26]. Thus, a beneficial role of EGF in the heat-stressed oviduct is feasible.

Materials and Methods

Isolation and culture of oviductal epithelial cells

Whole reproductive tracts from nonpregnant cows were collected at a local slaughterhouse and kept on ice. The oviducts were separated at the uterotubal junction; the surrounding connective tissue was trimmed and washed several times with Hank's balanced salt solution (HBSS; PAA Laboratories, Pasching, Austria). Isolation and culture of OECs were based on the methods described previously [27, 28]. Briefly, the oviductal lumen was flushed with 10 ml HBSS, and the OECs were mechanically dislodged while flushing with 15 ml HBSS. The collected OECs sheets were washed twice with HBSS by centrifuging (300 *g* for 10 min at 4 C). The resulting cell pellet was suspended in 4 ml HBSS, layered over 5 ml Easycoll (1.124 g/ml; Biochrom, Berlin, Germany) and centrifuged at 900 *g* for 20 min at 4 C. Cells (OEC sheets) at the interface were collected and washed twice with HBSS and cultured overnight in M199 medium with 5% fetal bovine serum (FBS; PAA) at 37 C in 5% CO_2 and 95% humidity. The OECs still in the suspension were washed twice with HBSS and trypsinized (0.05% trypsin; PAA) until single cells were obtained. Then, the OECs were plated in 6-well culture dishes (TPP, Trasadingen, Switzerland) and incubated under standard culture conditions. After monolayer formation, cells were again trypsinized and plated in 60 mm culture dishes (TPP) at a density of 3×10^4/ml and cultured until subconfluence.

EGF treatments

In a preliminary study, the subconfluent cells were incubated for 15 min with 5 doses (0, 0.5, 1, 5, 10 and 50 ng/ml) of EGF (Biomol, Hamburg, Germany) to evaluate the suitable concentration. To analyze the time-dependet response, 10 ng/ml EGF (highest amount of phosphorylated MAPK) was added to serum-free culture medium and incubated for 0, 5, 7.5, 15, 30 and 60 min to detect phosphorylated MAPK. In all experiments, cells were serum starved for 4 h before EGF treatment. After the desired period of incubations, cells were washed twice with PBS and stored at –80 C until protein purification or mRNA extraction.

Inhibition of MAPK phosphorylation

To decrease the level of phosphorylated MAPK, subconfluent second passage OECs were serum starved for 4 h and pretreated for 15 min with 50 μM MAPK inhibitor (PD98059, Sigma, Munich, Germany). Then, the OECs were stimulated with 10 ng/ml EGF for 15 min. OECs cultured with EGF but without the inhibitor and OECs cultured in M199 served as positive and negative controls.

Growth response to EGF

Cell proliferation in response to EGF treatment was carried out as described previously [29]. Basically, OECs in the second passage were seeded in 24-well culture plates (18000 cells/well) and incubated 24 h for attachment. Prior to stimulation, cells were serum starved for 4 h and pretreated for 15 min with 50 μM PD98059. Afterwards, the cells were stimulated with 10 ng/ml EGF for 48 h in accordance with the results of our preliminary study with OECs. OECs cultured with EGF but without the inhibitor and OECs cultured in M199 served as positive and negative controls. An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma, Munich, Germany) was used to determine cell proliferation.

Ovarian steroid treatments

For combined treatment of EGF (10 ng/ml for 15 min) with ovarian steroids, three different combinations of P4 and E2 (both from Sigma, Munich, Germany) were chosen based on their concentrations in the bovine oviduct during the normal estrous cycle [30] (luteal phase oviducts, high levels of P4 (500 ng/ml) and low levels of E2 (250 pg/ml); follicular phase oviducts, low levels of P4 (1 ng/ml) and high levels of E2 (1 ng/ml); postovulatory phase oviducts, low levels of P4 (1 ng/ml) and low levels of E2 (250 pg/ml)). Thus, to simulate the ovarian steroid environment in OECs, P4 and E2 were added to the culture media in the abovementioned combinations at the beginning of the second passage. The OECs cultured in M199 without ovarian steroids served as controls.

Mild/severe heat stress

In vitro heat stress was created according to a previous report [31] by culturing cells at 40 C (mild heat stress) and 43 C (severe heat stress). The cells were cultured at 40 C and 43 C for 24 h prior to stimulation experiments with EGF. Cells cultured at a homeothermic temperature (37 C) served as control. The OECs were collected after a 15 min stimulation with EGF.

Live/dead viability test

To detect degenerating cells due to heat treatment, a Live/Dead Viability/Cytotoxicity Kit (Invitrogen, Karlsruhe, Germany) was used. OECs in the second passage were seeded in 12-well culture plates (36000 cells/well) and incubated 24 h for attachment. Afterwards, the plates were placed for 24 h at 37 C, 40 C or 43 C in an atmosphere with 5% CO_2 and 95% humidified air. Subsequently, all samples were washed with PBS, and the above kit was used according to the supplier's instructions. OECs treated with 70% methanol for

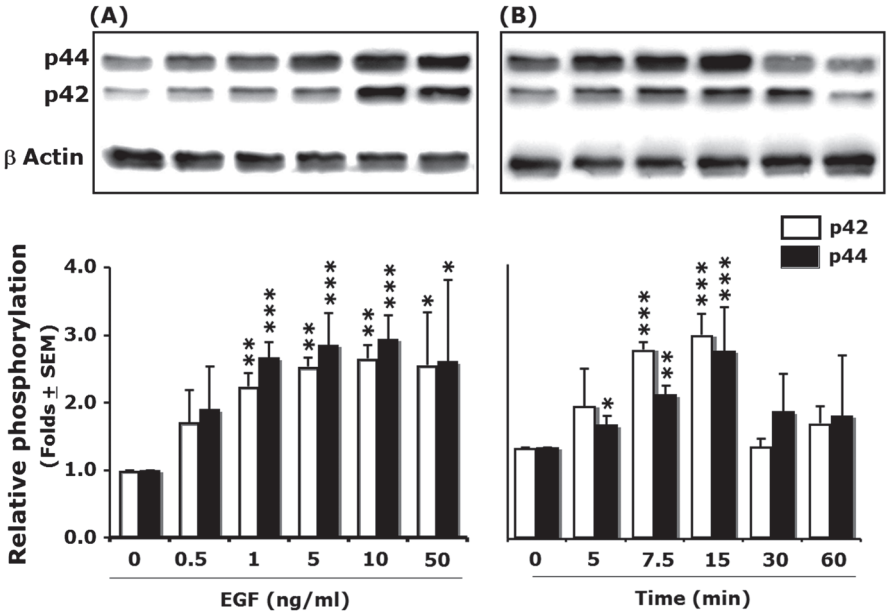

Fig. 1. Dose-dependent (A) and time-dependent (B) increase in phosphorylated MAPK (p42 = 42 kDa and p44 = 44 kDa) due to EGF in oviductal epithelial cells (OECs). Upper panel: A representative western immunoblot. Lower panel: Densitometric data from five experiments. Phosphorylated MAPK is normalized using β-actin. * $P < 0.05$ *vs*. controls (unstimulated); ** $P < 0.01$ *vs*. controls (unstimulated); *** $P < 0.001$ *vs*. controls (unstimulated).

30 min served as a control for degenerating cells. The probes were viewed under a Zeiss Axiovert 200M fluorescence microscope (Carl Zeiss, Jena, Germany).

Protein purification and Western blot analysis

Culture dishes stored at –80 C were placed on ice, and protein extraction and western blotting were carried out as previously described [29]. Briefly, the cells were lysed with a buffer containing 50 mM Tris-HCl at pH 7.4, 150 mM NaCl, 40 mM NaF, 5 mM EDTA, 1% (v/v) Nonidet P40, 0.1% (w/v) sodium deoxycholate and 0.1% (w/v) sodium dodecyl sulfate (SDS) supplemented with protease and phosphatase inhibitor cocktail (Sigma, Munich, Germany). Lysates were centrifuged for 5 min at 13.000 *g* and 4 C and the protein concentration of the supernatant was determined. Equal amounts of protein (15 μg/slot) were denatured in sample buffer (5 min at 95 C) and analyzed with SDS-PAGE. Western blots were performed using mouse anti-phospho-MAPK 42/44 (M8159, 1:10,000; Sigma, Munich, Germany), mouse anti-beta-actin (sc-47778, 1:5000; Santa Cruz, Heidelberg, Germany) and anti-mouse IgG horseradish peroxidase (HRP; 400; Pierce, Rockford, IL, USA) as suggested by the suppliers of the antibodies and detected with chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL, USA). The blots (n = 5–6 per treatment group) were analyzed by densitometric measurement and quantification (Bio-1D, Vilber Lourmat, Eberhardzell, Germany).

Statistical analysis

The data on dose- and time-dependent effects of EGF and the changes in phosphorylated MAPK due to EGF or/and ovarian steroids were analyzed using one-way ANOVA followed by the Dunnett's test for the mean separation (SAS® software). Data on the effects of EGF and the inhibitor (PD98059) on the level of phosphorylated MAPK and cell proliferation were separately analyzed using the simple paired t-test followed by the F-test. Probabilities less than 0.05 ($P < 0.05$) were considered significant.

Results

Dose- and time-dependent responses to EGF

A significant increase in phosphorylated MAPK (42 kDa and 44 kDa forms) was observed at a dose of 1 ng/ml ($P < 0.01$–0.001), while the highest effect was observed at 10 ng/ml (3 fold: $P < 0.01$–0.001) when compared with untreated controls (Fig. 1A). Elevation of phosphorylated MAPK 44 was observed after 5 min of EGF treatment ($P < 0.05$), whereas the increase in phosphorylated MAPK 42 was delayed and started after 7.5 min of stimulation ($P < 0.001$). Both kinases displayed the highest amount of phosphorylated protein at 15 min ($P < 0.001$), and the level then gradually decreased with time (Fig. 1B). The ratio of phosphorylated MAPK to total MAPK was not included in this study, since only the amount of phosphorylated (active) protein was of interest.

Inhibition of MAPK activity and cell proliferation

Addition of the MAPK inhibitor PD98059 to the culture medium abolished the significant EGF-triggered increase in phosphorylated MAPK 42 and 44 in OECs while significantly reducing the amount of phosphorylated protein to almost half the basal level ($P < 0.01$) (Fig. 2A). Incubation of OECs with EGF significantly elevated the cell proliferation after 48 h ($P < 0.01$). Addition of the MAPK

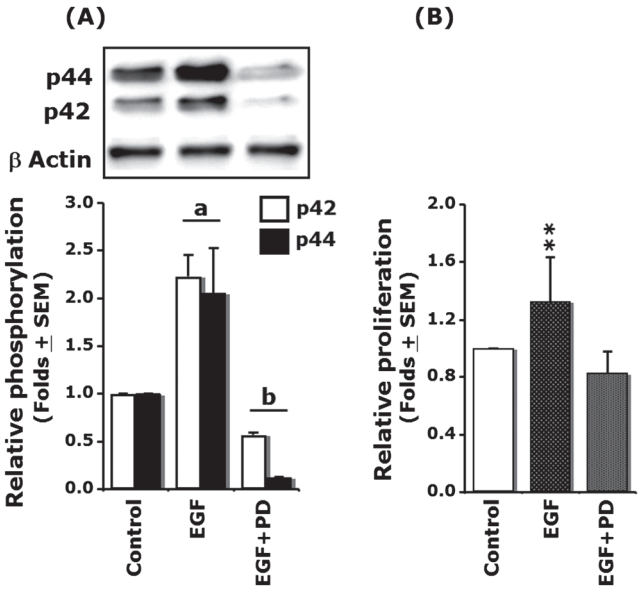

Fig. 2. Effect of EGF (10 ng/ml) and combined treatment with EGF and the PD98059 inhibitor (50 μM) on (A) phosphorylation levels of MAPK (p42 = 42 kDa and p44 = 44 kDa) after 15 min of stimulation. Upper Panel: A representative western immunoblot is shown. Lower panel: Densitometric data from five experiments. Phosphorylated MAPK is normalized using β-actin. (B) Proliferation of oviductal epithelial cells (OECs) over 48 h (n = 5, same concentrations as A). ** $P < 0.01$ *vs*. controls. [a] Signficantly higher than the controls at $P < 0.01$. [b] Signficantly lower than the controls at $P < 0.01$.

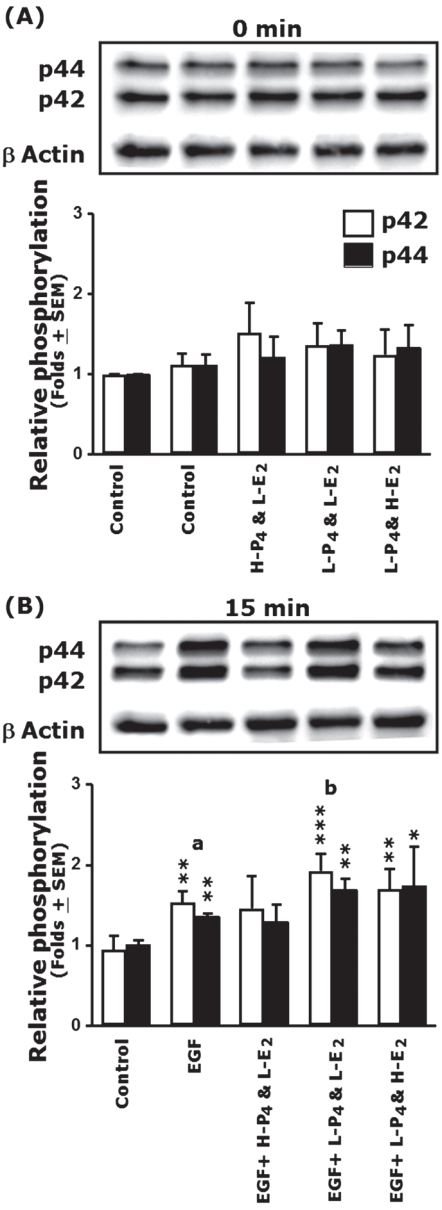

Fig. 3. Effect of EGF (10 ng/ml) and EGF with ovarian steroids simulating the normal estrous cycle (simulated luteal phase with H-P4 (500 ng/ml) and L-E2 (250 pg/ml); simulated follicular phase with L-P4 (1 ng/ml) and H-E2 (1 ng/ml) and simulated postovulatory phase with L-P4 and L-E2) on the amount of phosphorylated MAPK in oviductal epithelial cells (OECs) (n = 6) at 0 min (A, no EGF addition) and after 15 min of EGF stimulation (B). Upper Panel: A representative western immunoblot is shown. Lower panel: Densitometric data from six experiments. Phosphorylated MAPK is normalized using β-actin. * $P < 0.05$ *vs*. controls; ** $P < 0.01$ *vs*. controls; *** $P < 0.001$ *vs*. controls. [a, b] Different letters denote a significant difference in phosphorylated p44 due to the addition of $L\text{-}P_4/L\text{-}E_2$.

inhibitor PD98059 to the culture medium reduced the EGF-induced cell proliferation to the levels of untreated controls (Fig. 2B).

Effect of EGF and steroids

Exposure of OECs to different cyclic hormonal environments alone did not change the amount of phosphorylated MAPK (Fig. 3A). If the EGF stimulation was carried out for 15 min during the simulated postovulation phase (L-P4+L-E2; $P < 0.01$–0.001) or the simulated follicular phase (L-P4+H-E2; $P < 0.05$–0.01), a significant increase in phosphorylated MAPK 42 and 44 was detected. However, this effect of EGF was not observed in the simulated luteal phase (H-P4+L-E2). Interestingly, the amount of phosphorylated MAPK 44 was significantly higher when EGF was combined with the simulated postovulation steroidal environment (L-P4+L-E2) compared with EGF alone after 15 min of stimulation ($P < 0.01$; Fig. 3B).

Mild/severe heat stress

Exposure of OECs to mild *in vitro* heat stress (incubation at 40 C) and severe heat stress (incubation at 43 C) completely blocked the EGF-mediated increase in phosphorylated MAPK 42 and 44. Interestingly, severe heat stress exposed OECs showed significantly less phosphorylated MAPK 44 than the controls ($P < 0.01$; Fig. 4). To exclude the possibility that these observed changes were due to thermal damage, OECs under the two heat stress conditions were subjected to a live/dead viability test. Fluorescent calcein dye was retained in nearly all OECs independent of the temperature (green signal for viable cells; Fig. 5A–C). Damaged cells were indicated by a red signal, as the control pretreated with membrane-destroying

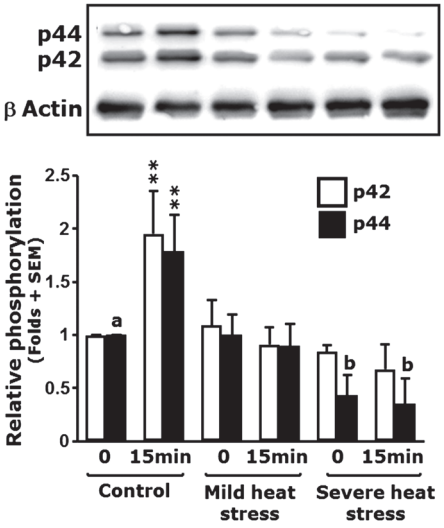

Fig. 4. Effect of mild and severe heat stress (incubation at 40 C and 43 C, respectively, for 24 h before EGF treatment) in EGF-stimulated oviductal epithelial cells (OECs) on the amount of phosphorylated MAPK (p42 = 42 kDa and p44 = 44 kDa) after 15 min, Upper Panel: A representative western immunoblot is shown. Lower panel: Densitometric data from six experiments normalized using β-actin. ** $P < 0.01$ *vs*. controls; *** $P < 0.001$ *vs*. controls. [a, b] Different letters denote a significant difference in phosphorylated p44 due to severe heat stress.

methanol (labeled with EthD-1; Fig. 5D).

Discussion

So far, the expression of EGF has not been reported for bovine oviductal epithelial cells. Yet our study clearly indicates that EGF is able to influence OECs behavior. It is known that in the normal bovine endometrium, EGF concentrations peak on days 2–4 of the estrous cycle [32, 33]. Since the local countercurrent transfer of uterine products to other parts of the reproductive tract is very well documented [34–36], the source of the EGF acting in the oviduct may be the locally transferred endometrial EGF. The results of the present *in vitro* study indicate that EGF stimulation increases the amount of phosphorylated MAPK in bovine oviductal epithelial cells and that the postovulatory phase ovarian steroidal environment (L-P4+L-E2) further amplifies this effect for MAPK 44 compared with EGF alone. The luteal phase steroidal environment (H-P4+L-E2) completely blocked the stimulatory effect of EGF on MAPK phosphorylation. It was reported previously that E2 and P4 are able to alter the phosphorylation status of MAPK. In rat cerebellar neurons [37], human lens epithelial cells [38] and human vascular endothelial cells [39], stimulation with E2 leads to activation of MAPK, while the P4 and E2 levels during pregnancy reduces MAPK 42/44 phosphorylation in the human myometrium [40]. It was also reported for the porcine oviduct that MAPK 42 activity is stimulated by E2 and decreased by P4 [10]. Taken together, it can be suggested that the steroid hormonal environment in the oviduct is able to alter the response of this signaling pathway to different stimuli and that therefore the MAPK system might play an important role in the control of the oviductal microenvironment.

Stimulation of cell motility and proliferation by EGF via activation of the MAPK pathway has been observed in various cell types including connective tissue-derived cells [41], the bovine mammary epithelium [42], spermatogonial stem cells [43], rat and mouse uterine stromal cells [44, 45], bovine trophoblast cells [29, 46] and bovine follicular granulosa cells [47, 48]. Consistent with this, EGF-stimulated OEC proliferation was observed in the present study. The observed temporal dynamics of phosphorylated MAPK are in line with the activation pattern of the canonical ERK MAP kinase cascade upon binding of EGF [49, 50]. It was previously reported that this stimulation of the proliferation of bovine OECs is dose dependent [51].

On the other hand, E2 plays a key role during the proliferation and differentiation of porcine oviductal tissue by activating the EGF system through its receptor [12]. In cultured OECs, the simulated postovulatory phase ovarian steroidal environment (L-P4+H-E2) also enhanced the increase in phosphorylated MAPK due to EGF. Furthermore, EGF-induced bovine trophoblast cell proliferation and activation of the MAPK signaling pathway were shown to be abolished by the MAPK inhibitor PD98059 [29, 46]. In the present study, addition of the inhibitor also suppressed the EGF-dependent increase in phosphorylated MAPK 42 and 44 and proliferation of OECs.

A high culture temperature (42 C) was shown to increase the uterine production of prostaglandin (PG) F2α by the endometrium collected at day 17 of the estrous cycle [31, 52] and to increase uterine PGF2α production in response to oxytocin at day 17 of pregnancy [53]. Moreover, the expressions of bovine oviductal microsomal

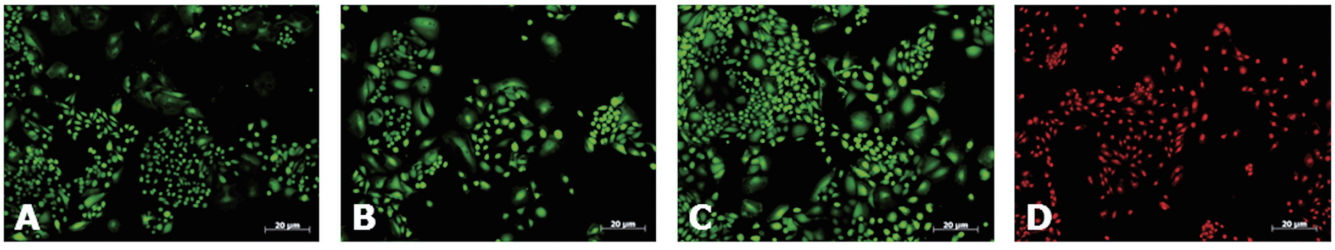

Fig. 5. Effect of mild and severe heat stress (incubation at 40 C or 43 C for 24 h) on cell viability determined with the fluorescent dyes calcein and EthD-1. OECs incubated under normal conditions (A, 37 C), mild heat stress (B, 40 C) or severe heat stress (C, 43 C) display membrane integrity and intracellular esterase activity (green staining, vital cells). In the control, cells were incubated for 30 min with 70% methanol and showed binding of EthD-1 (D, red staining) to nucleic acids due to disruption of cellular membranes.

PGE synthase 1 (mPGES1), cytosolic PGES (cPGES) and heat shock protein 90 (HSP90) mRNAs, and proteins were higher at 40.5 C than at 38.5 C [24]. It was also observed that the exposure of cultured day 17 conceptuses to a temperature of 43 C reduced secretion of interferon-tau [54] and that this may hinder the process of maternal recognition of pregnancy and lower the pregnancy rate. In the present study, it was observed that exposure of cultured OECs to temperatures of 40 C (mild heat stress) and 43 C (severe heat stress) abolished the EGF induced increase in phosphorylated MAPK. Furthermore, OECs exposed to severe heat stress (43 C) had a lower level of phosphorylated MAPK 44 but not MAPK 42 when compared with controls. The mechanism behind this differential phosphorylation cannot be explained, but varied activation of these two forms was also observed in human myometrial cells in response to treatment with ovarian steroids [40]. One possible explanation for the decrease in phosphorylated MAPK after continued exposure to heat is the activation of various phosphatases. For example, it has been shown that heat stress activates protein phosphatase 2A (PP2A) in HepG2 cells [55], which is a potent inhibitor of the MAPK pathway [56].

MAPK activation in ovarian granulosa cells is essential for female fertility [57] and the MAPK cascade is a principal regulator for the transition from the MII phase to pronuclear formation after fertilization [58]. Moreover, MAPK plays a vital role in the preparation of sperm for fertilization, including sperm maturation, activation, capacitation and the acrosome reaction, before fertilization of the oocyte [59]. This evidence confirms the importance of MAPK in many early reproductive events. Thus, the downregulation of the MAPK system by the heat stress observed in the present study may lead to infertility.

It has been repeatedly reported that the global surface temperature has increased by about 0.5 C since 1975 and that global warming will continue [60]. Thus, mild/severe heat stress, which compromises fertility in animals, may become progressively more important in certain areas where animals live unsheltered. As such, further comprehensive studies on the effect of high environmental temperature on oviductal function are vital to understand its negative effect on fertilization and embryo development and transport.

In conclusion, the results of the present study indicate that EGF has the potential to increase the amount of phosphorylated bovine oviductal MAPK and that this effect is highest during the simulated peri-ovulatory period *in vitro*. Thus, EGF may play a role in regulation of the oviductal microenvironment to facilitate fertilization and timely transport of the gamete/embryo. However, these regulatory mechanisms may be compromised at high temperature, which may result in low fertility and impaired embryo survival.

Acknowledgments

The authors thank Prof A Miyamoto, Obihiro University of Agriculture and Veterinary Medicine, Japan, for valuable suggestions concerning improvement of the manuscript. Furthermore, the excellent technical assistance of Mrs I Blume is gratefully acknowledged. MPBW is funded by a Georg Forster Research Fellowship for experienced researchers (Grant no. 1139085) granted by the Alexander von Humboldt Foundation. This study was supported by the National Research Council of Sri Lanka (Grant 11-198).

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