Heat stress induces oxidative stress and activates the KEAP1-NFE2L2-ARE pathway in bovine endometrial epithelial cells

Hirona MURATA[1], Hiroki KUNII[1], Kazuya KUSAMA[2], Toshihiro SAKURAI[3], Hanako BAI[1,*], Manabu KAWAHARA[1], Masashi TAKAHASHI[1, 4]

[1] Laboratory of Animal Genetics and Reproduction, Research Faculty of Agriculture, Hokkaido University, Kita-ku Kita 9 Nishi 9, Sapporo 060-8589, Japan

[2] Department of Endocrine Pharmacology, Tokyo University of Pharmacy and Life Sciences, Tokyo 192-0392, Japan

[3] School of Pharmaceutical Science, Ohu University, 31-1 Misumido, Tomita-machi, Koriyama-shi, Fukushima 963-8611, Japan

[4] Global Station for Food, Land and Water Resources, Global Institution for Collaborative Research and Education, Hokkaido University, Sapporo 060-0815, Japan

Funding: This work was supported by the Japan Society for the Promotion of Science (JSPS), KAKENHI Grant Number 20K15644

***Correspondence:** Hanako Bai, email: hbai@anim.agr.hokudai.ac.jp
ORCID: https://orcid.org/0000-0001-9940-1880
Laboratory of Animal Genetics and Reproduction, Research Faculty of Agriculture, Hokkaido University, Kita-ku Kita 9 Nishi 9, Sapporo 060-8589, Japan
Tel: +81 11 706 2544

Running title: Effect of heat stress on bovine endometrial epithelial cells

Summary sentence: Heat stress induces oxidative stress, whereas NFE2L2, a master regulator of the cellular environmental stress response, has a protective role in bovine endometrial epithelial cells cultured under heat stress conditions.

Key words: bovine uterus; endometrial epithelial cells; heat stress; oxidative stress

Abstract

Heat stress adversely affects the reproductive function in cows. Although a relationship between heat stress and oxidative stress has been suggested, it has not been sufficiently verified in bovine endometrial epithelial cells. Here, we investigated whether oxidative stress is induced by heat stress in bovine endometrial epithelial cells under high temperature. Luciferase reporter assays showed that the reporter activity of heat shock element (HSE) and antioxidant responsive element (ARE) was increased in endometrial epithelial cells cultured under high temperature compared to that in cells cultured under basal (thermoneutral) temperature. Also, nuclear factor, erythroid 2 like 2 (NFE2L2), a master regulator of cellular environmental stress response, stabilized and the expression levels of antioxidant enzyme genes increased under high temperature. Immunostaining confirmed the nuclear localization of NFE2L2 in endometrial epithelial cells cultured under high temperature. Quantitative polymerase chain reaction analysis showed that the expression levels of representative inflammatory cytokine genes, such as prostaglandin-endoperoxide synthase 2 (PTGS2) and interleukin 8, were significantly decreased in endometrial epithelial cells cultured under high temperature compared to those in cells cultured under basal temperature. Thus, our results suggest that heat stress induces oxidative stress, whereas NFE2L2 plays a protective role in bovine endometrial epithelial cells cultured under heat stress conditions.

Introduction

Heat stress negatively affects reproductive functions in both animals and humans. In the dairy industry, it causes significant economic losses [1]. Heat stress can affect reproductive systems, including endocrine functions, germ cells, fertilization, and embryonic development [1–6]. The negative effects of heat stress on the physiological conditions of cows have been well studied. In winter, bovine body temperature (rectal temperature) is 38.8 ± 0.04°C (38.5–39.1°C); this increases to 39.3 ± 0.03°C (38.0–41.7°C) in summer [7]. Thermoneutral (19°C) bovine body temperatures of 38.5–38.8°C and heat-stressed (29°C) body temperatures of 38.9–39.6 °C have also been reported [8]. In addition, uterine temperatures are approximately 0.2°C higher than the rectum [9]. Elevated temperature reduces blood flow to the uterus [10, 11], and maternal heat stress can ~~also~~ affect the fetus in utero, and the offspring [12−15]. Heat stress also affects the secretion of prostaglandin (PG) E2 and PGF2α in bovine endometrial tissues and stromal cells [16, 17]. A high culture temperature (43°C) stimulated endometrial PGF2α secretion [16], and heat stress (40.5°C) also increased PGE2 and PGF2α secretion in endometrial stromal cells [17]. Thus, heat stress may affect corpus luteum lifespan and/or function and cause infertility. For these reasons, the conception rate from artificial insemination decreases significantly in the summer [18–21]. However, the effect of heat stress on bovine endometrial epithelial cells remains unclear.

Heat shock proteins (HSPs) are transcriptionally activated under heat stress conditions and, therefore, can be used as heat stress markers [22]. HSPs, particularly HSP70 and HSP90, play a central role in the heat stress response and regulate protein misfolding in a variety of cells and tissues. HSP60 and HSP70 are the most important HSPs involved in reproduction. HSPs are expressed in various reproductive tissues, including the oocytes, placenta, and endometrium [23], and play essential roles in response to heat stress, protecting against induced cellular damage. The transcriptional activation is mediated by heat shock factors (HSFs), which bind to the conserved

78 heat shock responsive DNA element (HSE) [22, 24, 25]. The HSF was originally identified as a
79 heat shock-inducible regulator of HSP genes, but is now known to be induced by a variety of
80 stresses, including oxidative stress [26]. Indeed, heat stress induces oxidative stress and increases
81 the expression of its associated marker genes such as superoxide dismutase (SOD) and catalase
82 in plasma [27]. Additionally, heat stress-induced oxidative stress agents, such as reactive oxygen
83 species (ROS), disrupts cellular functions in bovine ovaries and embryos [28, 29]. Thus, although
84 studies have shown that oxidative stress and heat stress are closely related, the relationship
85 between them has not been clarified in bovine endometrial epithelial cells.

86 The transcription factor, nuclear factor, erythroid 2 like 2 (NFE2L2, also known as
87 NRF2), is a master regulator of cellular oxidative and electrophilic stress responses. NFE2L2
88 affects ROS homeostasis by regulating several antioxidant defense mechanisms. The NRF2 target
89 genes have a DNA sequence called the antioxidant response element (ARE), which is involved in
90 the homeostatic control of oxidants [30–32]. Under basal conditions, Kelch-like ECH-associated
91 protein 1 (KEAP1) binds to NFE2L2 and sequesters it from the nucleus, preventing the activation
92 of its target genes. Whereas, under stressed conditions, NFE2L2 is released from KEAP1, escapes
93 proteasomal degradation, stabilizes and translocates to the nucleus, and activates its target genes.
94 The KEAP1- NFE2L2 system is a conserved defense mechanism against oxidative stress. In
95 addition to protecting against oxidative stress, NFE2L2 controls inflammation [33]. The induction
96 of inflammatory biomarkers, such as interleukin (IL) 1b, IL6, tumor necrosis factor alpha (TNFA),
97 and prostaglandin-endoperoxide synthase 2 (PTGS2) was reported in *Nrf2* knockout mice [34].
98 Expression of these cytokine genes and NFE2L2 are closely related [35, 36]. In cows, the KEAP1-
99 NFE2L2 pathway respond to oxidative stress during embryonic development [37~~33~~]. Recently, it
100 has also been reported that activation of NFE2L2 attenuates the inflammatory response induced
101 by lipopolysaccharide in bovine endometrial epithelial cells [38]. Therefore, NFE2L2 may play a

protective role in bovine endometrial epithelial cells cultured under heat stress conditions.

It is hypothesized that the KEAP1–NFE2L2 pathway is involved in oxidative stress resistance by inducing antioxidant enzymes under heat stress in bovine endometrial epithelial cells. To better understand of uterine functions under heat stress, we examined the effect of heat stress on bovine endometrial epithelial cells, especially the induction of oxidative stress and the subsequent cellular responses.

Materials and Methods

Culture of bovine endometrial epithelial cells

Non-pregnant healthy cow uteri were obtained from a local slaughterhouse (Hokkaido Hayakita Meat Inspection Center, Japan). The isolation and culture of bovine endometrial epithelial cells from bovine endometrial tissues have been described previously [39, 40]. The epithelial cells were cultured in Dulbecco's Modified Eagle's medium (high glucose) (DMEM; Wako, Osaka, Japan) supplemented with 5 % (v/v) fetal bovine serum (FBS; ICN Bio-Source International, Camarillo, CA, USA) and Antibiotic-Antimycotic solution (Thermo Fisher Scientific, Waltham, MA, USA) at 38.5 °C with 5% CO_2, and were used within five passages. Cells were cultured at thermoneutral (38.5 °C: bovine body temperature) or heat-stressed (HS; 40.5 °C: bovine body temperature under high temperature) conditions for 3 h, 6 h, and 12 h. The maximum heat-stressed culture time of 12 h was selected as this is the length of the daytime period, and a previous time-course study reported that cow blastocysts were adversely affected by heat shocks of 9–12 h [41]. Cell proliferation assays were conducted using the Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) according to the manufacturer's protocol. Cells were seeded on a 96-well plate for proliferation assays. The stimulated index (SI) was calculated as the ratio of the average absorbance value of three wells that contained heat-stressed cells relative to wells containing non-stimulated cells (n = 8).

RNA extraction and analysis

Cells were cultured on 60 mm/Collagen-coated dishes (IWAKI AGC TECHNO GLASS Co. Ltd, Shizuoka, Japan) for RNA extraction. Each sample was analyzed in duplicate per group (n = 8). Total RNA was extracted from endometrial epithelial cells using ISOGEN II (Nippon Gene, Tokyo, Japan). For quantitative real-time PCR (qPCR) analysis, the isolated total RNA

(total 500 ng) was reverse-transcribed to cDNA using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan). The cDNA reaction mixture was diluted 5 times with molecular biology grade water, and 1 μL was taken for each amplification reaction. Target gene expression levels were determined by qPCR using a LightCycler® 96 (Roche Diagnostics, Basel, Switzerland) and THUNDERBIRD™ SYBR® qPCR Mix (Toyobo), with 0.5 μM primers (final conc.) listed in Table 1. The thermal cycling conditions were as follows: 1 cycle at 95 °C for 30 s, followed by 50 cycles at 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 30 s. Relative mRNA abundance was calculated based on the expression levels of bovine *H2AFZ*, which was used as a reference gene as it show stable expression in bovine endometrium [39, 40, 42], and was relatively stable under heat stress conditions (our preliminary observations). Each run was completed with a melting curve analysis to confirm the specificity of amplification and the absence of primer dimer formation. All qPCR experiments were performed following the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines [43].

Detection of intracellular reactive oxygen species (ROS)

Bovine endometrial epithelial cells were prepared as described above. Cells were seeded onto a 96-well dish (Thermo Fisher Scientific) (2.0×10^4 cells/well/200 μL), and cultured until they grew to 70–80% confluency. Cells were incubated at 38.5 °C or 40.5 °C for 3 h, 6 h, and 12 h. After culturing, the ROS levels in the cells were analyzed using the molecular probe, CellROX® Green Reagent (Thermo Fisher Scientific, C10444) (final concentration 5 μM) according to the manufacturer's instructions. Nuclei were stained with Hoechst 33342 (5 μg/mL). Fluorescence intensity was measured using multimode plate reader (ARVO™ X4; PerkinElmer, Waltham, MA, USA). Cells were observed under a microscope (LAS X with DMi8; Leica

157 Microsystems, Wetzlar, Germany). All assays were performed in triplicate (n = 8).

158

159 *Reporter assay*

160 Reporter constructs containing HSE and ARE were generated using the primers listed
161 in Table 2. The fragment was each inserted into a pNL[NLucP/minP/Hygro] vector (Promega,
162 Madison, WI, USA) and the nucleotide sequences of the constructs were confirmed by DNA
163 sequencing. Transient transfection and reporter assays were performed according to the
164 manufacturer's instructions. Bovine endometrial epithelial cells were cultured in DMEM
165 supplemented with 5% FBS and seeded onto a 4-well plate (Thermo Fisher Scientific). At 70–
166 80% confluency, 100 ng pNL reporter constructs, 5 ng pGL4.53 [luc2/PGK] firefly reporter (used
167 to correct for transfection efficiency), and 0.5 μL ViaFect™ Transfection Reagent (Promega) were
168 prepared in 10 μL DMEM without supplements. The pNLF1-NRF2 [CMV/neo] reporter vector
169 (Promega) was used to evaluate NFE2L2 (NRF2) stability. The pNLF1-NRF2 vector (100 ng)
170 along with the pKEAP1 vector (10 ng) and 0.5 μL ViaFect™ Transfection Reagent was prepared
171 in 10 μL DMEM without supplements. In addition, pNLF1-NRF2 were prepared in 10 μL DMEM
172 without supplements. After 15 min incubation, the plasmid mixture was added to the cells and
173 incubated at 38.5 °C under 5% CO_2 for 4 h. After 4 h incubation, the media were changed to
174 serum-containing media. After 48 h of transfection, cells were further incubated at 38.5 °C or
175 40.5 °C for 12 h under 5% CO_2. These cells were then lysed by the addition of 50 μL Passive
176 Lysis Buffer (Promega). The luciferase assay was performed using the Nano-Glo® Dual-
177 Luciferase® Reporter Assay System (Promega). Each sample was analyzed in duplicate per group
178 (n = 6).

179

180 *Immunocytochemistry*

Cells were plated onto 4-well dishes and analyzed for the localization of KEAP1, NRF2, PTGS2, or IL8 protein. Cells were grown to 70–80% confluence, fixed in 4% paraformaldehyde at room temperature for 10 min, and then permeabilized for 15 min with Tris buffered saline with Tween-20 (TBST). Non-specific binding was blocked by blocking the cells with Blocking One (Nacalai Tesque, Inc., Kyoto, Japan) for 20 min. The cells were incubated with the primary antibodies, KEAP1 (G-2) (sc-365626, Santa Cruz Biotechnology, Inc., TX, USA, 1:50), NRF2 (A-10) (sc-365949, Santa Cruz Biotechnology, Inc., 1:50), COX2 (ab23672, 1:100; Abcam), or IL8 (ab193818, Abcam, Cambridge, UK, 1:100) at room temperature overnight. The secondary antibody used was goat anti-mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (A11001; Invitrogen, Tokyo, Japan), Alexa Fluor 568 Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody (A10042; Invitrogen), or Donkey polyclonal Secondary Antibody to Goat IgG - H&L (Alexa Fluor 488) (ab150129; Abcam). Nuclei were counterstained with the 25 mg/mL Hoechst 33342 (Sigma-Aldrich, St Louis, MO, USA) prepared in phosphate-buffered saline (PBS). Fluorescence signals were visualized using a Leica: TCS SP5 confocal laser-scanning microscope. Results are representative of three independent experiments.

Western blotting

Western blotting

To detect PTGS2 protein, cell lysates were prepared in CelLyticTM M buffer (Sigma-Aldrich). The cell lysates (20 μg/lane) were separated through 10% SDS-PAGE and transferred onto a polyvinylidene difluoride (PVDF) membrane (n = 4) using an iBlot Gel Transfer Device system (Invitrogen). After blocking with PVDF blocking buffer for CanGetSignal (Toyobo), they were treated with goat polyclonal anti-COX2 antibody (ab23672, Abcam, 1:1000), or mouse monoclonal anti-beta actin antibody (internal control, 66009-1-Ig, Proteintech, IL, USA, 1: 5000).

Proteins were detected using the secondary antibody conjugated with horseradish peroxidase and ImmunoStar (Wako).

Statistical analyses

Results are expressed as the mean ± standard error of the mean (SEM). Data were analyzed using the Student's *t*-test to compare the control and heat stress (HS, 12h) group. For multiple comparisons to compare with the control and HS groups (3 h, 6 h, and 12 h), data were analyzed by one-way analysis of variance (ANOVA) followed by Dennett's test using the StatView statistical analysis software (version 5; SAS Institute Inc., Cary, NC, USA). Differences with P values < 0.05 were considered statistically significant.

220 **Results**

221 *Effect of heat-stressed culture on bovine endometrial epithelial cells*

222 The effect of culture temperature on bovine endometrial epithelial cells cultured under
223 thermoneutral (38.5°C, Cont.) and high temperature (40.5 °C, HS 3 h, 6 h, and 12 h) conditions
224 was examined (Figure 1). The gene expression levels of heat stress marker genes, *HSP60* and
225 *HSP90* were gradually increased under high temperature conditions, and increased significantly
226 at 6 h and 12 h compared to the control ($P < 0.01$). The *HSP70* gene expression levels were
227 significantly increased after 3 h heat stress culture compared to the control group. ($P < 0.05$)
228 (Figure 1A). Cell proliferation significantly decreased after 12 h at high temperature compared to
229 the control group ($P < 0.05$) (Figure 1B). Reactive oxygen species (ROS)-positive cells were
230 detected under both basal and high temperature conditions, and the fluorescence intensity in cells
231 was significantly higher after 12 h culture at high temperature compared to the control group (P
232 < 0.05) (Figure 1C).

233

234 *Effect of heat stress on HSE and ARE transcriptional activity, and expression levels of antioxidant*
235 *enzyme genes*

236 The effect of heat stress on the transcriptional activity of HSE and ARE in cells cultured
237 under thermoneutral or heat-stress conditions was measured with a luciferase reporter assay
238 (Figure 2). The HSE and ARE are crucial response elements for the heat stress and oxidative stress
239 responses, respectively (Figure 2A). Transcriptional activity of HSE was upregulated in the heat-
240 stressed group compared to the control group ($P < 0.01$). The transcriptional activity of ARE was
241 also upregulated under heat stress ($P < 0.05$). The stability of NFE2L2, the master regulator of
242 the oxidative stress response, was increased in cells cultured for 12 h under heat-stress condition
243 ($P < 0.05$) (Figure 2B). Furthermore, the expression levels of antioxidant enzyme genes, catalase,

244 copper/zinc superoxide dismutase (*CuZnSOD*), and manganese superoxide dismutase (*MnSOD*),
245 which are possible target of NFE2L2, gradually increased in endometrial epithelial cells under
246 high-temperature conditions, and were significantly higher at 12 h compared to the control group
247 ($P < 0.05$) (Figure 2C). Expression level of glutathione peroxidase 4 (*GPX4*) was not significantly
248 changed by heat stress.
249
250 *Expression of KEAP1 and NFE2L2 in bovine endometrial epithelial cells under heat-stressed*
251 *conditions*
252 To determine whether the KEAP1–NFE2L2 pathway, the major regulator of oxidative
253 stress responses, is induced by heat stress, the expression levels of *KEAP1* and *NFE2L2* mRNA
254 and their protein localizations, were examined in cultured bovine endometrial epithelial cells
255 under thermoneutral and high temperature conditions (Figure 3). *KEAP1* and *NFE2L2* mRNA
256 expression levels showed a slight increase under heat stressed conditions (Figure 3A). The
257 KEAP1 protein was detected in cells cultured under both basal and high temperature conditions,
258 and was predominantly localized in the cytoplasm (Figure 3B). The NFE2L2 protein was also
259 detected in cells under both basal and high-temperature conditions and was localized in the
260 cytoplasm under basal temperature conditions. Under heat-stress conditions, the NFE2L2 protein
261 was detected in both the cytoplasm and nucleus, but it was predominantly localized in the nucleus
262 (Figure 3C).
263
264 *Expression of inflammatory cytokines under heat stress conditions*
265 The expression of inflammatory cytokine genes under heat-stressed conditions were
266 examined, as the KEAP1–NFE2L2–ARE pathway is known to play a role in the anti-
267 inflammatory process. The mRNA expression levels of inflammatory cytokine genes were

compared between bovine endometrial cells cultured under basal and high temperature conditions (Figure 4, Supplemental Figure 1). The expression levels of *PTGS2* and *IL8* were lower under high temperature conditions than in the control group ($P < 0.05$) (Figure 4A, Supplemental Figure 1A). The PTGS2 protein was detected by western blotting and immunostaining in cells cultured under both basal and high temperature conditions (Figure 4B, 4C). The expression level and localization was not changed between the temperatures (Figure 4C). IL8 was also detected in the cytoplasm of cells cultured under both temperature conditions, and the localization was not changed by culture temperatures (Supplemental Figure 1B). The expression levels of PGF2α synthase (*PGFS*) and PGE2 synthase (*PGES*) (Figure 4D), and other cytokine genes (Supplemental Figure 1C), were not significantly affected by temperature.

Discussion

The effect of heat stress on cultured bovine endometrial epithelial cells, especially oxidative stress induction and the subsequent cellular responses, was investigated in this study. A possible schematic model of the effect of heat stress in bovine endometrial epithelial cells obtained from this study is shown in Figure. 5. Expression of *HSP70* significantly increased in cells cultured in heat-stressed condition for 3 h, while *HSP60* and *HSP90* expression significantly increased in heat stressed cells after 6–12 h (Figure 1A). The increase in *HSP* mRNA levels in this study is consistent with that shown in previous studies [17, 44]. Cell proliferation also significantly decreased after 12 h heat stress (Figure 1B). Heat stress induces oxidative stress and ROS generation in blood samples [27, 45], therefore, the induction of oxidative stress was investigated in bovine endometrial endothelial cells. CellROX-positive cells with ROS production were detected both under thermoneutral and heat-stressed conditions. Under high temperatures, more distinct fluorescence from CellROX staining was detected, and the fluorescence intensity significantly increased in cells subjected to heat stress for 12 h compared to the control group (Figure 1C). These results show that the heat stress response was induced after 3 h of incubation at 40.5°C, and 12 h was sufficient for heat stress loading and oxidative stress induction in endometrial epithelial cells.

The transcriptional activation of HSPs is mediated by HSFs, which bind to the HSE [22, 24, 25]. This study showed that the reporter activity of ARE, as well as HSE, was significantly activated in bovine endometrial epithelial cells cultured under heat-stressed conditions than that under thermoneutral conditions (Figure 2A). In addition, the reporter assay also showed that NFE2L2, a critical regulator of ARE-dependent transcription, was stabilized under heat-stressed conditions (Figure 2B). Furthermore, the mRNA expression levels of antioxidant enzymes, possible targets of NFE2L2, gradually increased and were significantly high after heat stress for

12 h (Figure 2C). These results suggest that the ARE-dependent antioxidant enzyme genes were upregulated via NFE2L2 stabilization in bovine endometrial epithelial cells undergoing heat stress.

Immunostaining showed that KEAP1 and NFE2L2 proteins were detected in bovine endometrial epithelial cells cultured under both basal and high temperature conditions (Figure 3B, 3C). NFE2L2 was localized in the cytoplasm under basal condition, whereas under heat-stress conditions, it was localized in both the cytoplasm and, predominantly, in the nucleus as expected. Although the number of NFE2L2-positive cells appeared to increase, there was no significant difference in gene expression levels at 3–12 h incubation under heat stress (Figure 3A). Under basal conditions, the NFE2L2 protein is maintained at low levels as it is suppressed by KEAP1 ubiquitin-proteasome degradation [46]. Upon exposure to oxidative stress, NFE2L2 escapes from KEAP1 and accumulates in the nucleus [33]. Thus, the increased NFE2L2 might not be due to increased gene expression, but to escape degradation. KEAP1 protein was predominantly localized in the cytoplasm, regardless of the culture temperature. This is consistent with a report that oxidative/electrophilic stress promotes NFE2L2 accumulation in the nucleus, without altering the cytoplasmic localization of KEAP1 [47]. These results further support the hypothesis that oxidative stress is induced by heat stress and that the KEAP1- NFE2L2 system could play a role in heat-stress protection mechanisms.

There have been several reports on oxidative stress and reproductive dysfunction. ROS affect multiple physiological processes from oocyte maturation to fertilization, embryonic development and pregnancy [48]. In the uterus, oxidative stress induces the release of cytokines and prostaglandins, resulting in endothelial cell dysfunction, which is involved in the development of preeclampsia. It has been also reported that excessive activation of ROS can cause preeclampsia [49–51]. In this study, we examined the effect of heat stress on the expression of inflammatory cytokine genes (Figure 4, Supplemental Figure 1), particularly on possible NFE2L2

target genes. In bovine endometrial epithelial cells, the expression of some inflammatory cytokines, such as *PTGS2* and *IL8,* decreased significantly under heat stress conditions. This is in agreement with previous reports of NFE2L2-mediated suppressions of inflammation [52–54]. These results suggest that NFE2L2 plays a role in the anti-inflammatory effect in bovine endometrial epithelial cells under heat stress.

Expression levels of *PTGS2*, a critical regulator of inflammation, decreased under heat stress culture. Whereas, the expression levels of *PGFS* and *PGES* was not significantly changed by heat stress. Sakai et al. reported that heat stress induced increase of PGE2 and PGF2α secretion in bovine endometrial stromal cells, but not epithelial cells [17]. Richter et al. reported that bovine fibroblasts produce PGE2 at basal temperatures (38.5°C), but PGE2 production rates decreases under extreme heat stress (45°C for 6 h), while PGFS protein expression was not affected by heat stress [55]. The mechanism of these differences in response to heat stress in each cell remains unknown. Studying the detailed expression of KEAP and/or NFE2L2 in these cells may help understand cell-dependent heat stress responses.

This study demonstrated that HSPs and antioxidant enzyme gene expression increases, whereas inflammatory cytokine expression decreases in cells after 12 h of heat stressed culture. In contrast, we previously reported that HSPs and antioxidant enzyme gene expression was lower, whereas that of inflammatory cytokines were higher, in bovine endometrium tissues collected in summer compared to those collected in winter [42]. These results suggest that chronic heat stress may reduce transcriptional activity of HSPs and antioxidant enzyme genes, thus affecting inflammatory cytokine levels in the uterine endometrium, which in turn might influence maternal receptivity and/or embryonic loss. This study only examined the effects of heat stress up to 12 h. Further studies are needed to elucidate the relationship between heat stress duration, the effect on the expression of these genes and proteins, and compensation mechanisms to protect uterine cells.

In particular, it will be necessary to verify the protein expression and its function for more target genes.

In conclusion, induction of oxidative stress by heat stress was observed in bovine endometrial epithelial cells. The reporter activity of HSE and ARE was increased by hear stress. NFE2L2 stabilized and accumulated in the nucleus under high temperature. Heat stress induced the expression of ARE-dependent antioxidant enzyme genes, while inflammatory cytokine genes were decreased. These results suggest that the KEAP1-NFE2L2-ARE pathway plays a protective role in bovine endometrial epithelial cells cultured under heat stress conditions. Modulating the KEAP1-NFE2L2-ARE pathway may help reduce the negative effects of heat and oxidative stress in bovine endometrial epithelial cells.

Acknowledgments

We thank the NICHIRO CHIKUSAN CO., LTD., and the Hokkaido Hayakita meat inspection center for providing bovine uterine tissues. We also thank Mr. Yusuke Yasue (Promega Japan, Tokyo, Japan) for the execution of the reporter assays. We would like to thank Editage (www.editage.com) for English language editing.

Author contributions

HM and HB designed the experiments. HM, HK, and HB, performed experiments and carried out data collection. KK, TS, MK, MT, and HB, provided resources, conducted data analysis, and wrote the manuscript. All authors have provided final approval for publication and agree to be held accountable for the work documented therein.

Data availability

All relevant data are included in the manuscript and its associated files.

Conflict of interest statement

The authors declare no conflicts of interest.

Figure legends

Figure. 1. Effect of heat stress on cultured bovine uterine endometrial epithelial cells.

(A) Relative gene expressions of heat shock proteins (*HSP60, HSP70,* and *HSP90*) under thermoneutral (38.5°C, Cont.) or high temperature (40.5 °C, HS 3 h, 6 h, and 12 h) conditions were determined by qPCR. The expression levels of *H2AFZ* were used as internal controls. Data are presented as means ± standard error of the mean (SEM). Asterisks indicates a significant difference (**$P < 0.01$, *$P < 0.05$). **(B)** The proliferation of bovine endometrial epithelial cells. SI: stimulation index (absorbance from stimulated wells/absorbance from non-stimulated wells). The asterisk (*) indicates a significant difference ($P < 0.05$). **(C)** Cells stained with the fluorogenic probe, CellROX® Green reagent, were visualized with a fluorescence microscope. Hoechst 33342 was used to stain nuclei. The fluorescence intensity was also measured. Average values were calculated from three wells. Data are presented as means ± SEM. *$P < 0.05$. Bar = 200 μm.

Figure. 2. Heat stress induces transactivation of the heat shock responsive element (HRE) and the antioxidant response element (ARE) in bovine uterine endometrial epithelial cells.

(A) Transcription of the reporter construct ~~when~~ transfected with HSE- or ARE-reporter construct into bovine endometrial epithelial cells and cultured under thermoneutral (38.5°C, Cont.) or heat stress (40.5°C, 12 h) conditions. **$P < 0.01$, *$P < 0.05$ **(B)** Stability of nuclear factor, erythroid 2 like 2 (NFE2L2) reporter construct co-transfected with KEAP1 construct into endometrial epithelial cells and cultured under basal or high temperature conditions. *$P < 0.05$ **(C)** Relative expression of antioxidant enzyme genes catalase, copper/zinc superoxide dismutase (*CuZnSOD*), manganese superoxide dismutase (*MnSOD*), and glutathione peroxidase 4 (*GPX4*), which are possible target of NFE2L2, under thermoneutral (38.5°C, Cont.) or high temperature (40.5 °C, HS 3 h, 6 h, and 12 h) conditions were determined using qPCR. The expression levels of *H2AFZ* were

used as internal control. Data are presented as means ± standard error of the mean (SEM). *, $P < 0.05$.

Figure 3. Expression levels and localization of Kelch-like ECH-associated protein 1 (KEAP1) and nuclear factor, erythroid 2 like 2 (NFE2L2), under thermoneutral and high temperature conditions.

Bovine endometrial epithelial cells were cultured under thermoneutral (38.5°C; Cont.) or high temperature (40.5°C; HS 3 h, 6 h, and 12 h.) conditions. Expression levels of (**A**) *KEAP1* and *NFE2L2* mRNA were examined using qPCR. Expression levels of *H2AFZ* were used as internal controls. Data are presented as means ± standard error of the mean (SEM). Protein localization of (**B**) KEAP1and (**C**) NFE2L2 was examined by immunostaining. Nuclear DNA was counterstained by Hoechst 33342. Scale bar = 50 μm. Negative Control (inset, without primary antibody).

Figure. 4. Expression levels and localization of PTGS2 in bovine endometrial epithelial cells.

(**A**) Expression levels of prostaglandin-endoperoxide synthase 2 (*PTGS2*), in endometrial epithelial cells under thermoneutral (38.5°C; Cont.) or heat stress (40.5°C; HS 3 h, 6 h, and 12 h) conditions examined using qPCR. Expression levels of *H2AFZ* were used as internal control. Data are presented as means ± standard error of the mean (SEM). Asterisks indicate significant differences: **, $P < 0.01$. (**B**) Western blotting of PTGS2 in endometrial epithelial cells under thermoneutral or heat stress conditions. (**C**) Protein localization of PTGS2 was examined by immunostaining. Nuclear DNA was counterstained by Hoechst 33342. Scale bar = 50 μm. Negative Control (inset, without primary antibody). (**D**) Expression levels of *PGES* and *PGFS* in endometrial epithelial cells under thermoneutral or heat stress conditions examined using qPCR. Expression levels of

426 *H2AFZ* were used as internal control. Data are presented as means ± standard error of the mean
427 (SEM).
428
429 **Figure. 5. A possible schematic model of the effect of heat stress in bovine endometrial**
430 **epithelial cells.**
431 In this study, bovine endometrial epithelial cells were cultured under thermoneutral (38.5°C) or
432 heat stress (40.5°C). Under heat stressed conditions, NFE2L2 proteins are translocated to the
433 nucleus, where they increase the expression of antioxidant enzymes genes and suppress the
434 expression of inflammatory cytokines vis ARE.
435
436 **Supplemental Figure. 1. Expression of inflammatory cytokines in bovine endometrial**
437 **epithelial cells.**
438 (**A**) Expression levels of interleukin (IL) 8 in endometrial epithelial cells under thermoneutral
439 (38.5°C; Cont.) or heat stress (40.5°C; HS 3 h, 6 h, and 12 h) conditions examined using qPCR.
440 The expression levels of *H2AFZ* were used as internal control. Data are presented as means ±
441 standard error of the mean (SEM). *, $P < 0.05$. (**B**) Protein localization of IL8 examined by
442 immunostaining. Nuclear DNA was counterstained by Hoechst 33342. Scale bar = 50 μm.
443 Negative Control (inset, without primary antibody). (**C**) Expression levels of *IL1b*, *IL6*, and tumor
444 necrosis factor alpha (TNFA) in endometrial epithelial cells under thermoneutral or heat stress
445 conditions examined using qPCR. The expression levels of *H2AFZ* were used as internal control.
446 Data are presented as means ± standard error of the mean (SEM).
447
448

References

[1] Boni R. Heat stress, a serious threat to reproductive function in animals and humans. *Mol Reprod Dev* 2019; **86**: 1307–1323.
DOI: 10.1002/mrd.23123

[2] Wolfenson D, Thatcher WW, Badinga L, Savio JD, Meidan R, Lew BJ, Braw-Tal R, Berman A. Effect of heat stress on follicular development during the estrous cycle in lactating dairy cattle. *Biol Reprod* 1995; **52**: 1106–1113.
DOI: 10.1095/biolreprod52.5.1106

[3] Burke JM, Spiers DE, Kojima FN, Perry GA, Salfen BE, Wood SL, Patterson DJ, Smith MF, Lucy MC, Jackson WG, Piper EL. Interaction of endophyte-infected fescue and heat stress on ovarian function in the beef heifer. *Biol Reprod* 2001; **65**: 260–268.
DOI: 10.1095/biolreprod65.1.260

[4] Hansen PJ, Drost M, Rivera RM, Paula-Lopes FF, al-Katanani YM, Krininger CE 3rd, Chase CC. Adverse impact of heat stress on embryo production: causes and strategies for mitigation. *Theriogenology* 2001; **55**: 91–103.
DOI: 10.1016/s0093-691x(00)00448-9

[5] Payton RR, Romar R, Coy P, Saxton AM, Lawrence JL, Edwards JL.Susceptibility of bovine germinal vesicle-stage oocytes from antral follicles to direct effects of heat stress in vitro. *Biol Reprod* 2004; **71**: 1303–1308.
DOI: 10.1095/biolreprod.104.029892

[6] Roth Z, Hansen PJ. Involvement of apoptosis in disruption of developmental competence of bovine oocytes by heat shock during maturation. *Biol Reprod* 2004; **71**: 1898–1906.
DOI: 10.1095/biolreprod.104.031690

[7] Sartori R, Sartor-Bergfelt R, Mertens SA, Guenther JN, Parrish, JJ, Wiltbank MC. Fertilization and early embryonic development in heifers and lactating cows in summer and lactating and dry cows in winter. *J Dairy Sci* 2002; **85**: 2803–2812.
DOI: 10.3168/jds.S0022-0302(02)74367-1.

[8] Gwazdauskas FC, Thatcher WW, Wilcox CJ. Physiological, environmental, and hormonal factors at insemination which may affect conception. *J Dairy Sci* 1973; **56**: 873–877.

485 DOI: 10.3168/jds.S0022-0302(73)85270-1
486
487 [9] El-Sheikh Ali H, Kitahara G, Tamura Y, Kobayashi I, Hemmi K, Torisu S, Sameshima H.,
488 Horii Y, Zaabel S, Kamimura, S. Presence of a temperature gradient among genital tract portions
489 and the thermal changes within these portions over the estrous cycle in beef cows. *J Reprod Dev*
490 2013; **59**: 59–65.
491 DOI: 10.1262/jrd.2012-017
492
493 [10] Gwazdauskas FC, Wilcox CJ, Thatcher WW. Environmental and managemental factors
494 affecting conception rate in a subtropical climate. *J Dairy Sci* 1975; **58**: 88–92.
495 DOI: 10.3168/jds.S0022-0302(75)84523-1
496
497 [11] Roman-Ponce H, Thatcher WW, Caton D, Barron DH, Wilcox CJ. Thermal stress effects on
498 uterine blood flow in dairy cows. *J Anim Sci* 1978; **46**: 175–180.
499 DOI: 10.2527/jas1978.461175x
500
501 [12] do Amaral BC, Connor EE, Tao S, Hayen MJ, Bubolz JW, Dahl GE. Heat-stress abatement
502 during the dry period: Does cooling improve transition into lactation? *J Dairy Sci* 2009; **92**: 5988–
503 5999.
504 DOI: 10.3168/jds.2009-2343
505
506 [13] Tao S, Monteiro AP, Thompson IM, Hayen MJ, Dahl GE. Effect of late-gestation maternal
507 heat stress on growth and immune function of dairy calves. *J Dairy Sci* 2012; **95**: 7128–7136.
508 DOI: 10.3168/jds.2012-5697
509
510 [14] Monteiro APA, Guo JR, Weng XS, Ahmed BM, Hayen MJ, Dahl GE, Bernard JK, Tao S.
511 Effect of maternal heat stress during the dry period on growth and metabolism of calves. *J Dairy*
512 *Sci* 2016; **99**: 3896–3907.
513 DOI: 10.3168/jds.2015-10699
514
515 [15] Monteiro APA, Tao S, Thompson IMT, Dahl DE. In utero heat stress decreases calf survival
516 and performance through the first lactation. *J Dairy Sci* 2016; **99**: 8443–8450.
517 DOI: 10.3168/jds.2016-11072
518

519 [16] Putney DJ, Malayer JR, Gross TS, Thatcher WW, Hansen PJ, Drost M. Heat stress-induced
520 alterations in the synthesis and secretion of proteins and prostaglandins by cultured bovine
521 conceptuses and uterine endometrium. *Biol Reprod* 1988; 39: 717–728.
522
523 [17] Sakai S, Hagihara N, Kuse M, Kimura K, Okuda K. Heat stress affects prostaglandin
524 synthesis in bovine endometrial cells. *J Reprod Dev* 2018; **64**: 311–317.
525 DOI: 10.1262/jrd.2018-051
526
527 [18] García-Ispierto I., López-Gatius, F., Santolaria, P., Yániz, J. L., Nogareda, C., López-Béjar,
528 M., & De Rensis, F. Relationship between heat stress during the peri-implantation period and
529 early fetal loss in dairy cattle. *Theriogenology* 2006; **65**: 799–807.
530 DOI: 10.1016/j.theriogenology.2005.06.011
531
532 [19] Huang C, Tsuruta S, Bertrand JK, Misztal I, Lawlor TJ, Clay JS. Trends for conception rate
533 of Holsteins over time in the southeastern United States. *J Dairy Sci* 2009; 92: 4641–4647.
534 DOI: 10.3168/jds.2008-1982
535
536 [20] De Rensis F, Lopez-Gatius F, García-Ispierto I, Morini G, Scaramuzzi RJ. Causes of
537 declining fertility in dairy cows during the warm season. *Theriogenology* 2017; **91**: 145–153.
538 DOI: 10.1016/j.theriogenology.2016.12.024
539
540 [21] Oikawa K, Yamazaki T, Yamaguchi S, Abe H, Bai H, Takahashi M, Kawahara M. Effects of
541 use of conventional and sexed semen on the conception rate in heifers: A comparison study.
542 *Theriogenology* 2019; **135**: 33–37.
543 DOI: 10.1016/j.theriogenology.2019.06.012
544
545 [22] Binder RJ. Functions of heat shock proteins in pathways of the innate and adaptive immune
546 system. *J Immunol* 2014; **193**: 5765–5771.
547 DOI: 10.4049/jimmunol.1401417
548
549 [23] Neuer A, Spandorfer SD, Giraldo P, Dieterle S, Rosenwaks Z, Witkin SS. The role of heat
550 shock proteins in reproduction. *Hum Reprod Update* 2000; **6**: 149–159.
551 DOI: 10.1093/humupd/6.2.149
552
553 [24] Wu C. Heat shock transcription factors: structure and regulation. *Annu Rev Cell Dev Biol*
554 1995; **11**: 441–469.

555 DOI: 10.1146/annurev.cb.11.110195.002301
556
557 [25] Morimoto RI. Regulation of the heat shock transcriptional response: cross talk between a
558 family of heat shock factors, molecular chaperones, and negative regulators. *Genes Dev* 1998; **12**:
559 3788–3796
560 DOI: 10.1101/gad.12.24.3788
561
562 [26] Barna J, Csermely P, Vellai T. (2018). Roles of heat shock factor 1 beyond the heat shock
563 response. *Cell Mol Life Sci* 2018; **75**: 2897–2916.
564 DOI: 10.1007/s00018-018-2836-6
565
566 [27] Bernabucci U, Ronchi B, Lacetera N, Nardone A. Markers of oxidative status in plasma and
567 erythrocytes of transition dairy cows during hot season. *J Dairy Sci* 2002; **85**: 2173–2179.
568 DOI: 10.3168/jds.S0022-0302(02)74296-3
569
570 [28] Roth Z. hysiology and endocrinology symposium: Cellular and molecular mechanisms of
571 heat stress related to bovine ovarian function. *J Anim Sci* 2015; **93**: 2034–2044.
572 DOI: 10.2527/jas.2014–8625
573
574 [29] Sakatani M. Effects of heat stress on bovine preimplantation embryos produced in vitro. *J*
575 *Reprod Dev* 2017; **63**: 347–352.
576 DOI: 10.1262/jrd.2017-045
577
578 [30] Rushmore TH, Morton MR, Pickett CB. The antioxidant responsive element. Activation by
579 oxidative stress and identification of the DNA consensus sequence required for functional
580 activity. *J Biol Chem* 1991; **266**: 11632–11639.
581
582 [31] Jin XL, Wang K, Liu L, Liu HY, Zhao FQ, Liu JX. Nuclear factor-like factor 2-antioxidant
583 response element signaling activation by tert-butylhydroquinone attenuates acute heat stress in
584 bovine mammary epithelial cells. *J Dairy Sci,* 2016; **99**: 9094–9103.
585 DOI: 10.3168/jds.2016-11031
586
587 [32] Liang Y, Batistel F, Parys C, Loor JJ. Glutathione metabolism and nuclear factor erythroid
588 2-like 2 (NFE2L2)-related proteins in adipose tissue are altered by supply of ethyl-cellulose
589 rumen-protected methionine in peripartal Holstein cows. *J Dairy Sci* 2019; **102**: 5530–5541.
590 DOI: 10.3168/jds.2018-15687

591 [33] Suzuki T, Yamamoto M. Stress-sensing mechanisms and the physiological roles of the
592 Keap1-Nrf2 system during cellular stress. *J Biol Chem* 2017; **292**: 16817–16824.
593 DOI: 10.1074/jbc.R117.800169
594
595 [34] Khor TO, Huang MT, Kwon KH, Chan JY, Reddy BS, Kong AN. Nrf2-deficient mice have
596 an increased susceptibility to dextran sulfate sodium-induced colitis.
597 *Cancer Res* 2006; **66**: 11580-11584.
598 DOI: 10.1158/0008-5472.CAN-06-3562.
599
600 [35] Loboda A, Stachurska A, Florczyk U, Rudnicka D, Jazwa A, Wegrzyn J, Kozakowska M,
601 Stalinska K, Poellinger L, Levonen AL, Yla-Herttuala S, Jozkowicz A, Dulak J. HIF-1 induction
602 attenuates Nrf2-dependent IL-8 expression in human endothelial cells.
603 *Antioxid Redox Signal* 2009; **11**: 1501-1517.
604 DOI: 10.1089/ars.2008.2211
605
606 [36] Breetha R, Ramaprasad Tr. Dietary n-3 but not n-6 fatty acids down-regulate maternal
607 dyslipidemia induced inflammation: A three-generation study in rats.
608 *Prostaglandins Leukot Essent Fatty Acids* 2018; **135**: 83-91.
609 DOI: 10.1016/j.plefa.2018.07.007
610
611 [37] Amin A, Gad A, Salilew-Wondim D, Prastowo S, Held E, Hoelker M, Rings F, Tholen E,
612 Neuhoff C, Looft C, Schellander K, Tesfaye D. Bovine embryo survival under oxidative-stress
613 conditions is associated with activity of the NRF2-mediated oxidative-stress-response pathway.
614 *Mol Reprod Dev* 2014; **81**: 497-513.
615 DOI: 10.1002/mrd.22316
616
617 [38] Fu K, Chen H, Wang Z, Cao R. Andrographolide attenuates inflammatory response induced
618 by LPS via activating Nrf2 signaling pathway in bovine endometrial epithelial cells.
619 *Res Vet Sci* 2021; **134**: 36–41.
620 DOI: 10.1016/j.rvsc.2020.11.022
621
622 [39] Shirozu T, Iwano H, Ogiso T, Suzuki T, Balboula AZ, Bai H, Kawahara M, Kimura K,
623 Takahashi H, Rulan B, Kim SW, Yanagawa Y, Nagano M, Imakawa K, Takahashi M. Estrous
624 cycle stage-dependent manner of type I interferon-stimulated genes induction in the bovine
625 endometrium.
626 *J Reprod Dev* 2017; **63**: 211–220.

627 DOI: 10.1262/jrd.2016-176. Epub 2017 Feb 26.
628
629 [40] Suzuki T, Sakumoto R, Hayashi KG, Ogiso T, Kunii H, Shirozu T, Kim SW, Bai H,
630 Kawahara M, Kimura K, Takahashi M. Involvement of interferon-tau in the induction of
631 apoptotic, pyroptotic, and autophagic cell death-related signaling pathways in the bovine uterine
632 endometrium during early pregnancy. *J Reprod Dev* 2018; **64**: 495–502.
633 DOI: 10.1262/jrd.2018-063
634
635 [41] Rivera RM, and Hansen PJ. Development of cultured bovine embryos after exposure to
636 high temperatures in the physiological range.
637 *Reproduction* 2001; **121**: 107-115.
638
639 [42] Bai H, Ukita H, Kawahara M, Mitani T, Furukawa E, Yanagawa Y, Yabuuchi N, Kim H,
640 Takahashi M. Effect of summer heat stress on gene expression in bovine uterine endometrial
641 tissues. *Anim Sci J* 2020; **91**: e13474.
642 DOI: 10.1111/asj.13474.
643
644 [43] Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T,
645 Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT. The MIQE guidelines: Minimum
646 information for publication of quantitative real-time PCR experiments. *Clin Chem* 2009; **55**: 611–
647 622.
648 DOI: 10.1373/clinchem.2008.112797
649
650 [44] Xiao Y, Rungruang S, Hall LW, Collier JL, Dunshea FR, Collier RJ. Effects of niacin and
651 betaine on bovine mammary and uterine cells exposed to thermal shock in vitro. *J Dairy Sci,*
652 2017; **100**: 4025–4037.
653 DOI: 10.3168/jds.2016-11876
654
655 [45] Trout JP, McDowell LR, Hansen PJ. Characteristics of the estrous cycle and antioxidant
656 status of lactating Holstein cows exposed to heat stress. *J Dairy Sci,* 1998; **81**: 1244–1250.
657 DOI: 10.3168/jds.S0022-0302(98)75685-1
658 [46] Ma Q. Role of nrf2 in oxidative stress and toxicity. *Annu Rev Pharmacol Toxicol* 2013; **53**:
659 401–426.
660 DOI: 10.1146/annurev-pharmtox-011112-140320
661
662 [47] Watai Y, Kobayashi A, Nagase H, Mizukami M, McEvoy J, Singer JD, Itoh K, Yamamoto M.

663 Subcellular localization and cytoplasmic complex status of endogenous Keap1. *Genes Cells* 2007;
664 **12**: 1163–1178.
665 DOI: 10.1111/j.1365-2443.2007.01118.x
666
667 [48] Agarwal A, Gupta S, Sharma RK. Role of oxidative stress in female reproduction. *Reprod Biol*
668 *Endocrinol* 2005; **3**: 28.
669 DOI: 10.1186/1477-7827-3-28.
670
671 [49] Malek A, Sager R, Schneider H: Effect of hypoxia, oxidative stress and lipopolysaccharides
672 on the release of prostaglandins and cytokines from human term placental explants. *Placenta*
673 2001; **22** (Suppl A): S45-50.
674
675 [50] Bilodeau JF, Hubel CA. Current concepts in the use of antioxidants for the treatment of
676 preeclampsia. *J Obstet Gynaecol Can* 2003; **25**: 742-750.
677
678 [51] Banerjee S, Smallwood A, Moorhead J, Chambers AE, Papageorghiou A, Campbell S,
679 Nicolaides K. Placental expression of Interferongamma (IFN-gamma) and its receptor (IFN-
680 gamma) R2 fail to switch from early hypoxic to late normotensive development in pre-eclampsia.
681 *J Clin Endocrinol Metab* 2005; **90**: 944-952.
682
683 [52] Ma Q, Kinneer K, Ye J, Chen BJ. Inhibition of nuclear factor kappaB by phenolic
684 antioxidants: interplay between antioxidant signaling and inflammatory cytokine expression.
685 *Mol Pharmacol* 2003; **64**: 211–219.
686 DOI: 10.1124/mol.64.2.211
687
688 [53] Li W, Khor TO, Xu C, Shen G, Jeong WS, Yu S, Kong AN. Activation of Nrf2-antioxidant
689 signaling attenuates NFkappaB-inflammatory response and elicits apoptosis. *Biochem Pharmacol*
690 2008; **76**: 1485–1489.
691 DOI: 10.1016/j.bcp.2008.07.017
692
693 [54] Thimmulappa RK, Scollick C, Traore K, Yates M, Trush MA, Liby KT, Sporn MB,
694 Yamamoto M, Kensler TW, Biswal S. Nrf2-dependent protection from LPS induced inflammatory
695 response and mortality by CDDO-Imidazolide. *Biochem Biophys Res Commun* 2006; **351**: 883–
696 889.
697 DOI: 10.1016/j.bbrc.2006.10.102
698

699 [55] Richter C, Viergutz T, Schwerin M, Weitzel JM. Prostaglandin E synthase interacts with
700 inducible heat shock protein 70 after heat stress in bovine primary dermal fibroblast cells.
701 *Cytometry A* 2015; **87**: 61–67.
702 DOI: 10.1002/cyto.a.22595.
703
704 [56] Harvey AJ, Santos AN, Kirstein M, Kind KL, Fischer B, Thompson JG. Differential
705 expression of oxygen-regulated genes in bovine blastocysts. *Mol Reprod Dev* 2007; **74**: 290–299.
706 DOI: 10.1002/mrd.20617
707
708 [57] Assidi M, Dufort I, Ali A, Hamel M, Algriany O, Dielemann S, Sirard MA. Identification of
709 potential markers of oocyte competence expressed in bovine cumulus cells matured with follicle-
710 stimulating hormone and/or phorbol myristate acetate in vitro. *Biol Reprod* 2008; **79**: 209–222.
711 DOI: 10.1095/biolreprod.108.067686
712
713 [58] Rodrigues-Cunha MC, Mesquita LG, Bressan F, Collado MD, Balieiro JC, Schwarz KR, de
714 Castro FC, Watanabe OY, Watanabe YF, de Alencar Coelho L, Leal CL. Effects of melatonin
715 during IVM in defined medium on oocyte meiosis, oxidative stress, and subsequent embryo
716 development. *Theriogenology* 2016; **86**: 1685–1694.
717 DOI: 10.1016/j.theriogenology.2016.05.026
718
719 [59] Nishimura R, Bowolaksono A, Acosta TJ, Murakami S, Piotrowska K, Skarzynski DJ, Okuda
720 K. Possible role of interleukin-1 in the regulation of bovine corpus luteum throughout the luteal
721 phase. *Biol Reprod* 2004; **71**: 1688–1693.
722 DOI: 10.1095/biolreprod.104.032151
723
724 [60] Shirasuna K, Jiemtaweeboon S, Raddatz S, Nitta A, Schuberth HJ, Bollwein H, Shimizu T,
725 Miyamoto A. Rapid accumulation of polymorphonuclear neutrophils in the Corpus luteum during
726 prostaglandin F(2α)-induced luteolysis in the cow. *PLoS One* 2012; **7**: e29054.
727 DOI: 10.1371/journal.pone.0029054
728
729 [61] Shirasuna K, Matsumoto H, Matsuyama S, Kimura K, Bollwein H, Miyamoto A. Possible
730 role of interferon tau on the bovine corpus luteum and neutrophils during the early pregnancy.
731 *Reproduction.* 2015; **150**: 217–225.
732 DOI: 10.1530/REP-15-0085
733
734 [62] Kawaguchi S, Bowolaksono A, Yoshioka S, Sakumoto R, Okuda K. Luteoprotective

735 mechanisms of prostaglandin F2alpha stimulated by luteinizing hormone in the bovine corpus
736 luteum. *J Reprod Dev.* 2013; **59**: 225-30.
737 DOI: 10.1262/jrd.2012-187. Epub 2013 Jan 25.
738

Table 1. Primers for real time PCR

Name (GenBank accession No.)	Sequence (5'-3')	Product length (bp)	References
Heat shock proteins			
HSP60	F: GTCTTCAAGTTGTGGCAGTCAA	145	[56]
(XM_015458500.1)	R: AAGTCATGAGGCTGAACATCTTC		
HSP70	F: GCAGTCGGACATGAAGGAGT	109	
(AY662497.1)	R: GATCTCCTCCGGGTAGAACG		
HSP90	F: CCGTTTGTTGTAAGGTGTGTATGTA	277	[57]
(AB072368.1)	R: GTATGGACAATGACTCCAATCAAGT		
Antioxidant enzymes			
Catalase	F: GGAAACGCCTGTGTGAGAAC	159	
(NM_001035386.2)	R: CTGCGTTCTTAGGTTTCTCCTC		
CuZnSOD	F: ACACAAGGCTGTACCAGTGC	105	[58]
(NM_174615.2)	R: TGTCACATTGCCCAGGTCTC		
MnSOD	F: TCCTGTTCAATCGCAGTTACAGA	162	[58]
(BT020988.1)	R: ACGGGGTGGTGACTATCAGA		
GPX4	F: TGTGGTGAAGCGGTATGGTC	192	[58]
(NM_174770.4)	R: CACGCCAGGTTCTCAGGTCT		
Inflammatory cytokine and related genes			
IL1b	F: AAACAGATGAAGAGCTGCATCCAA	394	[59]
(EU276067)	R: CAAAGCTCATGCAGAACACCACTT		
IL6	F: TAAGCGCATGGTCGACAAAA	150	
(EU276071)	R: TTGAACCCAGATTGGAAGCAT		
IL8	F: CCTCTTGTTCAATATGACTTCCA	189	[60]
(NM_173925)	R: GGCCCACTCTCAATAACTCTC		
TNFA	F: TGACGGGCTTTACCTCATCT	137	[61]
(NM_173966.3)	R: TGATGGCAGACAGGATGTTG		
PTGS2	F: TGTGAAAGGGAGGAAAGAGC	114	[62]
(AF004944.1)	R: GGCAAAGAATGCAAACATCA		
PGES	F: AGGACGCTCAGAGACATGGA	142	[62]
(NM_174443.2)	R: TTCGGTCCGAGGAAAGAGTA		
PGFS	F: TTCCCTTCAACCAGAGTTGG	113	[62]
(NM_1166224.1)	R: TCCCTGGCTTCAGAGACACT		

KEAP-NRF2 systems			
KEAP1	F: ACAACAGTGTGGAGAGGTATGAGC	108	[32]
(NM_001101142.1)	R: AGAGCAGACGGTTGAGGACAG		
NFE2L2	F: AGGACATGGATTTGATTGAC	272	[31]
(NM_001011678.2)	R: TACCTGGGAGTAGTTGGCA		
Internal control			
H2AFZ	F: AGAGCCGGTTTGCAGTTCCCG	116	
(NM_174809)	R: TACTCCAGGATGGCTGCGCTGT		

F: Forward, R: Reverse

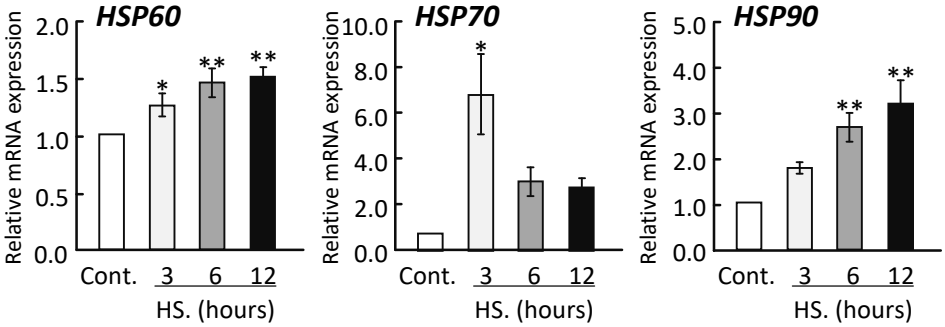
Table 2. Primers for reporter construction

Name	Sequence (5'-3')
Heat Shock Element	F: CTGGAAGATTCTAGAACGTTCTGGAAGATTCTAGAACGTTC
(HSE)	R: GAACGTTCTAGAATCTTCCAGAACGTTCTAGAATCTTCCAG
Antioxidant Responsive Element	F: TAGCTTGGAAATGACATTGCTAATGGTGACAAAGCAACTTTTAGCTTGGAAATGACATTGCTAATGGTGACAAAGCAACTTT
(ARE)	R: AA AGTTGCTTTGTCACCATTAGCAATGTCATTTCCAAGCTAAAAGTTGCTTTGTCACCATTAGCAATGTCATTTCCAAGCTA

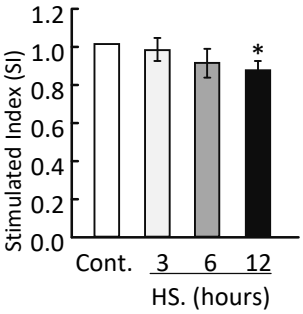
F: Forward, R: Revers

Figure 1.

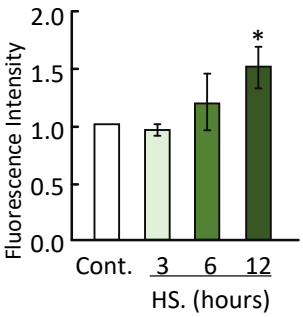
A


HSP60
HSP70
HSP90
Relative mRNA expression
Cont.
3
6
12
HS. (hours)

B


Stimulated Index (SI)
Cont.
3
6
12
HS. (hours)

C


Fluorescence Intensity
Cont.
3
6
12
HS. (hours)

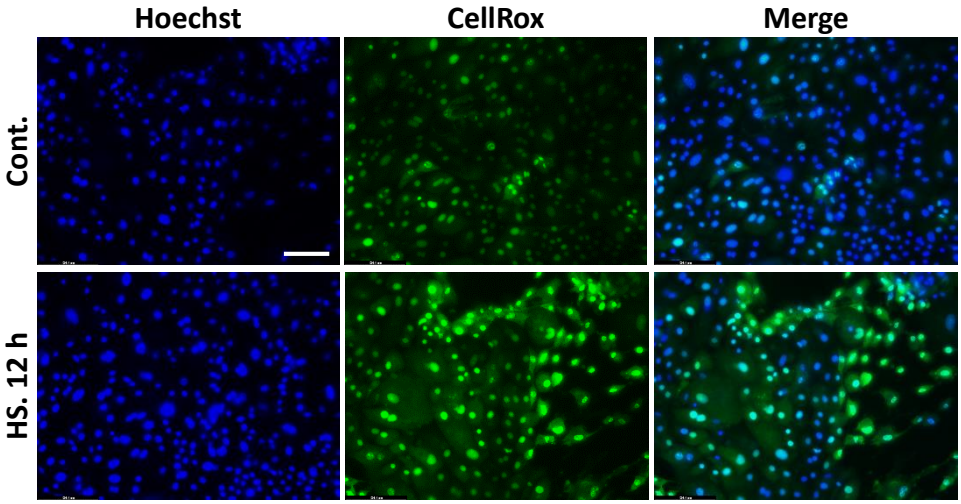

Hoechst
CellRox
Merge
Cont.
HS. 12 h

Figure 2.

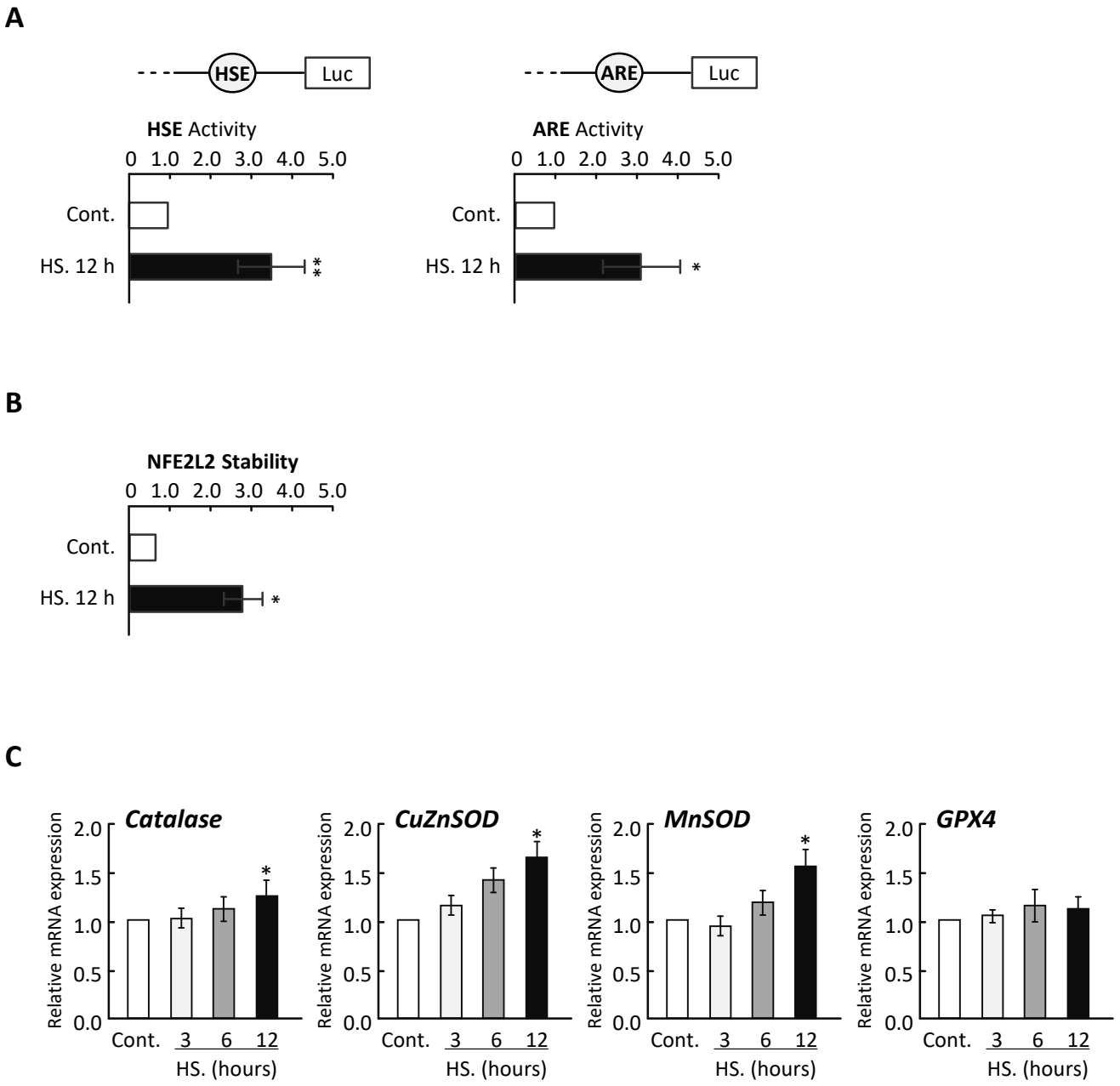
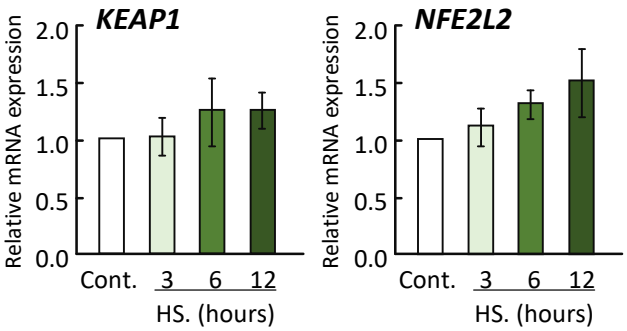
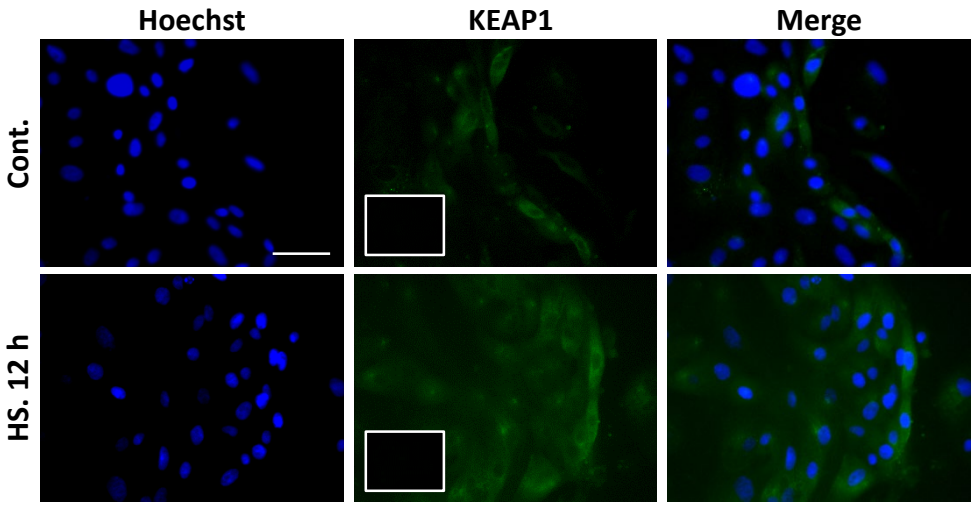

A
HSE
Luc
ARE
Luc
HSE Activity
0 1.0 2.0 3.0 4.0 5.0
Cont.
HS. 12 h
**
ARE Activity
0 1.0 2.0 3.0 4.0 5.0
Cont.
HS. 12 h
*
B
NFE2L2 Stability
0 1.0 2.0 3.0 4.0 5.0
Cont.
HS. 12 h
*
C
Catalase
CuZnSOD
MnSOD
GPX4
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
Cont. 3 6 12
HS. (hours)

Figure 3.

A


KEAP1
NFE2L2
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
Cont.
3
6
12
HS. (hours)

B


Hoechst
KEAP1
Merge
Cont.
HS. 12 h

C

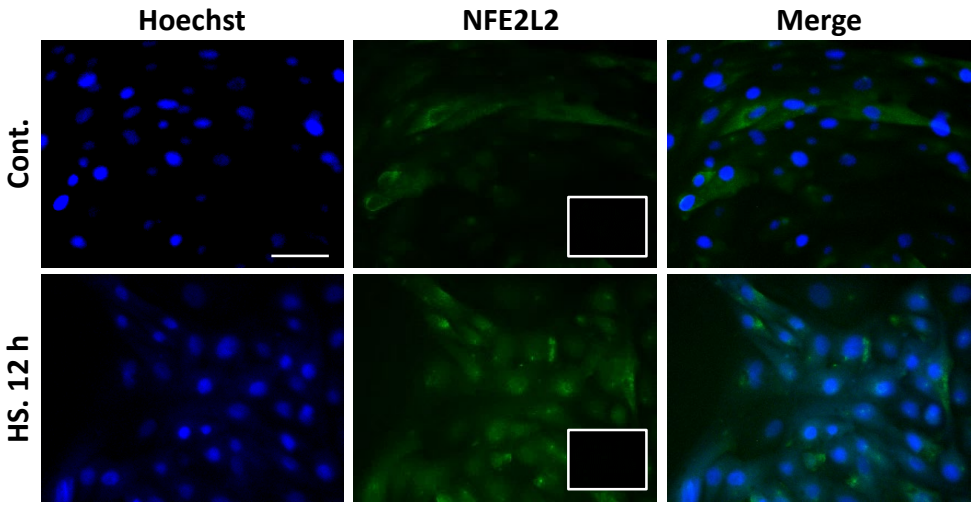
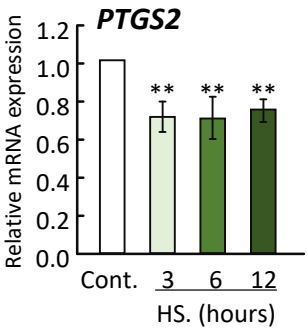
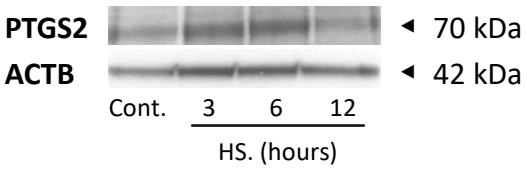

Hoechst
NFE2L2
Merge
Cont.
HS. 12 h

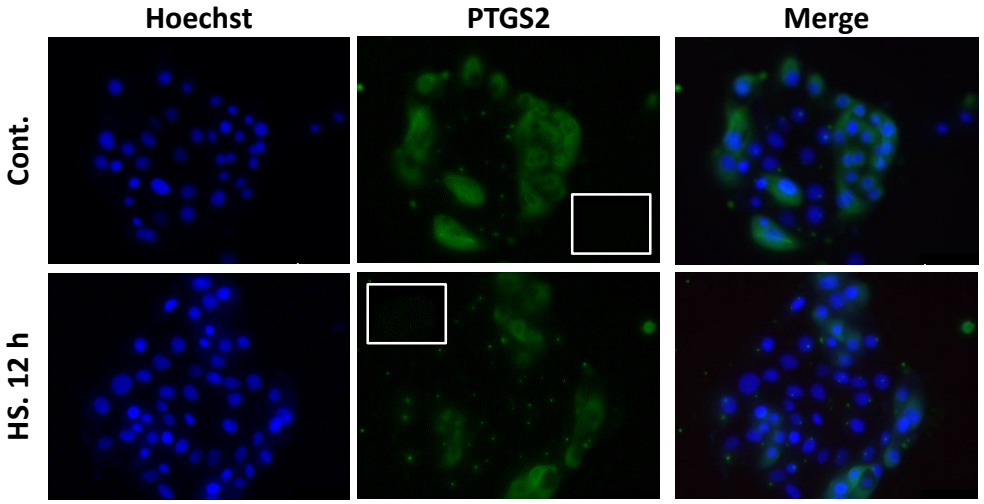
Figure 4.

A


PTGS2
Relative mRNA expression
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
**
**
Cont.
3
6
12
HS. (hours)


PTGS2
ACTB
70 kDa
42 kDa
Cont.
3
6
12
HS. (hours)

C


Hoechst
PTGS2
Merge
Cont.
HS. 12 h

D

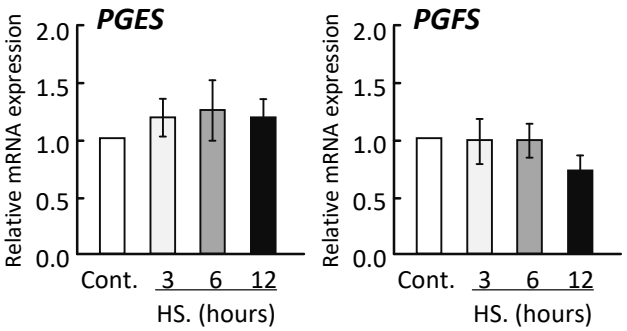
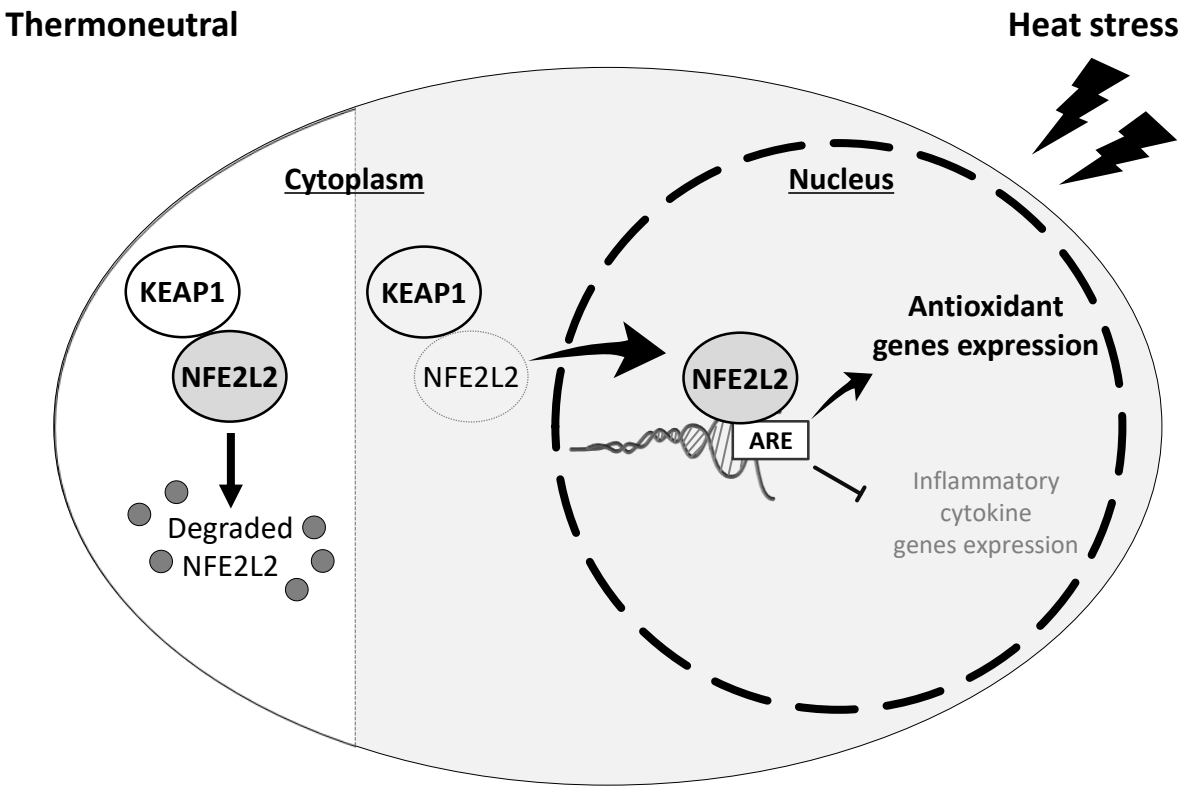

PGES
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
Cont.
3
6
12
HS. (hours)
PGFS
Relative mRNA expression
2.0
1.5
1.0
0.5
0.0
Cont.
3
6
12
HS. (hours)

Figure 5.


Thermoneutral
Heat stress
Cytoplasm
Nucleus
KEAP1
NFE2L2
KEAP1
NFE2L2
NFE2L2
ARE
Antioxidant
genes expression
Inflammatory
cytokine
genes expression
Degraded
NFE2L2