Factors affecting expression and transcription of uncoupling protein 2 gene

Doo Hyun KIM1,2), Hiroyuki SADAKANE1), Yuka NISHIKIORI1), Manami MATSUMURA1), Mayuko IKEDA1), Zhicheng DIAO1), Rajesh JHA1,3), Masaru MURAKAMI4), Tohru MATSUI1) and Masayuki FUNABA 1)*

1)Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan
2)FARMSCO, Gyeonggi 17599, Republic of Korea
3)Department of Human Nutrition Food and Animal Sciences, College of Tropical Agriculture and Human Resources, University of Hawaii at Manoa, HI 96822, USA
4)Laboratory of Molecular Biology, Azabu University School of Veterinary Medicine, Sagamihara, Kanagawa 252-5201, Japan

ABSTRACT. Previous studies suggest a negative relationship between hepatic oxidative stress and productivity in beef cattle. Uncoupling protein 2 (UCP2) is involved in the disappearance of reactive oxygen species, suggesting the defensive role of UCP2 against oxidative stress. The present study examined the relationship between oxidative stress and expression levels of UCP2/Ucp2 in cultured human and mouse liver-derived cells. We also explored factors regulating bovine Ucp2 transcription. As oxidative stress inducers, hydrogen peroxide, ethanol, and cumene hydroperoxide (CmHP) were used. Expression levels of hemoxygenase 1 (HMOX1), a representative gene induced by oxidative stress, were not affected by any oxidative stress inducers in HepG2 human liver-derived cells. The levels of UCP2 mRNA were also unaffected by the oxidative stress inducers. Treatment with CmHP increased expression of Hmox1 in Hepa1-6 mouse liver-derived cells, but Ucp2 expression was not changed. Stimulus screening for regulator of transcription (SSRT) revealed that expression of p50 or p65, transcription factors conferring response to oxidative stress, did not stimulate bovine Ucp2 transcription in HepG2 cells. SSRT also showed 11 molecules that induced Ucp2 transcription more than 4-fold; among them, endoplasmic reticulum (ER) stress-related transcription factors such as XBP1, c-JUN, JUNB, and C/EBPβ were identified. However, treatment with ER stress inducers did not increase Ucp2 expression in HepG2 and Hepa1-6 cells. The present results suggest that 1) neither oxidative stress nor ER stress induces Ucp2 expression in liver-derived cells, and 2) Ucp2 transcription is stimulated by several transcription factors.

KEY WORDS: expression, oxidative stress, transcription, uncoupling protein 2
glutathione S-transferase and Ucp2 was higher in cattle with low residual feed intake than in those with high residual feed intake [10, 30]. These results can be understood that higher expression of hepatic UCP2 is involved in reduced oxidative stress, leading to high feed efficiency (a ratio of body weight gain to feed intake). The oxidative stress is also suggested to be increased during fattening and by feeding dietary restriction of antioxidant reagents such as β-carotene and vitamin E in Japanese Black beef cattle [34]. The regulation of bovine Ucp2 transcription is not known so far.

The final objective of the study was to clarify whether oxidative stress directly induces Ucp2 expression in bovine hepatocytes. At present useful cell line originated from bovine hepatocyte is not available. Thus, as the first step of the study, we explored 1) whether oxidative stress induces UCP2/Ucp2 expression in human/murine liver-derived cells, and 2) factors affecting bovine Ucp2 transcription and expression.

MATERIALS AND METHODS

Cell culture

HepG2 human hepatoma cells (TKG0205) were provided by Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University. Hepa1-6 mouse hepatoma cells (RCB1638) was obtained from RIKEN BioResource Research Center. 3T3-L1 mouse preadipocytes (JCRB9014) were were obtained from JCRB Cell Bank.

HepG2, Hepa1-6, and 3T3-L1 cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum (Sigma, St. Louis, MO, USA, Lot: BCBV9382) and antibiotics; HepG2 and Hepa1-6 cells were cultured in the presence of high glucose (4,500 mg/l), whereas 3T3-L1 cells were in low glucose (1,000 mg/l). The cells were cultured with a new medium for further 24 hr. Luciferase activity in cells transfected with pcDNA3 (empty vector) was set at 1.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR)

Total RNA isolation and real-time RT-qPCR were performed as previously described [23]. Following qPCR primers were used: 5′-ggcagagggtgatagaagagg-3′ and 5′-agctcctgcaactcctcaaa-3′ to detect human HPRT1/Hprt1, 5′-ccagttaaagttgagagatcatctccaccaa-3′ to detect both human XBP1/Xbp1, 5′-tcctcctcagaccgctttt-3′ and 5′-cctggttcatcatcgctaatc-3′ to detect mouse XBP1/Xbp1, 5′-gggagctggaagcctggtatgag-3′ and 5′-gagtrgtgaagrtttttgattcttcctct-3′ to detect both human FOXA1/Foxa1 and mouse FOXA1/Foxa1, 5′-gccagggcacctgtggt-3′ to detect both human UCP2/UCP2 and mouse UCP2/UCP2.

Statistical analysis

Data are expressed as mean ± S.E. Data on relative gene expression were log-transformed to provide an approximation of a normal distribution before analysis. Differences between groups in each tissue were analyzed by one-way analysis of variance (ANOVA). When the effect of group in one-way ANOVA was significant, differences among groups were evaluated by Dunnett’s test or Tukey test. P<0.05 was considered significant.
RESULTS

Oxidative stress does not induce the expression of human and mouse UCP2/Ucp2

To clarify the relationship between oxidative stress and expression levels of UCP2/Ucp2, we first treated HepG2 cells with several oxidative stress inducers, including H2O2, EtOH, and CmHP for 1 hr [11, 14, 25, 33]. Expression levels of HMOX1, a representative gene induced by oxidative stress [17], were not affected by any oxidative inducers (Fig. 1A). We also treated with H2O2 and EtOH for various duration up to 24 hr, but expression levels of HMOX1 were not affected (data not shown). The long-term treatment with CmHP induced cell death in HepG2 cells (data not shown). The oxidative inducers did not affect UCP2 expression (Fig. 1B). Treatment with CmHP increased expression of Hmox1 in Hepa1-6 cells, another liver-derived cell line, whereas H2O2 and EtOH did not affect Hmox1 expression (Fig. 1C). These results suggest that unlike HepG2, CmHP induced oxidative stress in Hepa1-6 cells. However, Ucp2 expression was unaffected by CmHP treatment (Fig. 1D). The other oxidative stress inducers did not modify the expression level of Ucp2 in Hepa1-6 cells, either.

A previous study revealed that treatment with H2O2 induced FOXA1 expression in A549 human alveolar basal epithelial cells, which negatively regulates the excess expression of Ucp2 [27]. Thus, it is possible that the inability of H2O2 to induce Ucp2 expression results from increased expression of FOXA1/Foxa1. However, treatment with H2O2 had no effect on FOXA1/Foxa1 expression in HepG2 and Hepa1-6 cells (Fig. 2).

Next, we compared expression levels of HMOX1/Hmox1 and UCP2/Ucp2 between cells (Fig. 3); to enable the comparison, we designed primers for qPCR to detect both human and mouse HMOX1/Hmox1 and UCP2/Ucp2 mRNAs. Expression levels of HMOX1 in HepG2 cells were significantly higher than those of Hmox1 in Hepa1-6 cells. HMOX1 expression in HepG2 cells was also higher than that in another lineage cells, 3T3-L1 preadipocytes (Fig. 3A). The higher expression levels of UCP2 were also detected in HepG2 cells (Fig. 3B). In view of lower expression of Hmox1 in 3T3-L1 cells than in HepG2 cells, we hypothesized that the oxidative stress inducers could modulate expression levels of Hmox1 in 3T3-L1 cells; CmHP treatment increased Hmox1 expression (Fig. 4A). Thus, we consider that HepG2 cells are constitutively exposed to oxidative stress at a significant level under the basal condition of culture, even if cells were cultured by the recommended method. As a result, additional oxidative stress induction led to an unaffected expression of HMOX1. In 3T3-L1 cells, Ucp2 expression was not also affected by any oxidative stress inducers (Fig. 4B).

Fig. 1. Oxidative stress inducers do not induce uncoupling protein 2 (UCP2/Ucp2) in HepG2 cells and Hepa1-6 cells. HepG2 cells (A and B) and Hepa1-6 cells (C and D) were treated with or without H2O2 (0.5, 1, or 2 mM), ethanol (EtOH: 1, 2, or 3%), or cumene hydroperoxide (CmHP: 25 or 50 µM) for 1 hr. Expression levels of hemoxygenase 1 (HMOX1/Hmox1) (A and C) and UCP2/Ucp2 (B and D) were examined by reverse transcription-quantitative PCR (RT-qPCR) analysis, and the expression levels in the control cells were set at 1. The data are presented as the mean ± S.E. (n=3). **: P<0.01 vs. the control group.
SSRT reveals novel regulators for Ucp2 transcription

Recently, we developed a screening system for transcriptional regulators named as SSRT [23]. This screening enables us to identify molecule(s) to regulate transcription of the target gene; effect of 71 expression vectors including oxidative stress-related molecules was evaluated. Forced expression of p50 or p65, members of nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) transcription factor, did not stimulate bovine Ucp2 transcription (<4-fold)- the NF-κB pathway centrally regulates responses to oxidative stress [3] (Fig. 5). We also examined bovine Ucp2 transcription using oxidative stress inducer (hydrogen peroxide) and antioxidant agent (ascorbic acid phosphate), and observed no changes in luciferase activity in response to treatment with these reagents (data not shown).

SSRT also revealed 11 molecules that stimulated bovine Ucp2 transcription more than 4-fold; among them, spliced form of XBP1, c-JUN, JUNB, and C/EBPβ have been shown to be involved in the endoplasmic reticulum (ER) stress induction [13, 21].
Thus, we speculated that ER stress leads to the up-regulation of UCP2/Ucp2 expression in HepG2 and Hepa1-6 cells. As expected, treatment with ER stress inducers, thapsigargin, A23187, and tunicamycin, increased expression levels of spliced X-box binding protein 1 (XBP1/Xbp1), C/EBP homologous protein (CHOP/Chop), and UCP2/Ucp2 were examined by reverse transcription-quantitative PCR (RT-qPCR) analysis, and the expression levels in the control cells were set at 1. The data are presented as the mean ± S.E. (n=4). * and **: P<0.05 and P<0.01, respectively, vs. the control group.

Fig. 5. Stimulus screening for regulator of transcription (SSRT) identifies novel regulators of uncoupling protein 2 (Ucp2) transcription. HepG2 cells were transfected with various expression vector encoding transcription (co-) factors, cytosolic kinases, and extracellular ligands with Ucp2 (−3,000)-luc (n=2). Luciferase activity was measured and the expression level in cells transfected with pcDNA3 (empty vector) was set at 1. The data are presented as the mean ± S.E. (n=2).

Fig. 6. Uncoupling protein 2 (Ucp2) expression is not affected by induction of endoplasmic reticulum (ER) stress. HepG2 (A) and Hepa1-6 cells (B) were treated with thapsigargin (100 nM), A23187 (1 µM), or tunicamycin (2 µg/ml) for 6 hr. Expression levels of spliced form of X-box binding protein 1 (XBP1/Xbp1), C/EBP homologous protein (CHOP/Chop), and UCP2/Ucp2 were examined by reverse transcription-quantitative PCR (RT-qPCR) analysis, and the expression levels in the control cells were set at 1. The data are presented as the mean ± S.E. (n=4). * and **: P<0.05 and P<0.01, respectively, vs. the control group.
DISCUSSION

The present study explored 1) whether oxidative stress induces *Ucp2* expression in human/murine liver-derived cells, and 2) factors affecting bovine *Ucp2* transcription and expression. We show that oxidative stress does not directly increase *Ucp2* expression in HepG2, Hepa1-6, and 3T3-L1 cells. Results of SSRT also suggest that direct transactivation of *Ucp2* gene is not induced by oxidative stress, because molecules for the NF-κB pathway did not stimulate bovine *Ucp2* transcription. We also reveal several novel regulators to stimulate bovine *Ucp2* transcription; especially, molecules stimulated in response to ER stress induced the *Ucp2* transcription and expression. However, ER stress induction did not affect *UCP2/Ucp2* expression. The present results provide basic information on regulatory *Ucp2* expression. *UCP2* serves the removal of ROS [5, 6]. More factors than considered ever are likely to be involved in the control of ROS amount by UCP2.

*Ucp2* expression was not induced by CmHP in Hepa1-6 cells and 3T3-L1 cells (Figs. 1D and 4B), irrespective of the induction of Hmox1 gene. The results suggest that the defensive response to oxidative stress by lipid peroxidation is not achieved through the regulatory expression of *Ucp2*. Previous studies have shown that oxidative stress modulated *UCP2* expression. However, the responses were not consistent. The oxidative stress increased *UCP2* expression in A549 human alveolar basal epithelial cells [27], whereas oxidative stress-induced down-regulation of *UCP2* expression in ARPE-19 human retinal pigment epithelium cells line [16]. Expression levels of *Ucp2* in response to oxidative stress may be regulated in a cell-context dependent manner. Previous studies also showed post-translational regulation of uncoupling activity via UCP2 [9, 36]; the fatty acid and superoxide have been known to increase UCP2 activity, whereas GDP and genipin inhibit UCP2 activity. The cell defense to oxidative stress by CmHP may be achieved through modulation of UCP2 activity in Hepa1-6 cells and 3T3-L1 cells.

Gene induction of HMOX1/Hmox1 in response to oxidative stress inducers depended on cultured cells; HepG2 cells were resistant to HMOX1 expression to the oxidative stress inducers, whereas expression of Hmox1 was increased by CmHP treatment in Hepa1-6 cells, despite both being liver-derived cells. As the reason why expression levels of HMOX1 were unaffected by any oxidative stress inducers in HepG2 cells but not in Hepa1-6 cells, we speculate that the degree of oxidative stress is higher in HepG2 cells than in Hepa1-6 cells under the basal condition of culture; consequently, expression levels of HMOX1 may not be changed to the addition of the oxidative stress inducers to the culture medium in HepG2 cells. In fact, expression levels of HMOX1/Hmox1 were significantly higher in HepG2 cells than Hepa1-6 cells (Fig. 3). Previous studies showed that H2O2 (200 µM) or EtOH (2.5%) did not increase HMOX1 protein level in HepG2 cells [15, 19]. These results are conceptually consistent with the present study.

Up-regulation of Hmox1 expression depended on oxidative stress inducer in Hepa1-6 cells and 3T3-L1 cells; CmHP but not H2O2 and EtOH effectively increased expression of Hmox1. Previous studies also revealed that the extent of induced oxidative stress was different among oxidative stress inducers [2]. EtOH inhibited a mitochondrial electron transfer system, leading to increased production of superoxide production in gastric epithelial cells [29]. H2O2 is directly linked to superoxide production and damages the whole cell, including mitochondria. On the contrary, the primary action of CmHP occurred at the cell plasma membrane [32]. The mode to induce oxidative stress is likely to lead to the oxidative stress inducer-dependent Hmox1 expression. CmHP was a more potent oxidant than H2O2 in neurons [12], and this can be explained by the difference of hydrophobicity; CmHP with more hydrophobicity could initiate lipid peroxidation predominantly, leading to ROS-induced cell membrane injury [12]. In addition, considering that Hmox1 expression is transcriptionally regulated by transcription factor NF-E2-related factor 2 (NRF2) [17], the amount of nuclear NRF2 may vary depending on oxidative stress inducers.

Previous studies have shown that *UCP2/Ucp2* expression is regulated by various factors. *Ucp2* mRNA was increased by PPARα in rat primary hepatocytes [22]; our results showed that forced expression of PPARα did not increase bovine *Ucp2* transcription—hence the reason is unclear. *Ucp2* transcription was also stimulated by PPARγ [18], and SIRT1 inhibits PPAR-y-induced mouse *Ucp2* transcription in 293T cells [4]. Transcription of the human *UCP2* gene was increased by co-treatment with triiodothyronine and forced expression of PGC-1α, although the effect of PGC-1α expression alone was minimal (<2-fold) in INS-1E pancreatic β cells [24]. FOXA1 negatively regulated the mRNA level of mouse *Ucp2* probably through binding to the *Ucp2* promoter [27, 31]. Treatment with TGF-β also decreased expression of human *UCP2*, which may relate to SMAD4 binding to *UCP2* promoter [26]. Further, microRNAs have been reported to be involved in mouse *Ucp2* mRNA levels [7, 28]. These results are partly consistent with the present results on SSRT that showed stimulatory effects of PPARγ (4.1-fold) and PGC-1α (2.1-fold) on bovine *Ucp2* transcription. The present study revealed additional factors to stimulate bovine *Ucp2* transcription, i.e., C/EBPβ, C/EBPδ, ERα, ERRβ, SMAD3, FOXA3, TAZ, XBP1, c-JUN, and JUNB. The present results showed that induction of ER stress did not affect *UCP2/Ucp2* expression, irrespective of stimulation of bovine *Ucp2* gene transcription by ER stress-related molecules. It is possible that the molecules induced by ER stress other than XBP1, C-JUN, JUNB, and C/EBPβ have an inhibitory effect on *UCP2/Ucp2* expression.

In the present study, the effects of oxidative stress and ER stress were examined in human- and mouse-originated cells. Also, molecules to evaluate bovine *Ucp2* transcription were not always originated from the bovine gene transcript, and SSRT was performed in HepG2 human liver-derived cells; use of different cells may lead to different responses. Future studies are needed to clarify how these factors regulate *Ucp2* transcription using bovine gene transcript and bovine cells.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

ACKNOWLEDGMENT. This work was partially supported by the JSPS KAKENHI (17H03903 and 20H03130).
33. Wu, H., Zhang, G., Huang, L., Pang, H., Zhang, N., Chen, Y. and Wang, G. 2017. Hepatoprotective effect of polyphenol-enriched fraction from Folium Microcos on oxidative stress and apoptosis in acetaminophen-induced liver injury in mice. Oxid. Med. Cell. Longev. 2017: 3631565. [Medline] [CrossRef]

34. Yamada, T., Higuchi, M. and Nakanishi, N. 2013. Plasma 8-isoprostane concentrations and adipogenic and adipokine gene expression patterns in subcutaneous and mesenteric adipose tissues of fattening Wagyu cattle. J. Vet. Med. Sci. 75: 1021–1027. [Medline] [CrossRef]

35. Zhang, C. Y., Baffy, G., Perrot, P., Krauss, S., Peroni, O., Grujic, D., Hagen, T., Vidal-Puig, A. J., Boss, O., Kim, Y. B., Zheng, X. X., Wheeler, M. B., Shulman, G. I., Chan, C. B. and Lowell, B. B. 2001. Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, β cell dysfunction, and type 2 diabetes. Cell 105: 745–755. [Medline] [CrossRef]

36. Zhang, C. Y., Parton, L. E., Ye, C. P., Krauss, S., Shen, R., Lin, C. T., Porco, J. A. Jr. and Lowell, B. B. 2006. Genipin inhibits UCP2-mediated proton leak and acutely reverses obesity- and high glucose-induced beta cell dysfunction in isolated pancreatic islets. Cell Metab. 3: 417–427. [Medline] [CrossRef]