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Biochemistry

Factors affecting expression and transcription of uncoupling protein 2 gene

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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* transcrition 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

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Oxidative stress triggers the onset of various diseases in humans, including cancer, diabetes, cardiovascular diseases, and neurodegenerative diseases [20]. Upon induction of oxidative stress, various antioxidant responses are stimulated [8]; the defensive responses to oxidative stress include the disappearance of reactive oxygen species (ROS) via uncoupling protein 2 (UCP2) [5, 6]. Originally, UCP2 was suggested to be involved in nonshivering thermogenesis, regulatory secretion of insulin, and the onset of diabetes [35]. But the primary function of UCP2 is now thought to control the amount of mitochondria-derived ROS [5, 6]. It was revealed in a study in response to infection with *Toxoplasma gondii* where macrophages from *Ucp2*-knock out mice generated more ROS than those from wild-type mice [1]. UCP2-mediated removal of ROS is partly regulated by the transcript level of *UCP2* [18, 27]. Thus, oxidative stress may directly induce *Ucp2* expression, which leads to defensive responses to oxidative stress. However, previous results on regulatory expression of *UCP2* related to oxidative stress are not always consistent. Song *et al*. [27] reported that treatment with H_2O_2 increased *UCP2* expression in A549 human alveolar basal epithelial cells. In contrast, Liu *et al.* [16] showed down-regulation of *UCP2* expression in response to H_2O_2 treatment in ARPE-19 human retinal pigment epithelium cells.

Increased oxidative stress has been suggested in the liver of cattle utilizing feed inefficiently for its production (cattle with high residual feed intake), as compared with that of cattle utilizing feed more efficiently (cattle with low residual feed intake) [30]. Gene expression profile revealed that hepatic expression of molecules involved in detoxification of products of oxidative stress, including

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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 treated with H_2O_2 (0.5, 1, or 2 mM), ethanol (EtOH: 1, 2, or 3%), or cumene hydroperoxide (CmHP: 25 or 50 μM) for 1 hr. The mRNA levels of hemoxygenase 1 (*HMOX1/Hmox1*), *UCP2/Ucp2*, and forkhead box protein A1 (*FOXA1/Foxa1*) were examined. In addition, HepG2 and Hepa1-6 cells were treated with thapsigargin (100 nM), A23187 (1 μM), or tunicamycin (2 μg/ml) for 6 hr. The mRNA levels of the spliced form of X-box binding protein 1 (*XBP1/Xbp1*), C/EBP homologous protein (*CHOP/Chop*), and *UCP2/Ucp2* were examined. Further, transcriptional regulation of bovine Ucp2 was evaluated by luciferase-based reporter assays, as described below.

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 *HMOX1*, 5′-ttccgcatacaaccagtgagtgg-3′ and 5′-aaatcctggggcatgctgtcgg-3′ to detect mouse *Hmox1*, 5′-tgaaggaggccaccaaggaggt-3′ and 5′- ggtacaaggaggccatcaccagctt-3′ to detect both human *HMOX1* and mouse *Hmox1*, 5′-tgaaagccaacctcatgaca-3′ and 5′-gatgacagtggtgcagaagc-3′ to detect human *UCP2*, 5′-accggcagctttgaagaac-3′ and 5′-agaaacggggaccttcaatc-3′ to detect mouse *Ucp2*, 5′-tctgtcaaacagttctacaccaagg-3′ and 5′-gccagggcacctgtggt-3′ to detect both human *UCP2* and mouse *Ucp2*, 5′-cgactggaacagctactacg-3′ and 5′-tggtgttcatggtcatgtaggtgtt-3′ to detect both human *FOXA1* and mouse *Foxa1*, 5′-tctgctgagtccgctacaggtg-3′ and 5′-gctggcaggctctggggaag-3′ to detect spliced form of human *XBP1*, 5′-tctgctgagtccgctacaggtg-3′ and 5′-ccatgggaagatgttctggg-3′ to detect spliced form of mouse *Xbp1*, 5′-gggagctggaagcctggtatgag-3′ and 5′-gagtrgtgaagrtttttgattcttcctct-3′ to detect both human *CHOP* and mouse *Chop*, 5′-tgaccttgatttattttgcatacc-3′ and 5′-cgagcaagacgttcagtcct-3′ to detect human hypoxanthine phosphoribosyltransferase 1 (*HPRT1*), 5′-tcctcctcagaccgctttt-3′ and 5′-cctggttcatcatcgctaatc-3′ to detect mouse *Hprt1*, 5′- attcctatgactgtagattttatcagctgaagagcta-3′ and 5′-ccagttaaagttgagagatcatctccaccaa-3′ to detect both human *HPRT1* and mouse *Hprt1*. The ΔΔCt method was used to normalize the levels of target transcripts to *HPRT1/Hprt1* levels. Expression levels in control cells were set at 1.

Stimulus screening for regulator of transcription (SSRT)

We recently developed a screening to explore factors affecting the transcription of the target gene named as SSRT [23]. In this screening, the luciferase-based reporter gene is transfected with expression vectors to stimulate various signaling pathways: the expression vectors include plasmids encoding transcription (co-) factors, cytosolic kinases, and extracellular ligands [23]. Factors involved in the regulatory transcription of the target gene are explored by measuring luciferase activity. Evaluated expression vectors do not necessarily contain coding region originated from the bovine genes but human and mouse genes.

The reporter with a bovine *Ucp2* promoter fragment (nt −3,000 to +25, Ucp2 (−3,000)-luc) was prepared using pGL4, a luciferase-based reporter plasmid; the translation initiation site was numbered as +1. The nucleotide sequence of the constructs was verified by DNA sequencing. SSRT were performed in HepG2 cells, because plasmids can be easily transfected in HepG2; HepG2 cells were transiently transfected with the individual expression vector and Ucp2 (−3,000)-luc. At 4 hr post-transfection, cells were cultured with a new medium for further 24 hr. Luciferase activity in cells transfected with pcDNA3 (empty vector) was set at 1.

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 H_2O_2, 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 H_2O_2 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 H_2O_2 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 H_2O_2 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 H_2O_2 to induce *Ucp2* expression results from increased expression of FOXA1/Foxa1. However, treatment with H_2O_2 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 linage 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).

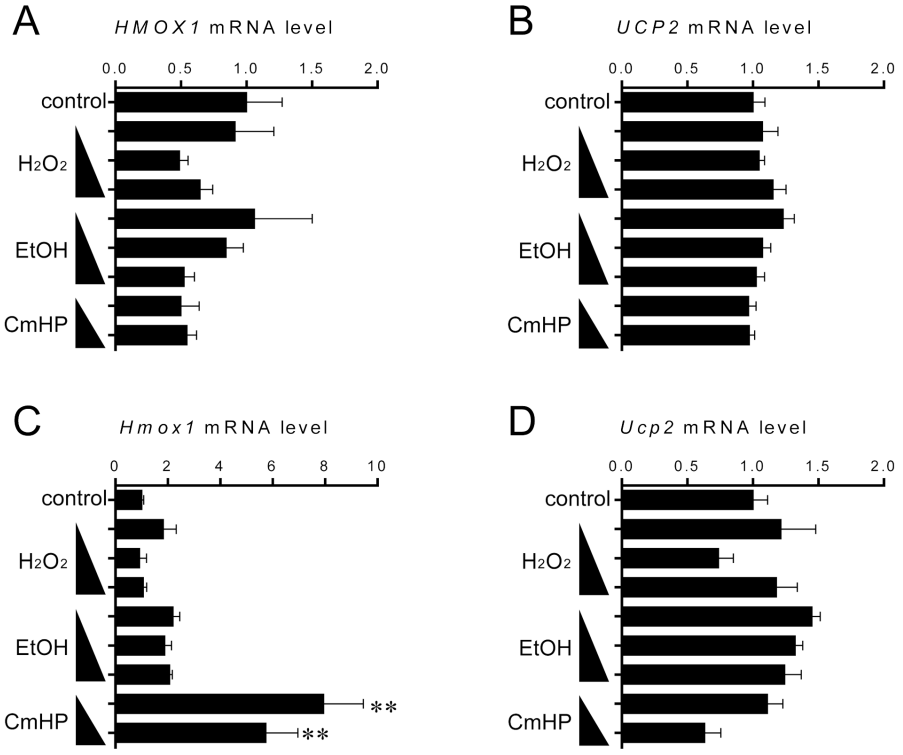

Fig. 1. Oxidative stress inducers do not induce uncoupling protein 2 (*UCP2/Ucp2*) in HepG2 cells and Hepa1-6 cells. HepG2 cells (A and B) and Hepa1-6 cells (C and D) were treated with or without H_2O_2 (0.5, 1, or 2 mM), ethanol (EtOH: 1, 2, or 3%), or cumene hydroperoxide (CmHP: 25 or 50 μM) for 1 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.

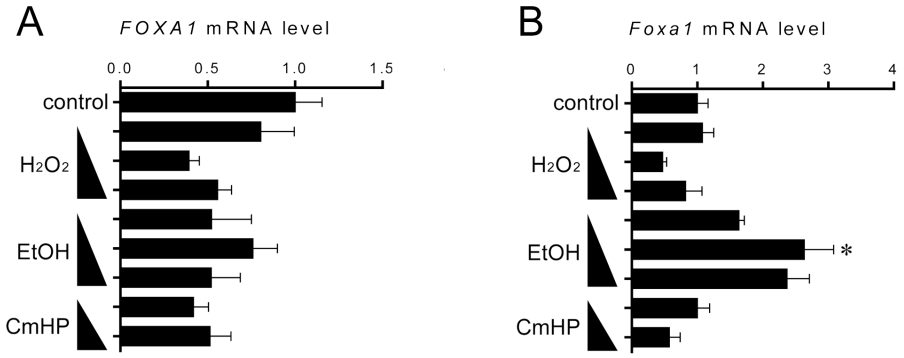

Fig. 2. Oxidative stress inducers do not apparently induce forkhead box protein A1 (*FOXA1/Foxa1*) in HepG2 cells and Hepa1-6 cells. HepG2 cells (A) and Hepa1-6 cells (B) were treated with or without H_2O_2 (0.5, 1, or 2 mM), ethanol (EtOH: 1, 2, or 3%), or cumene hydroperoxide (CmHP: 25 or 50 μM) for 1 hr. Expression levels of *FOXA1/Foxa1* 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). *: $P<0.05$ *vs.* the control group.

Fig. 3. Expression levels of hemoxygenase 1 (*HMOX1/Hmox1*) and uncoupling protein 2 (*UCP2/Ucp2*) gene are higher in HepG2 cells than in Hepa1-6 cells and 3T3-L1 cells. Expression levels of *HMOX1/Hmox1* (A) and *UCP2/Ucp2* (B) in HepG2, Hepa1-6, and 3T3-L1 cells were examined by reverse transcription-quantitative PCR (RT-qPCR) analysis, and the expression levels in HepG2 cells were set at 1. The data are presented as the mean ± S.E. (n=3). a, b, c: $P<0.05$.

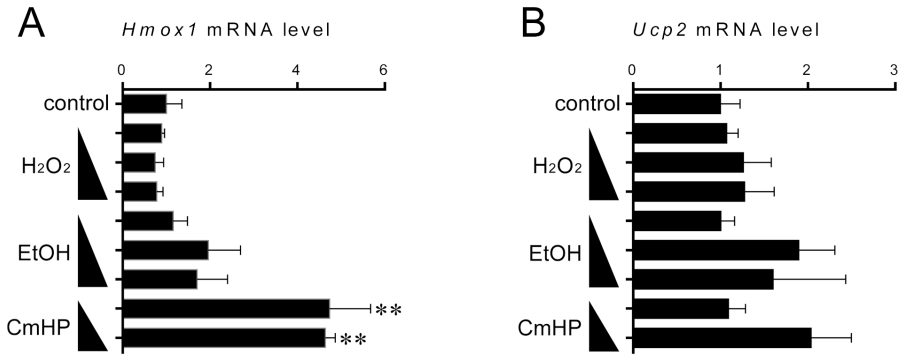

Fig. 4. Cumene hydroperoxide (CmHP) induces oxidative stress but does not affect expression level of uncoupling protein 2 (*Ucp2*) in 3T3-L1 cells. 3T3-L1 cells were treated with or without H_2O_2 (0.5, 1, or 2 mM), ethanol (EtOH: 1, 2, or 3%), or cumene hydroperoxide (CmHP: 25 or 50 μM) for 1 hr. Expression levels of *Hmox1* (A) and *Ucp2* (B) 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].

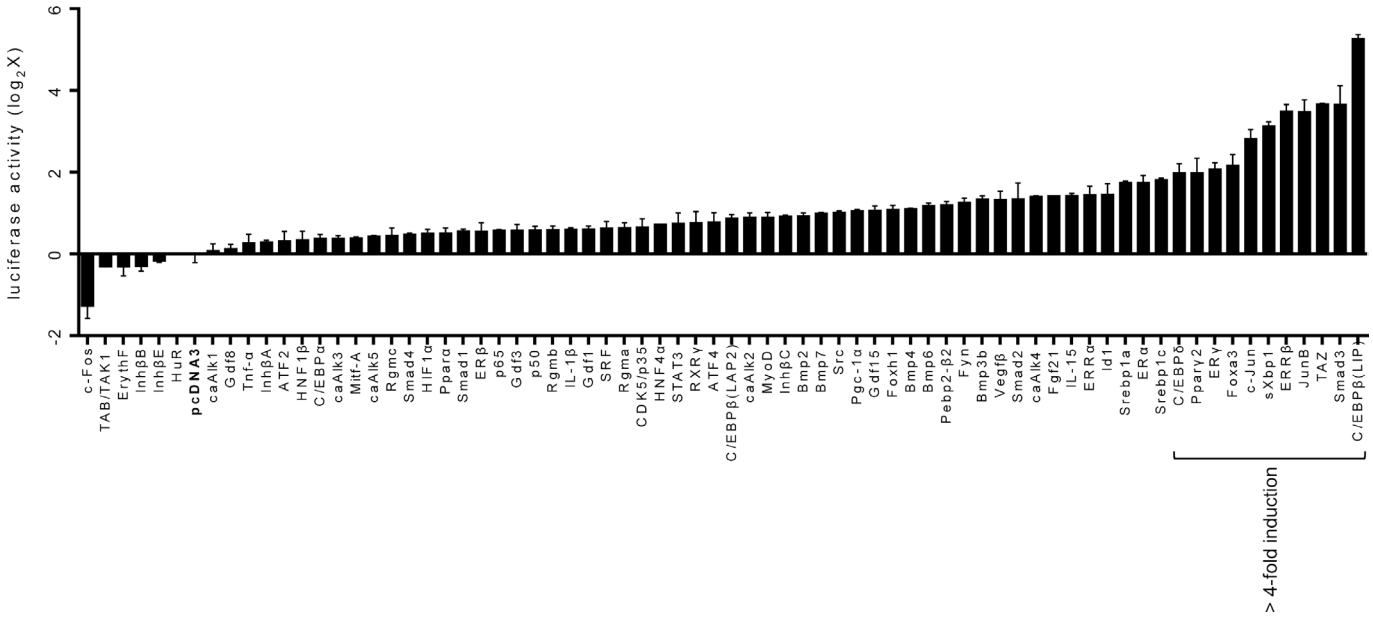

Fig. 5. Stimulus screening for regulator of transcription (SSRT) identifies novel regulators of uncoupling protein 2 (*Ucp2*) transcription. HepG2 cells were transfected with various expression vector encoding transcription (co-) factors, cytosolic kinases, and extracellular ligands with Ucp2 (−3,000)-luc (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).

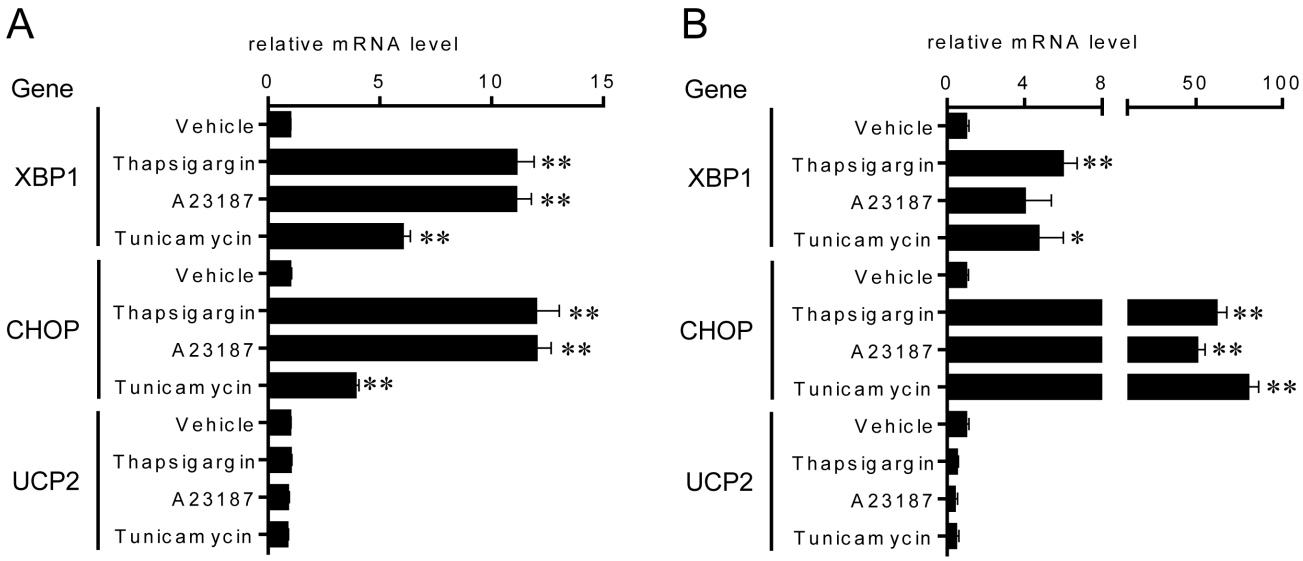

Fig. 6. Uncoupling protein 2 (*Ucp2*) expression is not affected by induction of endoplasmic reticulum (ER) stress. HepG2 (A) and Hepa1-6 cells (B) were treated with thapsigargin (100 nM), A23187 (1 μM), or tunicamycin (2 μg/ml) for 6 hr. Expression levels of spliced form of X-box binding protein 1 (*XBP1/Xbp1*), 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.

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 *XBP1/Xbp1* and *CHOP/Chop* (Fig. 6). In contrast, expression levels of *UCP2/Ucp2* were unaffected by ER stress induction in HepG2 cells (Fig. 6A) and Hepa1–6 cells (Fig. 6B).

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* transcrition. 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 H_2O_2 (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 H_2O_2 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]. H_2O_2 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 H_2O_2 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-the reason is currently unclear. *Ucp2* transcription was also stimulated by PPARγ [18], and SIRT1 inhibits PPARγ-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 PPARγ2 (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.

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