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A possibility of uncoupling protein 1 induction with the enhancement of myogenesis related to ruminal fermentation

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The expression of uncoupling protein 1 (UCP1), which regulates energy expenditure, is limited to brown/beige adipocytes in most mammals; however, it is also detected in the skeletal muscles of cattle. We previously observed a positive relationship between *Ucp1* and fast-twitch myosin heavy chain (*Myh*) expression in bovine skeletal muscles. In the present study, we explored the regulatory expression of *Ucp1* in bovine myogenic cells using cell culture. Vitamin C and high-dose capsaicin, which induce the formation of fast-twitch myotubes in murine myogenic cells, did not stimulate myogenesis in bovine myosatellite cells. Treatment with 4-phenylbutyric acid (PBA), a histone deacetylase inhibitor that enhances histone acetylation, upregulates the expression of all myogenic regulatory factors (MRFs), except *Myog*, in bovine myogenic cells. Consistent with this, PBA increased the expression levels of acetylated lysine 27 of histone 3 (H3K27), the fast-twitch component MYH1/2, and *Ucp1* in bovine myogenic cells. SB203580, an inhibitor of p38 MAP kinase, blocked PBA-induced myogenesis and *Ucp1* upregulation. PBA is a butyric acid-related molecule, and cattle produce large amounts of volatile fatty acids (VFAs), including acetic acid, propionic acid, and butyric acid, through ruminal fermentation. Propionic acid treatment stimulated H3K27 acetylation, myogenesis, and *Ucp1* induction. Thus, the upregulation of muscular *Ucp1* may be related to myogenic stimulation through the modulation of histone acetylation status in cattle; we propose that the cattle-specific expression of muscular UCP1 results from VFA production through ruminal fermentation.

Keywords Uncoupling protein 1, Myogenesis, Volatile fatty acid, 4-phenylbutyric acid, Cattle

Brown and beige adipocytes utilize chemical energy to produce heat. Energy expenditure in brown and beige adipocytes affects systemic energy metabolism in humans and rodents[1–3]. Uncoupling protein (UCP) 1 facilitates the uncoupling of proton influx into the mitochondrial matrix during ATP synthesis, leading to the stimulation of energy expenditure and thermogenesis. UCP1 expression is limited to brown/beige adipocytes in most mammals[4–6]. Therefore, UCP1 is responsible for heat production in brown/beige adipocytes and is a marker gene of brown/beige adipocytes.

Previously, human UCP1 was thought to be expressed under a limited (patho)physiological status such as in infants and patients with pheochromocytoma[4,7]. However, integrated positron emission tomography-computed tomography (PET-CT) studies using an ^{18}F-labeled glucose analog have shown the presence of functional brown/beige adipocytes expressing UCP1 in adults[8–11]. Similarly, brown/beige adipocytes were found to be absent in mature cattle, the expression level of adipose *Ucp1* in fetal calves decreased to the detection limit at birth[12,13], and *Ucp1* expression was not detected in fat depots of mature cattle[12,14–17]. However, we have previously detected UCP1 expression in the fat depots of mature cattle[18].

Our previous study also revealed that, unlike humans and rodents, UCP1 is expressed in the skeletal muscle of mature cattle[19]. This finding was unexpected because UCP1 expression was not detected in the intra- and intermuscular adipocytes[19]. The expression levels of *Ucp1* are positively correlated with those of myosin-heavy

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chain (*Myh*) *1*, *2*, and *4* which are predominantly expressed in fast-twitch myofibers, but not in *Myh7* (slow-twitch MYH)[20].

The information on the regulation of murine *Ucp1* expression may not be applicable to the regulation of muscular *Ucp1* in cattle. Murine *Ucp1* expression is directly increased by forskolin and all-*trans* retinoic acid in brown adipocytes[4,21], but these reagents do not affect *Ucp1* expression in bovine myogenic cells[22]. *Ucp1* expression is stimulated by the activation of the BMP pathway in murine brown adipocytes[23], whereas inhibition of the BMP pathway increased *Ucp1* expression in bovine myogenic cells[22]. These results suggest that further exploration of bovine myogenic cells is required to elucidate the factors affecting muscular *Ucp1*. The present study has examined how bovine *Ucp1* expression is regulated in cultured myogenic cells derived from bovine skeletal muscle.

Results

Vitamin C and high-dose of capsaicin do not stimulate myogenesis and *Ucp1* expression. Previously, we revealed that the expression levels of muscular *Ucp1* were positively correlated with those of *Myh1*, *Myh2*, and *Myh4* (fast-twitch myofibers), but not with those of *Myh7* (slow-twitch myofiber) in the skeletal muscle of cattle[20]. We also showed that the formation of fast-twitch myotubes was enhanced by the onset of endoplasmic reticulum (ER) stress by treatment with high-dose capsaicin before differentiation stimulation and subsequent treatment with vitamin C in murine C2C12 myogenic cells[24]. To characterize the regulation of *Ucp1* expression in bovine skeletal muscle, a similar protocol used to induce myogenesis in murine cells was first applied to bovine myosatellite cells.

Upon the onset of ER stress, the expression levels of the spliced forms of Xbp1 (*sXbp1*) and *Chop* increased[25]. High-dose capsaicin treatment increased the expression levels of *sXbp1* and *Chop* (Fig. 1A). Myogenesis is principally regulated by a series of expressions of myogenic regulatory factors (MRFs) such as *Myf5*, *Myod*, *Myog*, and *Mrf4*[26]. Although high-dose capsaicin significantly increased expression of *Myod*, this increase was relatively small (Fig. 1B). High-dose capsaicin treatment did not affect the expression of other MRFs (*Myf5*, *Myog*, and *Mrf4*). Vitamin C significantly increased the expression of *Myf5*, *Myod*, and *Myog*; however, a synergistic effect of co-treatment with high-dose capsaicin was not detected (Fig. 1B). The expression levels of *Myh2* and *Myh7* were increased by high-dose capsaicin, and *Myh1* expression was significantly decreased in cells treated with vitamin

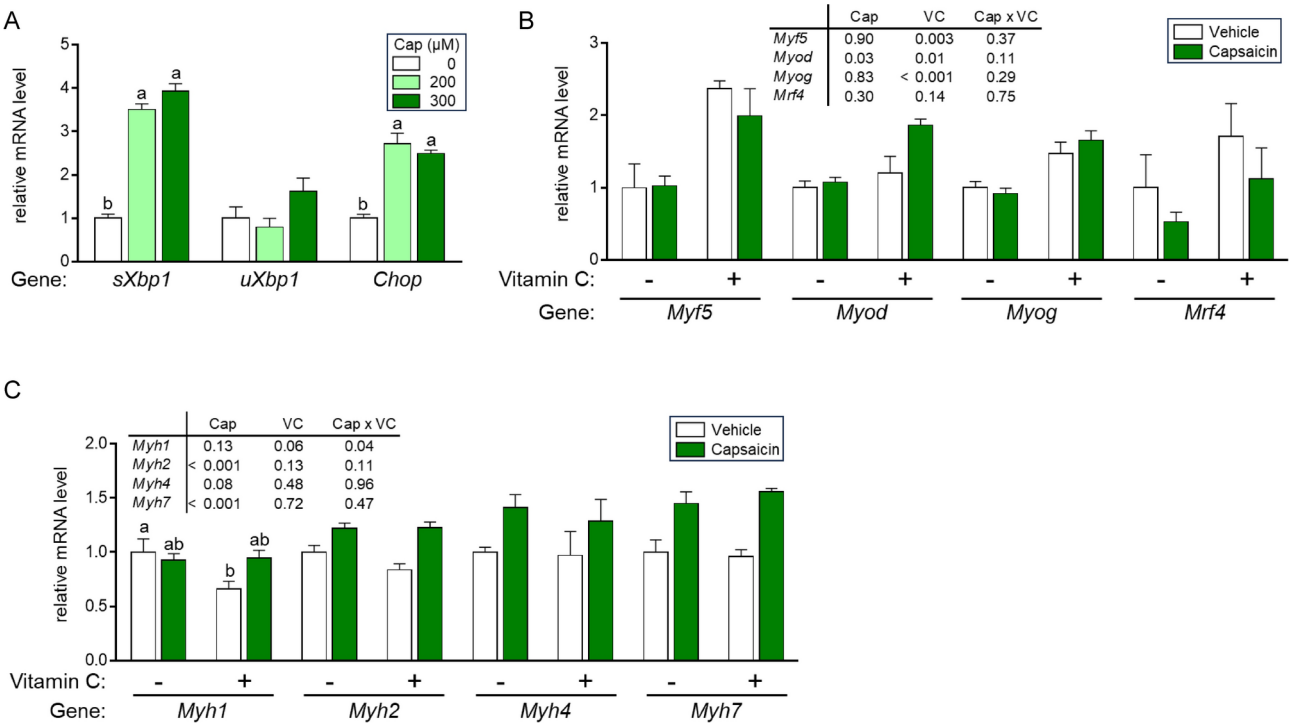

Fig. 1. Vitamin C and a high-dose of capsaicin do not stimulate differentiation of bovine myosatellite cells. (**A**) At confluence (day -1), bovine myosatellite cells were treated with or without capsaicin (Cap: 200 μM or 300 μM) for 12 h. Expression levels of genes related to ER stress were examined by RT-qPCR. (**B**, **C**) At confluence (day -1), bovine myosatellite cells were treated with or without capsaicin (Cap: 300 μM) for 24 h, followed by culture in DMEM containing 2% FBS supplemented with or without 100 μM vitamin C (VC) for 6 days. Expression levels of MRFs (**B**) and MYHs (**C**) were examined by RT-qPCR. The expression levels of the respective genes were normalized against *Hprt1* mRNA levels, and those in the control cells were set at 1. Data are presented as the mean ± SE (n = 3 for (**A**) or 4 for (**B**, **C**)). a, b: Means that do not have a common letter on the bar differ significantly ($P < 0.05$). Results of ANOVA are shown in inlet.

C alone (Fig. 1C). However, the effect of high-dose capsaicin and vitamin C was relatively small. Expression of *Ucp1* was not detected (data not shown).

PBA stimulates differentiation of bovine myosatellite cells to fast-twitch myotubes and *Ucp1* expression. Given that high-dose capsaicin and vitamin C did not greatly stimulate myogenesis in bovine myosatellite cells, we explored alternative treatments to increase myotube formation in bovine myogenic cells. Finally, we found that PBA (a butyric acid-related molecule) potentiated to enhance myogenesis in bovine myosatellite cells (Fig. 2, Supplementary Fig. S1–S3). Treatment with 1 mM PBA during myogenesis increased the formation of MYH1/2-positive thick and long myotubes (Fig. 2A, B). MYH1/2 protein levels were also upregulated in bovine muscle cells (Fig. 2C). Consistent with these results, treatment with 1 mM PBA increased the mRNA levels of fast-twitch *Myh1*, *Myh2*, and *Myh4* (Fig. 2D). When the gene expression was normalized against *Rps15a*, another reference gene, the similar results were obtained (Supplementary Fig. S2A). In contrast, the expression of the slow-twitch *Myh7* was unaffected by treatment with 1 mM PBA (Fig. 2D, Supplementary Fig. S2A). PBA (1 mM) enhanced the expression of *Myf5*, *Myod*, and *Mrf4* but not *Myog* (Fig. 2E, Supplementary Fig. S2B). These results suggest that PBA selectively stimulates the differentiation of bovine myosatellite cells into fast-twitch myotubes by inducing MRF expression.

Because PBA has been shown to inhibit ER stress[27] and histone deacetylase (HDAC)[28], we examined the expression levels of ER stress markers and histone acetylation (acetylated lysine 27 of histone 3 (H3K27)). PBA treatment did not decrease the expression of *sXbp1*, but increased unspliced Xbp1 (*uXbp1*) levels (Fig. 2F, Supplementary Fig. S2C). Although *Chop* expression was reduced by PBA treatment, the extent was relatively

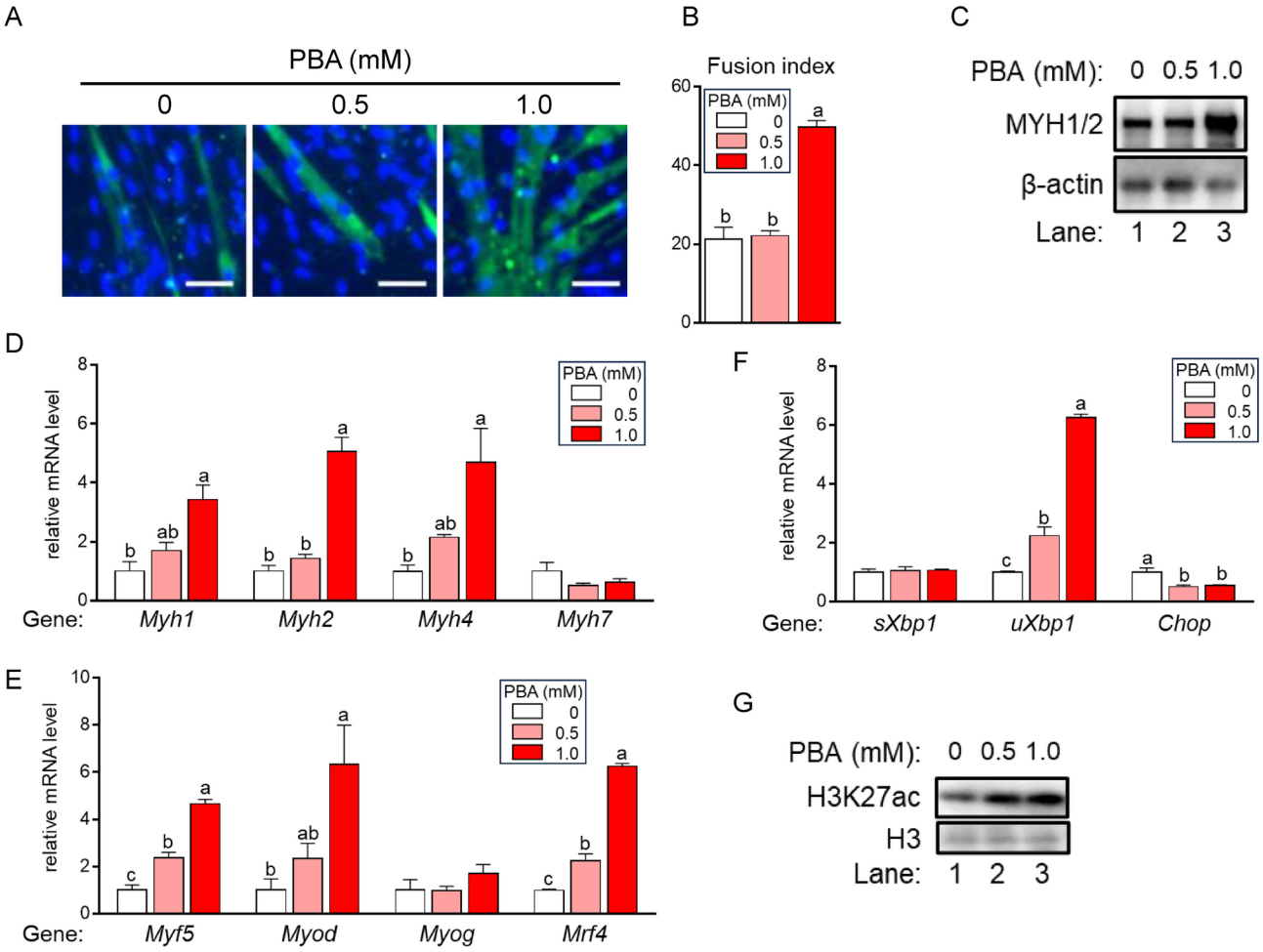

Fig. 2. PBA potentiates differentiation in bovine myosatellite cells. After reaching confluence (day 0), bovine myosatellite cells were cultured in myosatellite differentiation medium supplemented with or without PBA (0.5 mM or 1 mM) for 6 days. (**A**) A representative image of myosin heavy chain immunostaining in bovine myosatellite cells. Green: MYH1/2, blue: nuclei. Bar: 50 µm. (**B**) Fusion index. (**C**) The expression levels of MYH1/2 (**C**) and acetylated H3K27 (**G**) were examined by western blot analysis. As a reference, the expression levels of β-actin (**C**) and H3 (**G**) were examined. Expression levels of MYHs (D), MRFs (E), and ER stress-related genes (**F**) were quantified using RT-qPCR. The expression levels of the respective genes were normalized against *Hprt1* mRNA levels, and those in the control cells were set to 1. Data are presented as mean ± SE (n = 3). a-c: Means that do not have a common letter on the bar differ significantly ($P < 0.05$).

small (Fig. 2F, Supplementary Fig. S2C). PBA increased the levels of acetylated H3K27 (Fig. 2G, Supplementary Fig. S3). The enhancement of myogenesis by PBA is likely mediated by inhibition of HDAC activity.

Expression levels of *Ucp1* were below the detection limits in the absence of PBA, but PBA significantly induced *Ucp1* expression in bovine myogenic cells (Fig. 3A, Supplementary Fig. S4A): because we could not detect a significant expression of *Ucp1* in the control cells, statistical analysis was not performed. Brown adipogenesis is governed by transcriptional regulators such as *Ebf2*, *Prdm16*, *Nfia*, and *Pgc-1α*[29,30]. Expression levels of *Ebf2* and *Prdm16* were unaffected by PBA treatment, but *Nfia* and *Pgc-1α* expressions were increased in bovine myosatellite cells treated with PBA (Fig. 3B, Supplementary Fig. S4B). The expression levels of *Dio2* and *Cox*s, brown adipocyte-selective genes[31], were also upregulated by PBA treatment, except for *Cox2* and *Cox7a1* normalized against *Rps15a* (Fig. 3C, Supplementary Fig. S4C).

Activation of p38 MAP kinase is involved in PBA-induced myogenesis and *Ucp1* expression. Next, we explored the molecular mechanisms underlying PBA-stimulated myogenesis and *Ucp1* expression. Therefore, the phosphorylation levels of CREB, MAP kinases, and AMP kinase were evaluated (Fig. 4A, Supplementary Fig. S5). The treatment with PBA stimulated p38 MAP kinase phosphorylation (Fig. 4A). SB203580, an inhibitor of p38 MAP kinase[32], decreased PBA-induced MYH1/2 and *Ucp1* expression (Fig. 4B, C, Supplementary Fig. S6, S7). These results suggested that the p38 MAP kinase pathway is involved in myogenic stimulation and *Ucp1* induction in PBA-treated bovine myosatellite cells. PBA also slightly increased phosphorylated AMPK levels (Fig. 4A, Supplementary Fig. S5I, J). Although the hyperactivation of AMPK by use of synthetic reagent AICAR inhibits myogenesis[33], the physiological significance of weak phosphorylation of AMPK remains unclear.

Propionic acid stimulates myogenesis and *Ucp1* expression in bovine myosatellite cells but not in murine C2C12 myogenic cells. PBA is a butyric-acid-related compound. Volatile fatty acids (VFAs) such as acetic acid, propionic acid, and butyric acid are produced by ruminal fermentation[34]. We hypothesized that muscular *Ucp1* induction, specifically in cattle, is related to VFA production in the rumen. Bovine myosatellite cells were treated with acetic acid, propionic acid, or butyric acid, and the expression levels of MYH1/2 and *Ucp1* were evaluated (Fig. 5A-C, Supplementary Fig. S5 and S6). Acetic acid and propionic acid increased the levels of acetylated H3K27 (Fig. 5A, Supplementary Fig. S8) and MYH1/2 (Fig. 5B, Supplementary Fig. S9). Treatment with butyric acid did not affect the acetylation of H3K27 (Fig. 5A, Supplementary Fig. S8) but increased the expression of MYH1/2 (Fig. 5B, Supplementary Fig. S9). Propionic acid treatment also resulted in *Ucp1* induction in bovine

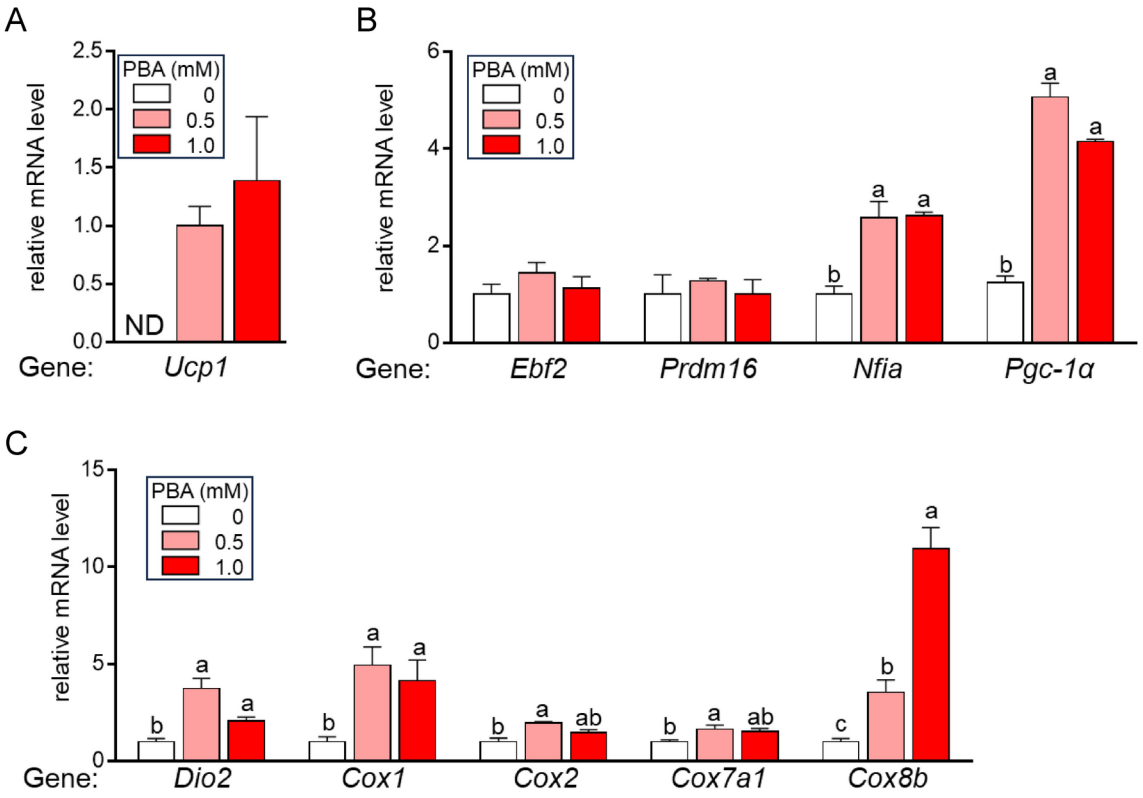

Fig. 3. PBA up-regulates expression of *Ucp1* and genes related to brown adipogenesis in bovine myosatellite cells. After reaching confluence (day 0), bovine myosatellite cells were cultured in differentiation medium supplemented with PBA (0.5 mM or 1 mM) for 6 days. The expression levels of *Ucp1* (**A**), transcriptional regulators related to brown adipogenesis (**B**), and mitochondrial respiration (**C**) were quantified using RT-qPCR. The expression levels of the respective genes were normalized against *Hprt1* mRNA levels, and those in the control cells were set to 1. The expression level of *Ucp1* in cells treated with PBA (0.5 mM) was set to 1. Data are presented as mean ± SE (n = 3). ND: not detected. a-c: Means that do not have a common letter on the bar differ significantly ($P < 0.05$).

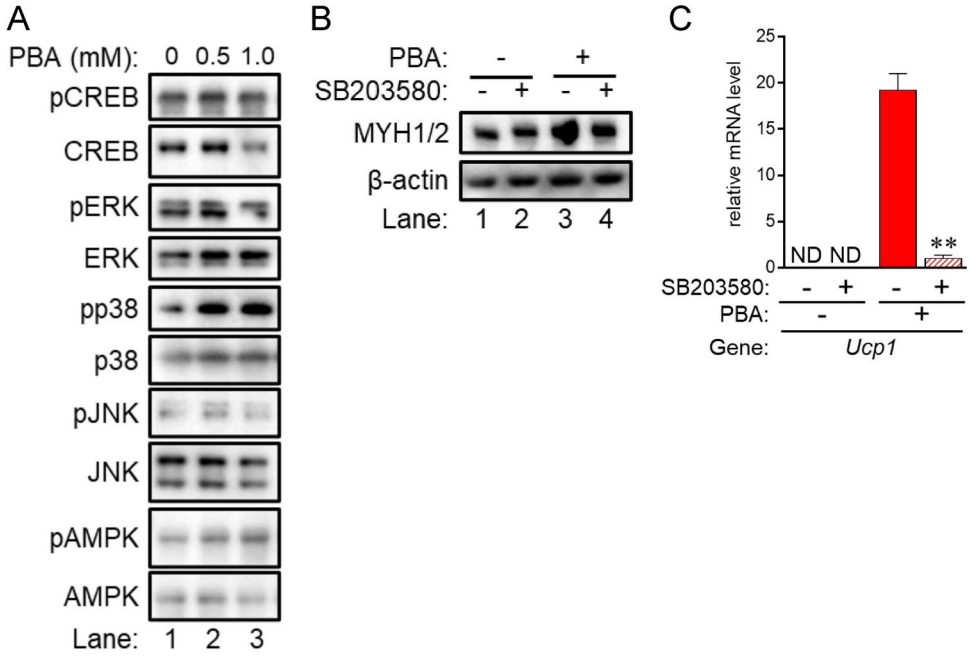

Fig. 4. p38 MAP kinase is involved in 4-PBA-induced *Ucp1* induction. (**A**) After reaching confluence (day 0), bovine myosatellite cells were cultured in myosatellite differentiation medium supplemented with or without PBA (0.5 mM or 1 mM) for 6 days. Expression levels of phosphorylated (p) CREB, CREB, pERK, ERK, pp38, p38, pJNK, JNK, pAMPK, and AMPK were examined by western blot analysis. (**B**, **C**) After reaching confluence (day 0), bovine myosatellite cells were cultured in myosatellite differentiation medium supplemented with or without PBA (1 mM) in the presence or absence of SB203580 (10 μM) for 6 days. Expression levels of MYH1/2 (B) were examined by western blot analysis. As a reference, the expression levels of β-actin were examined. (**C**) Expression levels of *Ucp1* were quantified by RT-qPCR. The expression level of *Ucp1* were normalized against *Hprt1* mRNA levels, and that in cells treated with PBA and SB203580 was set at 1. Data are presented as the mean ± SE (n = 4). ND: not detected. $^{**}P < 0.01$ *vs* PBA-treated cells.

myosatellite cells (Fig. 5C, Supplementary Fig. S10). In contrast, VFA and PBA stimulated neither acetylation of H3K27 or myogenesis in murine C2C12 myogenic cells (Fig. 5D and E, Supplementary Fig. S11 and S12). Treatment with PBA decreased the expression of acetylated H3K27 and MYH1/2 (Fig. 5D and E, Supplementary Fig. S11 and S12), which may be related to the decreased viability of PBA-treated C2C12 cells (Fig. 5F). Expression of *Ucp1* was below the detection limit in the C2C12 cells treated with either reagent (data not shown).

Discussion

UCP1 is not only a marker gene of brown/beige adipocytes in most mammals but is also responsible for brown/beige adipocyte-mediated thermogenesis[4–6]. UCP1 is uniquely expressed in the skeletal muscle tissues of cattle and is related to fast-twitch myofibers[19,20]. Here, we revealed that PBA stimulates myogenesis and *Ucp1* induction in bovine myogenic cells, but not in murine myogenic cells. Unlike humans and rodents, large amounts of VFAs are produced in cattle as a result of ruminal fermentation. Among the VFAs, propionic acid stimulated myogenesis and *Ucp1* expression in bovine myogenic cells but not in murine myogenic cells. The present results provide insight into why muscular UCP1 is detected, particularly in cattle.

We previously showed that murine myogenesis is stimulated by treatment with high-dose capsaicin prior to differentiation stimulation and subsequent treatment with vitamin C after differentiation stimulation[24]. Treatment did not increase myotube formation in bovine myosattelite cells. In contrast, treatment with PBA and VFA stimulated myogenesis in bovine myosatellite cells, but not in murine myoblasts. These results suggest species-dependent regulation of myogenesis. Previous studies have reported species-dependent differences in the myogenic activity and gene expression patterns[35–38]. The expression of desmin, a muscle-specific protein consisting of an intermediate filament, was detected in rat and goat myosatellite cells, but not in bovine myosatellite cells[35,36]. Gene expression patterns during myogenesis are distinct between human and mouse cells[37]. In addition, IL-1β stimulated and inhibited proliferation of mouse myosatellite cells and human myosatellite cells, respectively[37]. The regulation of muscle formation is distinct between mammals and fish[38]. The maximal growth of skeletal muscle is determined and finalized according to a fixed body size in mammals, whereas many fish have no limits on skeletal muscle growth due to both hyperplasia and hypertrophy of muscular cells[38].

Previous studies have shown that myogenic cells can differentiate into UCP1-positive adipocytes by forced expression of brown adipogenic genes, such as *Ebf2*, *Prdm16*, or *Nfia*[30,31,39]. Myogenic cells can also differentiate into UCP1-positive adipocytes in the absence of exogenous genes[40]. In this study, PBA treatment did not induce the emergence of bovine muscle cells with lipid droplets (data not shown). Instead, muscular *Ucp1* induction was

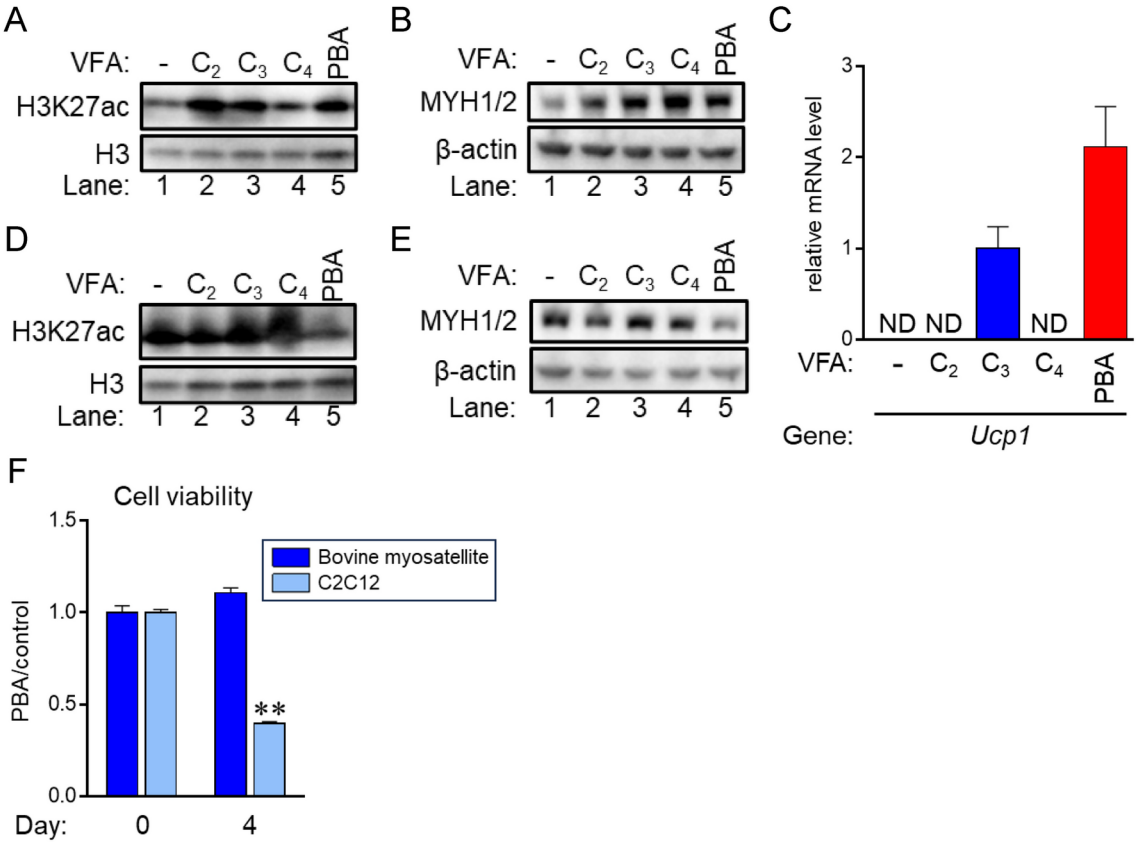

Fig. 5. Myogenic differentiation is stimulated by VFA and propionic acid induces *Ucp1* expression in bovine myosatellite cells. (**A**–**C**) After reaching confluence (day 0), bovine myosatellite cells were cultured in myosatellite differentiation medium supplemented with or without acetic acid (C_2: 1 mM), propionic acid (C_3: 1 mM), butyric acid (C_4: 1 mM), and PBA (1 mM) for 6 days. Expression levels of acetylated H3K27 (**A**) and MYH1/2 (**B**) were examined by western blot analysis. As a reference, the expression levels of H3 (**A**) and β-actin (**B**) were examined. (**C**) Expression levels of *Ucp1* were quantified by RT-qPCR. The expression level of *Ucp1* were normalized against *Hprt1* mRNA levels, and that in cells treated with propionic acid was set at 1. Data are presented as the mean ± SE (n = 4). ND: not detected. (**D**, **E**) On 1 day after confluence (day 0), murine C2C12 myoblasts were cultured in differentiation medium supplemented with or without acetic acid (C_2: 1 mM), propionic acid (C_3: 1 mM), butyric acid (C_4: 1 mM), and PBA (1 mM) for 6 days. Expression levels of acetylated H3K27 (**D**) and MYH1/2 (**E**) were examined by western blot analysis. As a reference, the expression levels of H3 (**D**) and β-actin (**E**) were examined. (**F**) Cell viability was evaluated in bovine myosatellite cells and murine C2C12 cells on day 0 and day 4. The results are shown as the ratio of cell viability in PBA-treated cells to that in control cells. Data are presented as the mean ± SE (n = 4). $^{**}P < 0.01$. *vs* bovine myosatellite cells.

positively correlated with the stimulation of myogenesis. These results suggest the possibility of *Ucp1* expression in bovine myotubes expressing fast-twitch MYHs. Brown adipocytes and muscle cells share a common lineage[39]. Both cell types are mitochondria-rich, and mitochondrial content increases during cold exposure in brown adipocytes as well as in muscular cells[41,42]. Furthermore, mitochondrial respiratory capacity is comparable between brown fat and skeletal muscles in humans[43]. Muscular UCP1 may be involved in energy expenditure in cattle.

The increase in murine *Myod* mRNA after differentiation stimulation is related to the abundance of acetylated H3K27 in the core enhancer region of *Myod* gene[44,45], resulting from the association of p300 histone acetyltransferase with this region[44] or the presence of the histone chaperone HIRA[45]. MYOD binds to the enhancer region of muscle-specific genes, such as *Myh*s, and recruits acetylated H3K27 to upregulate the expression of muscle-specific genes[46]. Thus, MYOD and acetylated H3K27 likely promote myogenesis through functional interactions. Mutual activation of MYOD and acetylation of H3K27 may also occur during the enhancement of myogenesis by PBA or VFA in bovine muscular cells.

Previous studies have shown that treatment with PBA does not increase the phosphorylation of p38 MAP kinase in human lung epithelial cells or murine breast cancer cells[47,48]. In contrast, the phosphorylation of p38 MAP kinase was increased by PBA in bovine myogenic cells (Fig. 4A), suggesting cell type- or animal species-dependent differences in p38 MAP kinase phosphorylation by PBA. p38 MAP kinase activation was required for PBA-induced myogenesis because inhibition of the p38 MAP kinase pathway decreased MYH1/2

expression in PBA-treated cells (Fig. 4B). Previous studies have revealed the stimulatory role of p38 MAP kinase in myogenesis[49,50].

This study has limitations, and the following points should be clarified in the future: 1. Molecular mechanisms underlying *Ucp1* induction by PBA and propionic acid; 2. Role of muscular UCP1 in thermogenesis in cattle; and 3. Relationship between muscular *Ucp1* levels and ruminal fermentation in vivo. We have previously shown that the expression levels of bovine *Ucp1* in brown adipocytes are modified by feeding[18,51,52]. The expression levels of *Ucp1* in subcutaneous fat depots were higher in beef cattle fed a high-concentrate diet than in those fed a high-roughage diet[18]. Ruminal propionic acid levels are increased by the ingestion of a high-concentrate diet[53]. In general, feed efficiency in beef cattle decreases with the progression of fattening[54]. Considering that a high-concentrate diet is usually provided during fattening in beef cattle, increased production of propionic acid in the rumen may decrease feed efficiency by increasing UCP1 expression in beef cattle. In addition, full characterization of the isolated cells from bovine muscles should also be done in the future: we prepared the myosatellite cells by the standard method, and the isolated cells were differentiated into myotubes. However, the skeletal muscle tissue is heterogenous, in which not only muscular cells but also fibroblasts, epithelial cells and endothelial cells are present. Furthermore, detailed relationships between H3K27 acetylation, myogenesis, and *Ucp1* expression should also be clarified, as the present study revealed that PBA or propionic acid stimulated H3K27 acetylation, myogenesis, and *Ucp1* expression in bovine muscular cells. However, acetic acid increased H3K27 acetylation and myogenesis, but not *Ucp1* expression. In addition, butyric acid stimulates myogenesis without increasing H3K27 acetylation.

Methods

Materials

Collagenase (Cat. 034–22,363), and dispase II (Cat. 383–02,281), capsaicin (Cat. 030–11,353), L-ascorbic acid phosphate magnesium salt *n*-hydrate (Cat. 013–12-61), SB203580 (Cat. 199–16,551), sodium acetate (Cat. 198–15,965), and sodium propionate (Cat. 194–03,012) were purchased from FUJIFILM Wako Pure Chemicals (Osaka, Japan), 4-phenylbutyric acid (PBA) (Cat. P0643) and sodium butyrate (Cat. S0519) from Tokyo Chemical Industry (Tokyo, Japan); rabbit monoclonal antibody against phospho-p38 (Thr180/Tyr182) (28B10) (Cat. #9216), rabbit polyclonal antibody against AMPKα (Cat. #2603), β-actin (Cat. #4967), ERK (Cat. #9102), JNK (Cat. #9252), phospho-ERK (Thr202/Tyr204) (Cat. #9101), or phospho-JNK (Thr183/Tyr185) (Cat. #9251), andrabbit monoclonal antibodies against acetylhistone H3 (Lys27) (D5E4) (Cat. #8173), CREB (D76D11) (Cat. #4820), histone H3 (D1H2) (Cat. #4499), p38 (D13E1) (Cat. #8690), phospho-AMPKα (Thr172) (40H9) (Cat. #2535), phospho-CREB (Ser133) (87G3) (Cat. #9198), from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal antibodies against myosin heavy chain (MYH) 1/2 (A4.1025) (Cat. sc-53088) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA).

Cell culture

All animal care and experiments were approved by the Animal Care Committee of Kyoto University (R4-21 and R5-21). All animal experiments were conducted following approved guidelines. The study is reported in accordance with ARRIVE guidelines (https://arriveguidelines.org). Bovine myosatellite cells were isolated from the *musculus longissimus cervicis* of Japanese Black steers aged 30 months. Tissues were minced into small pieces using surgical scissors and digested in Hank's balanced salt solution containing 1 mg/mL type I collagenase, 3.3 mg/mL dispase II, 100 U/mL penicillin, 100 µg/mL streptomycin, and 250 ng/mL amphotericin B for 1 h at 37 °C with shaking at 170 cycles/min. Subsequently, the cell suspension was sequentially filtered through 250-µm and 50-µm nylon meshes to remove undigested tissue fragments and debris. The filtrate was then centrifuged at 1200 g for 5 min. The digestion medium was then removed by decantation. The cell pellet was resuspended and washed twice with Dulbecco's modified Eagle's medium (DMEM) containing heat-inactivated 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 mg/mL streptomycin, 250 ng/mL amphotericin B, 30 µM ascorbic acid 2-phosphate (myosatellite growth medium). The collected cells were seeded on collagen-coated cell culture dishes and incubated in myosatellite growth medium at 37 °C under a humidified 5% CO_2 atmosphere. After reaching confluence (day 0), the medium was replaced with a myosatellite differentiation medium consisting of DMEM with 2% FBS supplemented with 60 nM insulin, 5 nM triiodothyronine (T_3), and antibiotics to induce differentiation from myoblasts to myotubes. Cells were harvested on day 6.

C2C12 myogenic cells[55] were obtained from RIKEN BioResource Research Center (Tsukuba, Japan). C2C12 myoblasts were differentiated into myotubes as described previously[56]. Briefly, one day after reaching confluence (day 0), C2C12 myoblasts were stimulated to induce differentiation from myoblasts to myotubes by culturing in DMEM containing 2% horse serum. During myogenesis, cells were treated with the indicated reagents.

Reverse transcription (RT)-quantitative (q) PCR

Total RNA isolation, cDNA synthesis, and qPCR were performed as previously described[56]. The nucleotide sequences of the qPCR primers are listed in Supplementary Table S1. Relative gene expression was determined using the ΔΔCt method[57], and the levels of target transcripts were normalized to those of *Hprt1*, because *Hprt1* is a stable gene during myogenesis[58]. We also used *Rps15a* as a reference gene, which was shown to be better in bovine cells[59].

Western blot

Western blotting was performed as previously described[56]. Because western blot is a semi-quantitative assay[60], we judged the relative abundance of a protein of interest based on apparent band intensity. We performed western blot at least two independent samples. Comparable results were obtained, and a representative result is shown.

Immunofluorescence

Immunofluorescence staining was performed as described previously[24,61]. The fusion index was calculated as the ratio of the number of nuclei incorporated into MYH1/2-positive myotubes to the total number of nuclei[24,61].

Cell viability assay

Cell viability was assessed as described previously[62]. The viability ratio of PBA-treated cells to control cells was evaluated.

Statistical analysis

Data are presented as the mean ± standard error of the mean (SEM). Gene expression data were log-transformed to approximate a normal distribution before analysis. Statistical analyses of gene expression were performed using GraphPad Prism software (San Diego, CA, USA). Data were analyzed using one-way or two-way analysis of variance (ANOVA): the factor considered was PBA in one-way ANOVA, and the factors considered were capsaicin, vitamin C, and the interaction between capsaicin and vitamin C in two-way ANOVA. When the effect of PBA was statistically significant in one-way ANOVA, and when the effect of the interaction between capsaicin and vitamin C was significant in two-way ANOVA, comparisons between groups were performed using Tukey's test. Student's *t*-test was used to evaluate effect of SB203580 on *Ucp1* expression levels in PBA-treated cells and differences of cell viability between bovine myosatellite cells and C2C12 cells. Differences were considered statistically significant at $P < 0.05$.

Data availability

All data included in this study are available upon reasonable request by contact with the corresponding author.

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Author contributions

Z.D. designed and performed experiments, and contributed to the interpretation of results and manuscript writing. S.J. performed experiments. E.I. and H.Y. designed sampling in animal studies. M.M. prepared samples in cell culture studies. M.F. designed and supervised whole studies, performed experiments, contributed to the interpretation of results, and wrote manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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