Modulation of brown adipocyte activity by milk by-products: Stimulation of brown adipogenesis by buttermilk

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Brown adipocytes dissipate chemical energy in the form of heat through the expression of mitochondrial uncoupling protein 1 (Ucp1); Ucp1 expression is further upregulated by the stimulation of β-adrenergic receptors in brown adipocytes. An increase in energy expenditure by activated brown adipocytes potentially contributes to the prevention of or therapeutics for obesity. The present study examined the effects of milk by-products, buttermilk and butter oil, on brown adipogenesis and the function of brown adipocytes. The treatment with buttermilk modulated brown adipogenesis, depending on the product tested; during brown adipogenesis, buttermilk 1 inhibited the differentiation of HB2 brown preadipocytes. In contrast, buttermilk 3 and 5 increased the expression of Ucp1 in the absence of isoproterenol (Iso), suggesting the stimulation of brown adipogenesis. In addition, the Iso-induced expression of Ucp1 was enhanced by buttermilk 2 and 3. The treatment with buttermilk did not affect the basal or induced expression of Ucp1 by Iso in HB2 brown adipocytes, except for buttermilk 5, which increased the basal expression of Ucp1. Conversely, butter oil did not significantly affect the expression of Ucp1, irrespective of the cell phase of HB2 cells, ie, treatment during brown adipogenesis or of brown adipocytes. The results of the present study indicate that buttermilk is a regulator of brown adipogenesis and suggest its usefulness as a potential food material for antiobesity.

KEYWORDS
activation, brown adipocytes, buttermilk, differentiation, Ucp1

1 INTRODUCTION

Brown adipocytes dissipate chemical energy in the form of heat against cold exposure or excess feeding, which is achieved through activation of sympathetic nerve activity.1–3 The thermogenic function of brown adipocytes results from the expression of a series of genes related to a high mitochondrial content and elevated cellular respiration, which is largely uncoupled from ATP synthesis.4 This uncoupling occurs through mitochondrial uncoupling protein 1 (Ucp1), a mammalian brown adipocyte-specific protein that promotes proton leakage across the inner mitochondrial membrane.2,5

Previous findings showing that adult humans have functional brown adipocytes6–8 prompted research that focused on the activity of brown adipocytes as a novel therapeutic for obesity.9 The activation of human brown adipocytes is responsible for energy expenditure during acute cold exposure.10 The clarification of factors affecting the function of brown adipocytes or brown adipogenesis will be helpful for establishing a practical strategy to prevent or develop therapeutics for obesity.

Milk or milk by-products may contain factor(s) that modulates the function of brown adipocytes through direct effects without affecting sympathetic nerve activity or the regulation of preadipocyte differentiation. Thermal imaging revealed that the ingestion of semiskimmed milk significantly increased body temperature within 5 minutes in the supraclavicular region of 8- and 13-year-old children11,12; this location corresponds to the distribution of brown adipocytes in humans6,8 suggesting the activation of brown adipocytes. In addition, human milk stimulated the differentiation of white preadipocytes.13,14 In view of overlapping mechanisms between brown adipogenesis and white adipogenesis,15 milk may also have the potential to modulate brown adipogenesis.

The objective of this study was to clarify the effects of buttermilk and butter oil on brown adipogenesis as well as the function of brown adipocytes. To achieve this, we used a brown adipogenesis model of HB2 preadipocytes that differentiate into brown adipocytes in the presence of insulin16,17 and added buttermilk or butter oil during the differentiation process of preadipocytes or to brown adipocytes.
2 | MATERIALS AND METHODS

2.1 | Materials

The following reagents were purchased: A mouse monoclonal antibody against Srebp1 (A-4, polyclonal antibody against Ucp1 [ab10983]) and a mouse monoclonal antibody against β-actin (AC-15, ab6276) were from Abcam (Cambridge, Massachusetts); bovine insulin and isoproterenol (Iso) were from Sigma (St Louis, Missouri).

Five buttermilk powder types (designated as 1 to 5) and 3 butter oil types (designated as A to C) were used; buttermilk was manufactured in Megmilk Snow Brand Co, Ltd (Sapporo, Japan). Buttermilk 1 was made in Isobun-nai factory, whereas the others were made in Bekkai factory; buttermilk 2 to 5 were different lots. Butter oil A was obtained from Murray Goulburn Co-operative Co, Ltd (Southbank, Australia), whereas butter oil B and C were from Synlait Milk Ltd (Rakaia, New Zealand); butter oil B and C were different lots.
FIGURE 3  Effects of a treatment with buttermilk or butter oil during brown adipogenesis on the expression of brown adipocyte–selective genes (experiment 1). HB2 brown preadipocytes were differentiated in the presence or absence of buttermilk or butter oil. On day 8, the expression of Prdm16, Pgc-1α, Cidea, and Cox7a1, was evaluated by RT-qPCR and expressed as a ratio to Hprt1, with the level in control HB2 brown adipocytes being set to 1. Data shown are the mean ± SE (n = 4). RT-qPCR indicates reverse transcriptase–quantitative real-time polymerase chain reaction.

FIGURE 4  Effects of a treatment with buttermilk or butter oil during brown adipogenesis on the expression of Ucp1 (experiment 1). HB2 brown preadipocytes were differentiated in the presence or absence of buttermilk or butter oil. On day 8, cells were treated with or without Iso for 4 h, and the expression of Ucp1 was evaluated by RT-qPCR and expressed as a ratio to Hprt1, with the level in control HB2 brown adipocytes being set to 1. Data shown are the mean ± SE (n = 4). *P < .05, significantly different from the expression level in Iso-untreated cells with the respective milk by-product. †P < .05, significantly different from the expression level in cells treated with the respective Iso, but not with milk by-product. Ucp1 expression was also evaluated by Western blotting B. Iso indicates isoproterenol; RT-qPCR, reverse transcriptase–quantitative real-time polymerase chain reaction.
Buttermilk is the liquid fraction obtained when churning butter from cream, which is the upper layer of centrifuged whole milk. Butter oil is obtained from cream through sequential concentrations of a water-insoluble fraction, and the composition of butter oil is similar to that of butter. Approximate analyses indicate that buttermilk contains approximately 30% protein, approximately 50% carbohydrate, approximately 7% minerals, and approximately 8% fat, while more than 99.3% of butter oil is fat.

2.2 | Cell culture

HB2 brown preadipocytes, which were kindly provided by Dr M. Saito (Tenshi University), were cultured in Dulbecco modified eagle medium with 10% fetal bovine serum and antibiotics as described previously; HB2 cells were stimulated to differentiate into brown adipocytes by insulin (20nM) 2 days after confluence (day 0). To examine the effects of buttermilk and butter oil during brown adipogenesis, we added buttermilk or butter oil extracts prepared as described below to the culture medium from day 0 to day 8. In the evaluation of their effects on brown adipocytes, cells were treated with the extracts from day 8 to day 12. On day 8 in experiments to examine brown adipogenesis, or on day 12 in experiments to examine effects in brown adipocytes, a treatment with a β-adrenergic receptor agonist Iso (10μM) was conducted for 4 hours before harvesting.

2.3 | Preparation of buttermilk powder and butter oil extracts

Buttermilk powder was suspended in 70 vol of culture medium, followed by centrifugation at 36 000g at 4°C for 60 minutes (SRX-201, Tomy, Tokyo, Japan). The supernatant was sterilized by filtration through a 0.22-μm filter (Bottle top filter, Iwaki, Tokyo, Japan) and used as culture medium containing buttermilk extracts. Buttermilk extracts were prepared on days 0, 4, and 8.

Two grams of butter oil was mixed with 40 mL of ethanol and vigorously shaken for 60 minutes at room temperature followed by...
filtration through filter paper. The ethanol extracts were concentrated to approximately 150 mg ethanol extract per milliliter using a centrifugal evaporator (RD-400, Yamato Scientific, Tokyo, Japan) and applied to cells at a concentration of 150 μg/mL. Samples were stored under N₂ gas at -20°C until analysis.

2.4 Western blotting

Western blot analyses were performed as described previously. The reacted proteins were visualized using the ECL Advance Western blotting detection system (GE Healthcare, Buckinghamshire, UK) according to the manufacturer’s protocol. After stripping the antibodies and detection reagents, the membranes were reprobed with an antibody against β-actin.

2.5 Reverse transcriptase–quantitative real-time polymerase chain reaction

RNA isolation and reverse transcriptase–quantitative real-time polymerase chain reaction (RT-qPCR) were performed as described previously. The oligonucleotide primers for RT-qPCR were described previously, except for Prdm16, which was 5'-GTGCTTAATTCCACCTTAGTTCTG-3' and 5'-AGGGACAGCATCATTGCATA-3' (GenBank accession number NM_027504). The Ct value was determined, and the abundance of gene transcripts was analyzed using the ΔΔCt method with Hprt1 as the corrected gene.

2.6 Statistical analyses

Data are expressed as the mean ± SE. Data were analyzed by analysis of variance using the GLM procedures of SAS. Data on gene expression were log-transformed to provide an approximation of a normal distribution before analyses. Comparisons between control cells and cells treated with milk by-product were performed using the Dunnet test. As for Ucp1 expression, comparison between Iso-untreated cells and Iso-treated cells with the respective milk by-product (un)treatment was also performed using Student t test. P < .05 was considered significant.

![FIGURE 6](image-url) Effects of a treatment with buttermilk during brown adipogenesis on the expression of brown adipocyte–selective genes (experiment 2). HB2 brown preadipocytes were differentiated in the presence or absence of buttermilk or butter oil. On day 8, the expression of Prdm16 A, Pgc-1α B, Cidea C, and Cox7a1 D, was evaluated by RT-qPCR. Cells were also treated with or without Iso for 4 h, and the expression of Ucp1 E, was evaluated by RT-qPCR. Gene expression levels were expressed as a ratio to Hprt1, with that in control HB2 brown adipocytes being set to 1. Data shown are the mean ± SE (n = 4). *P < .05, significantly different from the expression level in Iso-untreated cells with the respective milk by-product. †P < .05, significantly different from the expression level in cells treated with the respective Iso, but not with milk by-product. Iso indicates isoproterenol; RT-qPCR, reverse transcriptase–quantitative real-time polymerase chain reaction.
3 | RESULTS

3.1 | Stimulatory effects of buttermilk on the differentiation of brown preadipocytes

Dairy products may modulate brown adipogenesis as well as brown adipocyte function. We first examined the effects of buttermilk or butter oil on the differentiation of HB2 brown preadipocytes.

As shown in Figure 1A, lipid accumulation on day 8, which was evaluated by oil red O staining, was lower in cells treated with buttermilk 1 than in control cells; buttermilk 2 and 3 did not affect lipid accumulation. Ppary is a transcription factor that plays a central role in adipogenesis; the expression level of Ppary reflects the degree of differentiation of white adipocytes and may also be related to brown adipogenesis. The expression of Ppary2 was not statistically affected by buttermilk or butter oil (Figure 1B). Fabp4, a carrier protein for fatty acids, is strongly expressed in mature adipocytes. Neither buttermilk nor butter oil affected the expression level of Fabp4 (Figure 1C).

We previously reported that Srebp1c is involved in brown adipogenesis in HB2 cells. Thus, the expression level of Srebp1c was examined in RT-qPCR analyses (Figure 2A). Similar to the results of oil red O staining, the expression level of Srebp1c seemed to be decreased in HB2 cells treated with buttermilk 1, but it was not significant. The expression of Srebp1 at the protein level was decreased by the treatment with buttermilk 1 (Figure 2B).

Prdm16 is a master regulator of brown adipogenesis and is predominantly expressed in brown adipocytes. Pgc-1α and Cox7a1 stimulate mitochondrial biogenesis and function, and Cidea is involved in the regulation of Ucp1 expression in brown adipocytes; all of these genes are more strongly expressed in brown fat depots than in white fat depots. The expression of Prdm16, Pgc-1α, Cidea, and Cox7a1 was not significantly affected by the treatment with buttermilk or butter oil, but the expression level of these genes was numerically lower in cells treated with buttermilk 1 than in the control cells (Figure 3).

The treatment with buttermilk 3 increased Ucp1 mRNA levels in the absence of Iso over those in control cells (Figure 4A). The Iso treatment for 4 hours strongly induced Ucp1 expression, and the treatment with buttermilk 2 or 3 enhanced Iso-induced Ucp1 expression. In contrast, the treatment with buttermilk 1 inhibitedIso-induced Ucp1 expression. Butter oil had no effect on Ucp1 expression, irrespective of the Iso stimulation. The changes observed in mRNA levels were basically repeated in analyses performed at the protein level; Western blotting revealed that the expression of Ucp1 was stronger in cells treated with buttermilk 2 or 3 than in control cells under basal conditions, and also that Iso-induced Ucp1 expression was enhanced in cells treated with buttermilk 3 (Figure 4B). The gene expression of Ppary2, brown adipocyte–selective genes, and Ucp1 suggest that buttermilk 1 potentially inhibits the differentiation of brown preadipocytes, and that buttermilk 2 and 3 enhance brown adipogenesis.

Since the effects of buttermilk on brown adipogenesis varied depending on the products tested, we also examined 2 additional products of buttermilk in experiment 2. The treatment with buttermilk 1 decreased the expression of Ppary2, Fabp4, and Srebp1c (Figure 5), although it was not statistically significant in experiment 1. In addition, the expression of Prdm16, Pgc-1α, Cidea, and Cox7a1 was also significantly decreased by the treatment with buttermilk 1 (Figure 6A-D), which was also statistically insignificant in experiment 1. The tendency of inhibitory effect of buttermilk 1 on brown adipogenesis was detected in 3 independent experiments (data not shown). The new buttermilk in experiment 2 affected the expression levels of genes related to brown adipogenesis; a significant decrease in the expression of Srebp1c, Prdm16, and Pgc-1α by buttermilk 4 was detected, whereas buttermilk 5 significantly increased Cidea expression (Figures 5C and 6A-C). Although Srebp1c mRNA levels were decreased by the treatment with buttermilk 4 (Figure 5C), its protein levels were not (Figure 5D). Consistent with experiment 1, buttermilk 1 decreased Iso-induced Ucp1 expression. Buttermilk 5 increased Ucp1 expression in the absence of Iso (Figure 6E). These results confirm the inhibitory effects of buttermilk 1 on brown adipogenesis and suggest the stimulatory effects of buttermilk 5.

![Figure 7](image-url)
3.2 | Enhanced maturation of brown adipocytes induced by the buttermilk type

We next investigated whether milk by-products modulate the function of brown adipocytes. The treatment with buttermilk (nos. 1-3) or butter oil (A to C) for 4 days, ie, from day 8 to day 12, did not affect lipid accumulation or the expression level of Fabp4 in HB2 brown adipocytes (Figure 7). In addition, neither buttermilk nor butter oil affected the expression of brown adipocyte-related genes including Ucp1; the expression level of Ucp1 was not modulated irrespective of the Iso treatment (Figure 8). We examined the effects of buttermilk 4 and 5 in more detail. The treatment with buttermilk 5 did not affect the expression of Fabp4 but significantly upregulated that of Prdm16, Pgc-1α, Cidea, and Cox7a1 (Figure 9A-E). Furthermore, buttermilk 5 exhibited the ability to upregulate Ucp1 expression in the absence of Iso but did not have any effect on Iso-induced Ucp1 expression (Figure 9F). These results suggest that butter oil did not modulate brown adipocyte activity whereas buttermilk 5 potentially activated it.

4 | DISCUSSION

The present study evaluated milk by-products, buttermilk and butter oil, as modulators of brown adipogenesis and the function of brown adipocytes. Ucp1 mRNA levels reflect the activity of brown adipocytes. Therefore, we evaluated the expression level of Ucp1 in brown adipocytes treated with milk by-products. We found that several products of buttermilk (nos. 2, 3, and 5) have a tendency to stimulate brown adipogenesis or function of brown adipocytes. Thus, they may have potential as a food material for antiobesity. Specific factors present in buttermilk to modulate brown adipocyte activity should be explored in future studies.

Previous studies reported that milk strongly stimulates the differentiation of 3T3-L1 preadipocytes, a cell model for adipocyte differentiation. In a standard differentiation program, growth-arrested confluent 3T3-L1 preadipocytes are treated with appropriate hormonal agents, which induce synchronous reentrance into the cell cycle and undergo at least 2 rounds of mitosis, referred to as mitotic clonal expansion. During mitotic clonal expansion, the expression of the adipogenic transcription factors C/ebpβ and C/ebpδ is induced, which leads to the induction and activation of Pparγ, a master regulator of adipogenesis. A treatment with the lipid fraction of milk, even in the absence of appropriate hormonal reagents, was found to induce C/ebpβ expression during mitotic clonal expansion and increase lipid accumulation in 3T3-L1 adipocytes. In addition, milk has been shown to significantly activate Pparγ as a transcription factor. Although the mechanisms underlying the stimulatory effects of buttermilk on brown adipogenesis currently remain unclear, the C/ebp-Pparγ axis may be modulated in view of the common cascade between white adipogenesis and brown adipogenesis.

Dairy products have the ability to enhance diet-induced thermogenesis; energy expenditure was found to be elevated for 4 hours after the additional ingestion of a yoghurt drink in humans. Postprandial energy expenditure was higher in humans who ingested a diet with whey protein as a protein source than in those who ingested a diet with soy protein. Thermography of children revealed a rapid increase in body temperature in the supraclavicular region within 5 minutes of milk ingestion, which corresponded to the distribution of brown

![FIGURE 8](image.png) Effects of a treatment with buttermilk or butter oil in brown adipocytes on the expression of brown adipocyte-selective genes (experiment 3). HB2 brown adipocytes on day 8 were cultured for 4 days in the presence or absence of buttermilk or butter oil. On day 12, the expression of Prdm16 A, Pgc-1α B, Cidea C, and Cox7a1 D, was evaluated by RT-qPCR. Cells were also treated with or without Iso for 4 h, and the expression of Ucp1 E, was evaluated by RT-qPCR. Gene expression levels were expressed as a ratio to Hprt1, with that in control HB2 brown adipocytes being set to 1. Data shown are the mean ± SE (n = 4). Iso indicates isoproterenol; RT-qPCR, reverse transcriptase−quantitative real-time polymerase chain reaction.
adipocytes in humans. In view of the time scale of changes, these findings appear to reflect the activation of brown adipocytes. Since one type of buttermilk (no. 5) strongly increased Ucp1 expression in brown adipocytes (Figure 9F), factor(s), which contributes to brown adipocyte activation, is also present in buttermilk. A meta-analysis of randomized controlled clinical trials indicates that the inclusion of dairy products decreases body fat mass under energy-restricted conditions. Dairy products contain as-yet-uncharacterized factors that decrease body fat accumulation. Since the ability of skimmed milk to decrease adiposity in obese rats was shown to be superior to that of whey, various milk fractions exhibit the ability to modulate body fat accumulation. The stimulation of brown adipogenesis by buttermilk, as shown in this study, may be also involved in dairy product–induced decreases in adiposity.

Buttermilk contains the milk fat globule membrane, which is a special membrane composed of a lipid bilayer and proteins and encircles lipid droplets of milk fat. The milk fat globule membrane contains conjugated linoleic acid in a fraction of phospholipids; conjugated linoleic acid modulates the proliferation/differentiation of brown preadipocytes and brown adipocyte activity. Thus, since the phospholipid fraction is extractable in culture media, conjugated linoleic acid as the form of phospholipids in buttermilk may be responsible for the modulation of brown adipogenesis and function of brown adipocytes. Fatty acids of buttermilk contained 0.83% of conjugated linoleic acid, and percentage of fatty acids in buttermilk was 5.49%. Thus, the content of conjugated linoleic acid in buttermilk is estimated as 0.46 mg/g. Future studies are needed to evaluate effect of conjugated linoleic acid at the level contained in buttermilk.

Conway et al recently reported that the consumption of buttermilk decreased blood pressure in hypercholesterolemic patients, suggesting a potential beneficial effect of buttermilk. In addition to its stimulatory activity on brown adipogenesis shown in the present study, butter milk may have preferential activities other than those expected for health.

**CONFLICT OF INTEREST**

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