Cow's milk increases the activities of human nuclear receptors peroxisome proliferator-activated receptors α and δ and retinoid X receptor α involved in the regulation of energy homeostasis, obesity, and inflammation

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ABSTRACT

The nuclear peroxisome proliferator-activated receptors (PPAR) have been shown to play crucial roles in regulating energy homeostasis including lipid and carbohydrate metabolism, inflammatory responses, and cell proliferation, differentiation, and survival. Because PPAR agonists have the potential to prevent or ameliorate diseases such as hyperlipidemia, diabetes, atherosclerosis, and obesity, we have explored new natural agonists for PPAR. For this purpose, cow's milk was tested for agonistic activity toward human PPAR subtypes using a reporter gene assay. Milk increased human PPAR α activity in a dose-dependent manner with a 3.2-fold increase at 0.5% (vol/vol). It also enhanced human PPAR δ activity in a dose-dependent manner with an 11.5-fold increase at 0.5%. However, it only slightly affected human PPAR γ activity. Ice cream, butter, and yogurt also increased the activities of PPAR α and PPAR δ , whereas vegetable cream affected activity of PPAR δ but not PPAR α . Skim milk enhanced the activity of PPAR to a lesser degree than regular milk. Milk and fresh cream increased the activity of human retinoid X receptor (RXR) α as well as PPAR α and PPAR δ , whereas neither affected vitamin D3 receptor, estrogen receptors α and β , or thyroid receptors α and β . Both milk and fresh cream were shown by quantitative real-time PCR to increase the quantity of mRNA for uncoupling protein 2 (UCP2), an energy expenditure gene, in a dose-dependent manner. The increase in UCP2 mRNA was found to be reduced by treatment with PPAR δ -short interfering (si)RNA. This study unambiguously clarified at the cellular level that cow's milk increased the activities of human PPAR α , PPAR δ , and RXR α . The possible role in enhancing the activities of PPAR α , PPAR δ , and RXR α , and the health benefits of cow's milk were discussed.

Key words: milk, peroxisome proliferator-activated receptor α , peroxisome proliferator-activated receptor δ , retinoid X receptor α

INTRODUCTION

The peroxisome proliferator-activated receptor (PPAR) family, consisting of 3 subtypes—PPAR α , PPAR δ , and PPAR γ —belongs to the nuclear receptor superfamily (Michalik et al., 2006). As with most nuclear receptors, PPAR exhibit a modular structure consisting of an N-terminal region, a DNA binding domain, a flexible hinge region, a ligand-binding domain, and a C-terminal region (Schmidt et al., 1992; Sher et al., 1993; Mukherjee et al., 1997). The PPAR subtypes heterodimerize with another nuclear receptor, retinoid X receptor (RXR), and these PPAR-RXR heterodimers bind to PPAR response elements located in the target gene's promoter via the DNA binding domain of the PPAR (Kliwer et al., 1992; Feige et al., 2005). Upon the binding of a ligand to the ligand-binding domain, PPAR undergo conformational changes that result in the dissociation of corepressors and recruitment of coactivators (Nolte et al., 1998). These interactions with coregulators allow PPAR subtypes to modulate the expression of their target genes. Recent studies on these target genes have advanced understanding of the crucial roles of PPAR subtypes in regulating 1) energy homeostasis including lipid and carbohydrate metabolism (Wang et al., 2003; Lefebvre et al., 2006), 2) inflammatory responses (Devchand et al., 1996), and 3) cell proliferation, differentiation, and survival (Tan et al., 2001; Michalik et al., 2004). Thus, PPAR subtypes have become important drug targets for intervention in diseases such as hyperlipidemia, diabetes, atherosclerosis, obesity, cancer, and Alzheimer's disease (Walczak and Tontonoz, 2002; Bragt and Popeijus, 2008; Jiang et al., 2008; Tachibana et al., 2008).

A noteworthy feature of PPAR subtypes is their broad range of agonists presumably because of their large ligand-binding pockets (Nolte et al., 1998). In re-

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cent years, a large number of natural compounds have been identified as agonists for PPAR, including polyunsaturated fatty acids and herbal compounds (Huang et al., 2005; Grimaldi, 2007). Such compounds have the potential to be used as therapeutic or preventive agents for PPAR-related diseases. This led us to explore novel agonistic compounds among natural products including dairy products such as milk. Cow's milk has been demonstrated to have several health benefits including the strengthening of bone, prevention of bone loss from arthritis and menopause, protection from metabolic syndrome, prevention of obesity, and protection against cardiovascular diseases (Hayashida et al., 2004; Sun and Zemel, 2004; Huth et al., 2006; Jauhiainen and Korpela, 2007; Pfeuffer and Schrezenmeir, 2007; Bonjour et al., 2008). As some of these benefits overlap with the pharmacological effects of agonists for PPAR, we examined whether milk exerts PPAR agonistic activity. This paper clarified for the first time that milk has PPAR agonistic activity and enhances the expression of uncoupling protein 2 (UCP2), important for energy expenditure, via the activation of PPAR δ .

MATERIALS AND METHODS

Cells and Reagents

The CV-1 cell line derived from African green monkey kidney was obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan), and the L6 cell line derived from rat myoblast was obtained from ATCC (Manassas, VA). The cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin, and 1% streptomycin at 37°C in a humidified atmosphere of 5% CO₂. The cow's milk, skim milk, and fresh cream were purchased from Nippon Milk Community Co. Ltd. (Tokyo, Japan), the butter from Snow Brand Milk Products Co. Ltd. (Tokyo, Japan), the ice cream from Morinaga Milk Industry Co. Ltd. (Tokyo, Japan), the yogurt from Meiji Dairies Corporation (Tokyo, Japan), and the vegetable fresh cream from Nippon Milk Community. The lipid contents of milk, skim milk, fresh cream, ice cream, and vegetable fresh cream were 38, 6, 463, 80, and 416 mg/mL; those of butter and yogurt were 810 and 30 mg/g, respectively. The agonist WY14643 used as a positive control agonist for PPAR α was obtained from Tocris Bioscience (Ellisville, MI), and GW501516 used as a positive control agonist for PPAR δ and pioglitazone used as a positive control agonist for PPAR γ were purchased from Alexis Biochemicals (San Diego, CA). The positive control agonists for other nuclear receptors were obtained as follows; 1 α , 25-dihydroxyvitamin D3

for vitamin D3 receptor (VDR) from Funakoshi Co. Ltd. (Osaka, Japan), β -estradiol for estrogen receptors (ER) α and β from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and 3,3',5'-triiodo-L-thyronine (T3) for thyroid receptors (TR) α and β and 9-*cis*-retinoic acid for RXR α from Sigma-Aldrich (St. Louis, MO).

Plasmids

The Gal4 fusion PPAR-ligand-binding domain (LBD) expression plasmids pCMXGAL4-PPAR α LBD, pCMXGAL4-PPAR δ LBD, and pCMXGAL4-PPAR γ LBD were constructed by inserting subcloned PPAR-LBD into the pCMXGAL4 expression vector. The Gal4 reporter plasmid pG5-Luc and control *Renilla* luciferase expression plasmid pGL4.75hRluc-CMV were purchased from Promega Corporation (Madison, WI).

Luciferase Reporter Assays

The CV-1 cells were seeded at 2×10^5 per well in 6-well culture dishes and after overnight culture, transiently transfected with 1 μ g of the expression plasmid pGal4DBD/PPAR-LBD and 0.9 μ g of pG5-Luc together with 0.1 μ g of pGL4.75hRluc-CMV per well using FuGENE HD (Roche, Diagnostics, Indianapolis, IN) as a transfection reagent. Cells were harvested 4 h after transfection, washed once with PBS, and plated again at 1.6×10^4 per well in 96-well plates. The cow's milk, other dairy products, and positive control drugs were added to the culture as described in the following section, and the cells were incubated at 37°C for 48 h. The diluted milk and other dairy products used for the preparation of the culture containing 0.005, 0.05, and 0.1% (vol/vol) products were made by adding 10, 50, and 100 μ L of the original sterile products to 990, 450, and 400 μ L of distilled water in microtubes, respectively, and dispersing the mixtures by vibrating the microtubes on a vortex. In the case of butter, the original sterile butter was transferred to a microtube with a sterile spatula and melted by incubation at 37°C before it was diluted. To make medium containing the dairy products, 4.65 μ L of the original or diluted dairy product was added to 460.35 μ L of medium in microtubes, and the mixtures were dispersed by vibrating on a vortex. Each 50 μ L of medium containing the dairy products was finally added to 50 μ L of the culture in a well of the culture plate. The positive control drugs were diluted with dimethyl sulfoxide, and the original and diluted positive controls were added to the culture to make a 2:100 dilution. After incubation with the dairy products or positive control drugs, cells were washed with PBS and lysed with passive lysis buffer. The re-

sulting lysates were used for the reporter assay. The expression of the reporter was evaluated by measuring the activity of firefly luciferase using the dual luciferase reporter assay system (Promega Corp.) and an ARVO HTS 1420 multilabel counter (PerkinElmer, Waltham, MA) as a luminometer according to the instructions of the manufacturer. Firefly luciferase activity was corrected for transfection efficiency based on the activity of internal control *Renilla* luciferase.

Short Interfering RNA Treatment of Cells

The L6 cells were seeded at 1×10^5 per well in 6-well culture dishes and after overnight culture, the culture medium was replaced with fresh medium. The cells were transfected with 50 pmol of PPAR δ -short interfering (si)RNA cocktail or control-siRNA (B-Bridge International, Inc., Mountain View, CA) by FuGENE HD. After 6 h, the cells were subjected to a treatment with trypsin, collected, and centrifuged. The cell pellet was resuspended with fresh medium and plated again in a 6-well culture dish. After incubation overnight, the culture medium was replaced with fresh medium containing milk, fresh cream, or GW501516 at 0.5 μ M (final concentration). After 48 h of incubation, cells were harvested, washed with PBS, and supplied for preparation of total RNA.

Quantitative Real-Time PCR

Total RNA was prepared from cells cultured in the presence of a test substance, harvested, and washed with PBS using RNAqueous-4PCR (Ambion, Austin, TX) according to the manufacturer's protocol. The contaminating genomic DNA in total RNA samples was removed by treatment with RNase-free DNase, and the DNase was removed by DNase Removal Reagent. Real-time PCR was carried out with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc., Foster City, CA) using 2 μ g of total RNA and random primer according to the manufacturer's directions. The reaction mixtures were prepared with a QuantiTect SYBR Green PCR kit (Qiagen Inc., Valencia, CA) using 100 ng of cDNA and 0.1 μ mol each of the forward and reverse primers. To normalize the expression of target genes, reaction mixtures containing primers for GAPDH, a housekeeping gene, were also prepared. Quantitative real-time PCR was performed and analyzed using an ABI Prism 7900 HT Real-Time PCR System (Perkin-Elmer Applied Biosystems Inc., Norwalk, CT).

Statistical Analyses

Statistical analyses to assess the comparisons between multiple treatment groups were based on Dun-

Figure 1. Selectivity of the agonistic activity of milk. The agonistic effect of cow's milk on peroxisome proliferator-activated receptor (PPAR) subtypes was evaluated by conducting reporter gene assays using PPAR agonists WY14643, GW501516, and pioglitazone (PGZ) as positive controls for PPAR α , PPAR δ , and PPAR γ , respectively. Details are described in the text. All values are means $\pm$ SD for 3 tests. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with controls (distilled water 0.5%).

Figure 2. Selectivity of the agonistic activity of various dairy products. The agonistic effects of ice cream, butter, yogurt, and vegetable cream were determined as described in Figure 1. All values are means $\pm$ SD for 3 tests. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with controls (distilled water 0.5%). WY14643, GW501516, and pioglitazone (PGZ) are peroxisome proliferator-activated receptor (PPAR) agonists.

Figure 3. Effect of defatting on the agonistic activity of milk. The agonistic effects of milk and skim milk were determined as described in Figure 1. All values are means $\pm$ SD for 3 tests. ** $P < 0.01$; *** $P < 0.001$ compared with controls (distilled water 0.5%). WY14643, GW501516, and pioglitazone (PGZ) are peroxisome proliferator-activated receptor (PPAR) agonists.

nett's multiple comparisons test using GraphPad Prism for Windows (GraphPad Software Inc., La Jolla, CA). Comparisons between 2 treatment groups were based on Student's *t*-test.

RESULTS

Selectivity of the Agonistic Activity of Milk

As part of our efforts to find natural products with PPAR agonistic activity, we investigated whether cow's milk has activity for human PPAR family subtypes using reporter gene assays (Figure 1). The milk increased human PPAR α activity in a dose-dependent manner with a 3.2-fold increase at 0.5% (vol/vol) relative to the vehicle control. The positive control WY14643 at 100 μ M caused a 17.6-fold increase. Furthermore, milk also strengthened human PPAR δ activity in a dose-dependent manner with an 11.5-fold increase at 0.5%. The positive control GW501516 at 1 μ M achieved a 75.5-fold increase. However, milk had little effect on human PPAR γ activity.

Selectivity of the Agonistic Activity of Various Dairy Products

Next, we investigated whether other dairy products have the ability to increase PPAR activity (Figure 2). We tested ice cream, butter, and yogurt as dairy products, and vegetable cream as a nondairy product. All of the dairy products enhanced PPAR α activity, whereas vegetable cream did not. Ice cream, butter, and yogurt each at 0.5% increased the activity 2.3, 1.8, and 1.5-fold more than the vehicle control, respectively. Meanwhile, the dairy and nondairy products all increased PPAR δ activity. Ice cream, butter, and yogurt increased PPAR δ activity 12.2-, 8.1-, and 8.3-fold, respectively, whereas vegetable cream increased it 8.4-fold. None of these products enhanced PPAR γ activity. Of note, the profile of activity against PPAR α and PPAR δ differed between the dairy products and vegetable cream.

Effect of Defatting on the PPAR Agonistic Activity of Cow's Milk

Because polyunsaturated fatty acids such as arachidonic acid, linoleic acid, and eicosapentaenoic acid were reported to activate PPAR (Grimaldi, 2007), we examined whether defatting, which may reduce the amount of polyunsaturated fatty acid in milk, affects the increase in PPAR activity (Figure 3). Cow's milk at 0.5% increased PPAR α activity 3-fold more than the vehicle control, whereas skim milk increased it 2.2-fold. Meanwhile, cow's milk at 0.5% increased PPAR δ activity 6.8-fold, whereas skim milk increased it 2.2-

fold. The defatting of the milk reduced the increase in PPAR α activity to 60%, whereas it reduced the increase in PPAR δ activity to 21%.

Effect of Milk and Fresh Cream on the Activities of Various Nuclear Receptors

The ability of cow's milk to promote PPAR α and PPAR δ activities led us to investigate whether milk can influence the activities of other nuclear receptors. We evaluated the effect of milk and fresh cream on the transcriptional activities of 9 human nuclear receptors, PPAR α , PPAR δ , PPAR γ , VDR, ER α , ER β , TR α , TR β , and RXR α (Figure 4). Both products increased the activity of human RXR α as well as PPAR α and PPAR δ , whereas neither affected the other nuclear receptors. Milk and fresh cream each at 0.5% increased RXR α activity 1.8- and 10.0-fold more than the vehicle control, respectively.

Effect of Milk and Fresh Cream on the Expression of UCP2 Gene

Expression of the UCP2 gene is known to be regulated by PPAR δ (Wolf, 2003). Accordingly, we examined whether milk and fresh cream enhanced the expression of rat UCP2 in rat myoblast L6 cells by quantitative real-time PCR (Figure 5). Both the milk and cream increased the quantity of mRNA for UCP2 in a dose-dependent manner, and at 0.5%, the increase was 1.7- and 3.5-fold, respectively.

Effect of siRNA on the Expression of UCP2

Furthermore, we examined by knockdown of the PPAR δ gene using PPAR δ -siRNA whether the increase in mRNA for UCP2 on treatment of L6 cells with milk or fresh cream was mediated by the activation of PPAR δ (Figure 6). The PPAR δ -siRNA treatment was confirmed to reduce by 61% the quantity of mRNA for UCP2, which was increased by the positive control GW501516. Consistent with this rate of reduction, the PPAR δ -siRNA treatment was also found to reduce by 55 and 36% the quantity of UCP2 mRNA that was increased by the milk and fresh cream, respectively.

DISCUSSION

In this report, we elucidated for the first time that cow's milk and related dairy products increase the activities of PPAR α , PPAR δ , and RXR α . Functional roles of PPAR α include 1) the regulation of energy homeostasis by activating fatty acid catabolism, and stimulating gluconeogenesis and ketone body synthesis (Lefebvre et al., 2006) and 2) the attenuation of

Figure 4. Effect of milk and fresh cream on the activities of various nuclear receptors. The agonistic effect of milk and fresh cream on peroxisome proliferator-activated receptor (PPAR) α , δ , γ , vitamin D3 receptor (VDR), estrogen receptors (ER) α and β , thyroid receptors (TR) α and β , and retinoid X receptor (RXR) α were determined as described in the text using 1 α ,25-dihydroxy-vitamin D3 as a positive control for VDR, β -estradiol for ER α and ER β , 3,3',5'-triiodo-L-thyronine (T3) for TR α and TR β , and 9-*cis*-retinoic acid (9cRA) for RXR α . All values are means $\pm$ SD for 3 tests. ** $P < 0.01$; *** $P < 0.001$ compared with controls (distilled water 0.5%). WY14643, GW501516, and pioglitazone (PGZ) are peroxisome proliferator-activated receptor (PPAR) agonists.

inflammatory responses (Devchand et al., 1996). The activation of fatty acid catabolism induces a decrease in circulating triglyceride levels, a reduction in liver and muscle steatosis, and adiposity, which consequently ameliorates insulin resistance. Meanwhile, functional roles of PPAR δ include 1) the regulation of energy homeostasis by activating fatty acid catabolism and adaptive thermogenesis, 2) the retardation of weight increase (Wang et al., 2003), and 3) the control of cell proliferation, differentiation, and survival (Tan et al., 2001; Michalik et al., 2004). Last, RXR α functions in the regulation of developmental and metabolic processes by forming heterodimers with other nuclear receptors such as retinoic acid receptor, PPAR subtypes, liver X receptor, farnesoid X receptor, and VDR (Germain et al., 2006). Transactivation of RXR α enables heterodimerized nuclear receptors to activate their target genes. Cow's milk and related dairy products are expected to exhibit the functions of PPAR α , PPAR δ , and RXR α as activators of nuclear receptors.

Among the reported health benefits of cow's milk, an acceleration of weight and fat loss (Sun and Zemel, 2004; Pfeuffer and Schrezenmeir, 2007) attracted our interest, because this pharmacological effect is similar to that of a PPAR δ activator. The major factor exerting this effect was identified as calcium, a typical con-

stituent of cow's milk. However, because dairy sources of calcium have substantially greater antiobesity effects than calcium carbonate, dairy products are thought to contain additional bioactive compounds that provide an attenuation of adiposity beyond that found with calcium alone (Sun and Zemel, 2004). Furthermore, dairy sources of calcium were elucidated to increase basal adipose lipolysis, decrease the activity and expression of fatty acid synthase, and increase the expression of UCP2 in white adipose tissue, PPAR α in muscle, and PPAR γ in adipocyte tissue more than calcium alone (Sun and Zemel, 2004). Factors other than calcium responsible for the antiobesity-related effects of dairy products have been discussed previously. Candidates included conjugated linoleic acid (Pariza et al., 2001), branched amino acids (leucine, isoleucine, and valine; Layman, 2003), and peptide(s) exhibiting angiotensin-converting enzyme inhibitory activity (FitzGerald et al., 2004). However, further study seems to be required to conclude which constituents are responsible. Our findings that cow's milk increases the activity of PPAR α and PPAR δ indicated a novel mechanism behind the antiobesity activity of cow's milk. Namely, a direct effect on PPAR α and PPAR δ by milk leads to the expression of targets such as fatty acid synthase and UCP2, and thus antiobesity activity. Our findings using a myoblast

Figure 5. Effect of milk and fresh cream on the expression of uncoupling protein 2 (UCP2). The effect of milk and fresh cream on the expression of UCP2 in L6 cells was determined by real-time PCR. All values are means $\pm$ SD for 3 tests. ** $P < 0.01$; *** $P < 0.001$ compared with controls (distilled water 0.5%). GW501516 is a peroxisome proliferator-activated receptor (PPAR) δ agonist.

cell line indicated that the expression of UCP2 was enhanced by treatment with cow's milk, and further, using siRNA, that this enhancement was dependent on PPAR δ . The constituents of milk responsible for the effect on PPAR α and PPAR δ remain unknown, although one of the most likely candidates is polyunsaturated fatty acids, for several reasons: 1) Cow's milk contains 3.5 to 4.0% lipid and approximately 3.4% of the fatty acids are polyunsaturated, mainly linoleic acid and linolenic acid (Soyeurt et al., 2006); 2) polyunsaturated fatty acids were elucidated to activate PPAR subtypes (Grimaldi, 2007); 3) preference for nuclear receptors and PPAR subtypes of some polyunsaturated fatty acids including linoleic acid resemble closely that of cow's milk (Vanden-Heuvel et al., 2006); namely, they exert agonistic activity against PPAR α , PPAR δ , and RXR α ; and 4) defatting decreased the activation of transcriptional activity of PPAR α and PPAR δ , whereas fresh cream exhibited stronger agonistic activity against PPAR subtypes than cow's milk. Nonetheless, there is still the possibility that unidentified factors are responsible for the agonistic activity of cow's milk.

Our finding of agonistic activity toward PPAR and RXR α seems to require reconsideration of the health

benefits of cow's milk. The ameliorating effects of milk on arthritis, sleep disorders, and cognitive dysfunction are related to PPAR α and PPAR δ . Accordingly, the role that the agonistic activity of milk plays in these effects should be examined although the factors responsible have been presumed to be lactoferrin, tryptophan, and vitamin B₁₂ (Selhub et al., 2000; Hayashida et al., 2004; Cubero et al., 2006). Furthermore, the agonistic activity of milk toward RXR α implies a possible ameliorating effect on diseases involving heterodimerized nuclear receptor permissive partners such as liver X receptors and farnesoid X receptor (Forman et al., 1995). In this respect, it is of interest whether the RXR α agonistic activity of milk affects cholesterol homeostasis.

Finally, it should be noted that further study is required to verify whether the agonistic activity of cow's milk against PPAR α , PPAR δ , and RXR α plays a role in the health benefits of milk, although this study showed unambiguously at the cellular level that milk has agonistic activity against PPAR α , PPAR δ , and RXR α . Further study should include 1) identification of a constituent of milk that exhibits the agonistic activity against PPAR α , PPAR δ , and RXR α , which enables the elucidation of pharmacokinetics and in vivo

Figure 6. Effect of short interfering (si)RNA on the expression of uncoupling protein 2 (UCP2). The effect of control siRNA and peroxisome proliferator-activated receptor (PPAR) δ -siRNA on the expression of UCP2 in L6 cells was determined as described in the text. All values are means $\pm$ SD for 3 tests. * $P < 0.5$, *** $P < 0.001$ compared with respective control siRNA. GW501516 is a peroxisome PPAR agonist.

pharmacodynamics of the active constituent, and 2) comparative studies on efficacy of milk in the disease models using normal and PPAR α -, PPAR δ -, or RXR α -knockout mice.

CONCLUSIONS

Our results showed for the first time that milk and other dairy products increase the activities of human PPAR α , PPAR δ , and RXR α . Milk was further proven to enhance the expression of UCP2 through the activity of PPAR δ . These results should help to verify whether the increases in the activity of PPAR α , PPAR δ , and RXR α play a role in the health benefits of cow's milk such as antiobesity.

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