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Citrus auraptene acts as an agonist for PPARs and enhances adiponectin production and MCP-1 reduction in 3T3-L1 adipocytes

Kayo Kuroyanagi [a,1], Min-Sook Kang [a,1], Tsuyoshi Goto [a], Shizuka Hirai [a], Kana Ohyama [a], Tatsuya Kusudo [a], Rina Yu [b], Masamichi Yano [c], Takao Sasaki [d], Nobuyuki Takahashi [a], Teruo Kawada [a,*]

[a] *Laboratory of Molecular Function of Food, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Uji, Kyoto 611-0011, Japan*
[b] *Department of Food Science and Nutrition, University of Ulsan, Ulsan 680-749, South Korea*
[c] *Department of Citriculture, National Institute of Fruit Tree Science, Ministry of Agriculture, Forestry and Fisheries, Shimizu, Shizuoka 424-0292, Japan*
[d] *ARKRAY Inc., Kyoto 601-8045, Japan*

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Abstract

Citrus fruit compounds have many health-enhancing effects. In this study, using a luciferase ligand assay system, we showed that citrus auraptene activates peroxisome proliferator-activated receptor (PPAR)-α and PPARγ. Auraptene induced up-regulation of adiponectin expression and increased the ratio of the amount of high-molecular-weight multimers of adiponectin to the total adiponectin. In contrast, auraptene suppressed monocyte chemoattractant protein (MCP)-1 expression in 3T3-L1 adipocytes. Experiments using PPARγ antagonist demonstrated that these effects on regulation of adiponectin and MCP-1 expression were caused by PPARγ activations. The results indicate that auraptene activates PPARγ in adipocytes to control adipocytekines such as adiponectin and MCP-1 and suggest that the consumption of citrus fruits, which contain auraptene can lead to a partial prevention of lipid and glucose metabolism abnormalities.

Keywords: Auraptene; Adipocyte; PPAR; Adiponectine; MCP-1; Inflammation; Metabolic syndrome

In Western culture, excess visceral fat accumulation or obesity has reached epidemic proportions, resulting in metabolic syndrome [1]. However, more than 10 years of research has shown that adipocytes also function as endocrine cells that release various bioactive substances, so called ''adipocytokines'' (or ''adipokines'') [2] that play a major role in the regulation of food intake, insulin sensitivity, energy metabolism, and the vascular microenvironment [3]. Adiponectin, an adipocytokine, is considered as a physiologically active protein that not only improves insulin sensitivity but also inhibits the inflammatory processes [4,5]. Recently, monocyte chemoattractant protein (MCP)-1 has been reported to be a novel adipocytokine involved in the development of obesity-associated insulin resistance and atherosclerosis [6,7]. MCP-1 inhibits insulin-dependent glucose uptake and the expression of adipogenic genes, thus, MCP-1-deficient mice lack insulin resistance and are resistant to atherosclerosis [6,7]. Recent studies have shown that adipose-tissue-derived MCP-1 induces macrophage infiltration into adipose tissues and thus augments inflammatory responses in obesity [8]. These suggest that the modulation of adipocytokines is a useful strategy for preventing not only obesity-induced inflammation but also the development of obesity-related pathologies.

* Corresponding author. Fax: +81 774 38 3752.
E-mail address: fat@kais.kyoto-u.ac.jp (T. Kawada).
[1] These authors contributed equally to this work.

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Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that regulate the metabolism of glucose and lipids [9]. PPARγ is strongly expressed in adipocytes and responsible for growth and differentiation [10]. PPARγ plays a significant role in the transcriptional activation of adipocytokines including adiponectin [11]. PPARα, another PPAR isoform, is involved in the control of lipid metabolism in the liver and skeletal muscle [12,13]. PPARα activation causes lipid clearance via β-oxidation enhancement in peripheral tissues [14].

Citrus fruit compounds have many beneficial bioactivities (for example, anticarcinogenesis, antihypertention and anti-cardiac effects) [15,16]. Here, a luciferase ligand assays showed that auraptene, a citrus fruit compound contained mainly in the peel, activated both PPARα and PPARγ. In this study, it was shown that auraptene regulates the transcription of PPARγ target genes, induces the expression and secretion of adiponectin, and inhibits those of MCP-1 in differentiated 3T3-L1 adipocytes. These findings suggest that auraptene acts as an agonist for PPARγ in adipocytes and is a valuable medical and food component for the gradual improvement of metabolic syndrome.

Materials and methods

Chemical reagents. The citrus components used except auraptene were kindly provided by the National Institute of Fruit Tree Science, Ministry of Agriculture, Forestry and Fisheries (Shizuoka, Japan). Auraptene was purchased from LKT Lab (MN, USA). All the other chemicals used were from Sigma (MO, USA) or Nacalai Tesque (Kyoto, Japan).

Luciferase ligand assay. Luciferase ligand assay was performed using an advanced highly sensitive system, which was developed by modifying the dual luciferase system (Promega, WI, USA), as previously described [17,18]. Briefly, for assay using the GAL4/PPAR chimera system, we transfected p4xUASg-tk-luc (a reporter plasmid), pM-hPPARα, pM-hPPARγ or pM-hPPARδ (an expression plasmid for a chimera protein for a GAL4 DNA binding domain and each human PPAR ligand binding domain) and pRL-CMV (an internal control) into CV1 cells cultured on 24-well plates. The transfection was performed using LipofectAMINE (Invitrogen, Corp., Carlsbad, CA, USA) according to the manufacturer′s protocol. Twenty-four hours after the transfection, the transfected cells were cultured in a medium containing each compound for an additional 24 h. Luciferase ligand assay was performed using the dual luciferase system in accordance with the manufacturer′s protocol.

3T3-L1 adipocyte culture. Adipocyte differentiation assay was performed as previously described [18]. In brief, 3T3-L1 murine preadipocytes (from ATCC) were cultured in Dulbecco′s modified Eagle′s medium containing 10% fetal bovine serum, and 10 mg/ml penicillin/streptomycin at 37 °C in 5% CO_2. Three days after confluence was reached, the cells were incubated in the differentiation medium (DM) containing 0.25 μM dexamethazone, 10 μg/ml insulin, and 0.5 mM 1-methyl-3-isobutylxanthine as in the maintenance medium. After 48 h, the cell culture medium was changed to post-DM containing 5 μg/ml insulin as in the maintenance medium, and fresh post-DM was supplied every 2 days. Cell viabilities were determined calorimetrically using CellTiter 96 (Promega) in accordance with the manufacturer's protocol.

RNA preparation and real-time fluorescence monitoring RT-PCR. By using M-MLV reverse transcriptase (Invitrogen), total RNA was reverse-transcribed in accordance with the manufacturer's instructions using a thermal cycler (Takara PCR Thermal Cycler SP: Takara Shuzo Co., Shiga, Japan). To quantify mRNA expression, real-time RT-PCR was performed with a LightCycler System (Roche Diagnostics, Mannheim, Germany) using SYBR Green fluorescence signals, as described previously [18,19]. The oligonucleotide primers of mouse PPAR target genes were designed using a PCR primer selection program shown in the website of the Virtual Genomic Center from the GenBank database as follows: mouse 36B4 (BC011291, Fwd: TGTGTGTCTGCAGATCGGGTAC, Rev: CTTTGGCGGGATT AGTCGAAG), mouse adiponectin (NM_009605, Fwd: ACAACCA ACAGAATCATTATGACGG, Rev: GAAAGCCAGTAAATGTAGA GTCGTTGA), mouse MCP-1 (NM_011333, Fwd: ATGCAGGT CCCTGTCATGCTTC, Rev: GGCATCACAGTCCGAGTCACAC). All the other oligonucleotide primer sets used were previously described [17–19].

Analysis of adiponectin and MCP-1 secretion. Aliquots of the conditioning medium were taken from the 3T3-L1 adipocyte culture 8 days after differentiation. Aliquots of the culture medium were also taken from culture of 3T3-L1 adipocytes at different periods and stored at −80 °C until analysis. Adiponectin level was determined using a mouse adiponectin ELISA development kit (R&D, USA). Multimers of adiponectin were detected as described previously [20]. In brief, the culture medium taken was subjected to 5–15% SDS–PAGE (Bio-Rad, USA) under non-reducing and non-heat-denaturing conditions. Adiponectin was detected using rabbit polyclonal anti-adiponectin antibodies for mouse adiponectin (Affinity BioReagents, USA), followed by incubation with a secondary antibody conjugated to horseradish peroxidase, anti-rabbit IgG (Promega). Secondary-antibody binding was visualized using by chemiluminescence with the ECL Western blotting detection system (Amersham Bioscience, USA). MCP-1 secretion was quantified with a mouse MCP-1 ELISA set (BD Bioscience, USA) in accordance with the manufacturer's instructions.

Statistical analysis. The data were presented as means ± SEM and statistically analyzed using an unpaired *t*-test, the Welch *t*-test and one-way ANOVA when variances were heterogeneous. Differences were considered significant at $p < 0.05$.

Results

Auraptene stimulates the activities of both PPARα and PPARγ

First, to find novel PPAR ligands in citrus fruit compounds, we screened the PPARs activities of using the luciferase ligand assay system. In assays using GAL4 and each PPAR isoform-chimera protein, of seven compounds, auraptene activated GAL4/PPARα and GAL4/PPARγ chimera transactivations as shown in Fig. 1A. Auraptene belongs to the chemical class of coumarin, also called 7-geranyloxy coumarin (the structure of this chemical is shown in Fig. 1B). The PPARα and PPARγ activation levels in the case of 50 μM auraptene were approximately 4.1- and 2.7-fold, respectively, higher than those in the case of the vehicle control. However, none of the compounds had any effect on GAL4/PPARδ transactivation (data not shown). Furthermore, in the case of auraptene, dose-dependent increases in both PPARα and PPARγ transactivation levels were observed using the chimera assay system (Fig. 1C and D). These data indicate that auraptene can activate both PPARα and PPARγ.

Auraptene up-regulates the gene and protein expression levels of adiponectin and induces high-molecular-weight (HMW) multimers of adiponectin

To determine the effects of auraptene as a PPARγ agonist on the expression of adiponectin in adipocytes, 3T3-L1

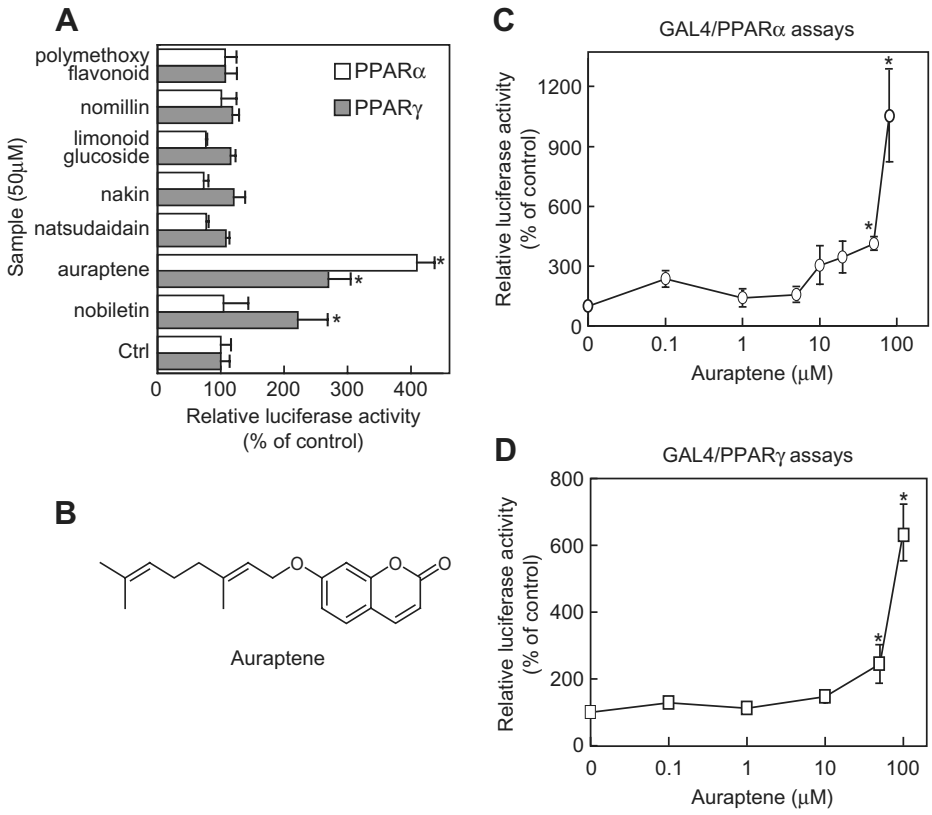

Fig. 1. Effects of various citrus fruit compounds on activations of PPARα and PPARγ in luciferase ligand assay system using GAL4/PPAR chimera proteins. (A) Results of luciferase assays using various citrus fruit compounds. p4xUASg-tk-luc and pRL-CMV were transfected into CV1 cells together with pM-hPPARα or pM-hPPARγ. Twenty-four hours after the transfection, the cells were treated with several citrus fruit compounds at 50 μM for 24 h. (B) Chemical structure of auraptene showing PPARα and PPARγ activations. Auraptene is a coumarin. (C and D) Results of luciferase assays showing dose-dependencies of auraptene on PPARα and PPARγ activities. p4xUASg-tk-luc and pRL-CMV were transfected into CV1 cells together with pM-hPPARα (C) or pM-hPPARγ (D). Twenty-four hours after the transfection, the cells were treated with auraptene at various concentrations for 24 h. The activity of vehicle control was set at 100%, and relative luciferase activity was presented as fold induction relative to that of the vehicle control. The values are the means ± SEM of 3–4 tests. $^{*}p < 0.05$ compared with vehicle controls.

cells were treated with auraptene throughout both an induction and a promotion period of differentiation or only differentiation induction. The auraptene treatment throughout both an induction and a promotion period of differentiation dose-dependently increased the mRNA expression level of adiponectin (Fig. 2A). Furthermore, the results of ELISA showed that the amount of adiponectin protein secreted into the supernatant also increased by auraptene (Fig. 2A). The auraptene treatment during only differentiation induction also increased both mRNA expression and protein secretion of adiponectin (Fig. 2B).

Adiponectin characteristically forms multimers [21,22]. It has recently been reported that an increase in the ratio of the high molecular weight (HMW) forms to the total amount of adiponectin correlates with an improvement in insulin sensitivity induced by thiazolidinedione treatment [23]. Here, it was examined whether auraptene affects the multimers of adiponectin. As shown in Fig. 2C and D, the ratio of the amount of HMW multimers to the total amount of adiponectin was increased by the auraptene treatment. This effect was similar to that of pioglitazone treatment. The results indicate that auraptene addition increases the levels of adiponectin mRNA and protein in adipocytes, and as well the ratio of the amount of HMW multimers of adiponectin to the total amount of adiponectin.

Auraptene inhibits the gene expression and secretion of MCP-1

Next, it was studied whether auraptene affects MCP-1 mRNA synthesis and secretion in differentiated 3T3-L1 adipocytes. In the presence of auraptene throughout both an induction and a promotion of 3T3-L1 adipocyte differentiation, the mRNA and secretion levels of MCP-1 decreased dose-dependently (Fig. 3A). Moreover, the treatment with auraptene during only differentiation induction also decreased both levels of MCP-1 dose-dependently (Fig. 3B). These data suggest that auraptene decreases the MCP-1 levels of both mRNA and protein. Auraptene at the concentration used in this study did not affect cell viability (data not shown).

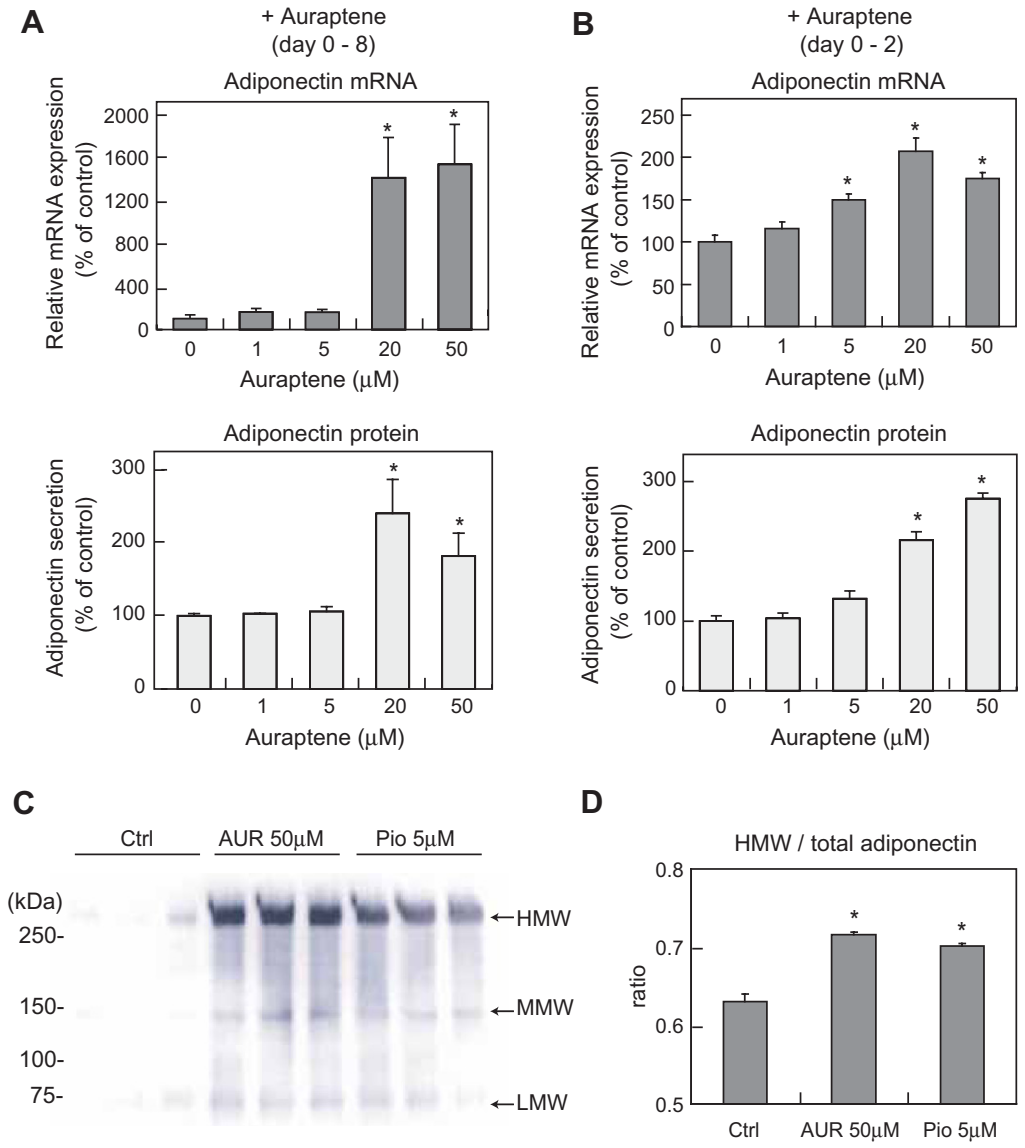

Fig. 2. Auraptene induces adiponectin expression in 3T3-L1 adipocytes. 3T3-L1 cells were treated with 1, 5, 20, and 50 μM auraptene throughout a promotion and/or an induction period of differentiation. The mRNA and protein expression levels of adiponectin with auraptene treatment throughout both an induction and a promotion period of differentiation are shown in (A). Similar effects of auraptene treatment during only differentiation induction are shown in (B). mRNAs were prepared 8 days after differentiation induction. Adiponectin secretion level was measured using aliquots collected from differentiated 3T3-L1 adipocyte culture medium 8 days after differentiation induction. The expression level of a vehicle control in each experiment was set at 100%, and relative expression level was presented as fold induction relative to that of a vehicle control. (C) Culture medium of differentiated 3T3-L1 adipocytes treated with auraptene (AUR) or pioglitazone (pio) during differentiation induction and subjected to SDS–PAGE under non-reducing and non-heat-denaturing conditions. Multimer forms of adiponectin were detected using an anti-adiponectin antibody. (D) Quantitative results of SDS–PAGE. The values are means ± SEM of 3–4 tests. $^{*}p < 0.05$ compared with vehicle controls.

Auraptene acts as a PPARγ ligand in 3T3-L1 adipocytes

To determine whether the effects of auraptene is caused by PPARγ activation, GW9662 [24], a PPARγ antagonist, was added together with auraptene to the culture medium of 3T3-L1 adipocytes. As a result of auraptene and GW9662 addition during only differentiation induction, GW9662 significantly decreased the mRNA expression levels of aP2 and adiponectin (Fig. 4). These data indicate that auraptene induces the expression of PPARγ target genes in 3T3-L1 differentiation as a ligand and suggest that 3T3-L1 differentiation is enhanced by auraptene treatment through the PPARγ-dependent pathway. Moreover, these results indicate that auraptene acts as a PPARγ ligand to regulate MCP-1 mRNA expression during 3T3-L1 adipocyte differentiation. Interestingly, MCP-1 mRNA expression level increased after the addition with auraptene and GW9662, although no dose-dependent increase was observed.

Discussion

This study showed that auraptene acts as a dual agonist for PPARα and PPARγ in luciferase ligand assays. Several natural compounds such as phytol and abietic acid func-

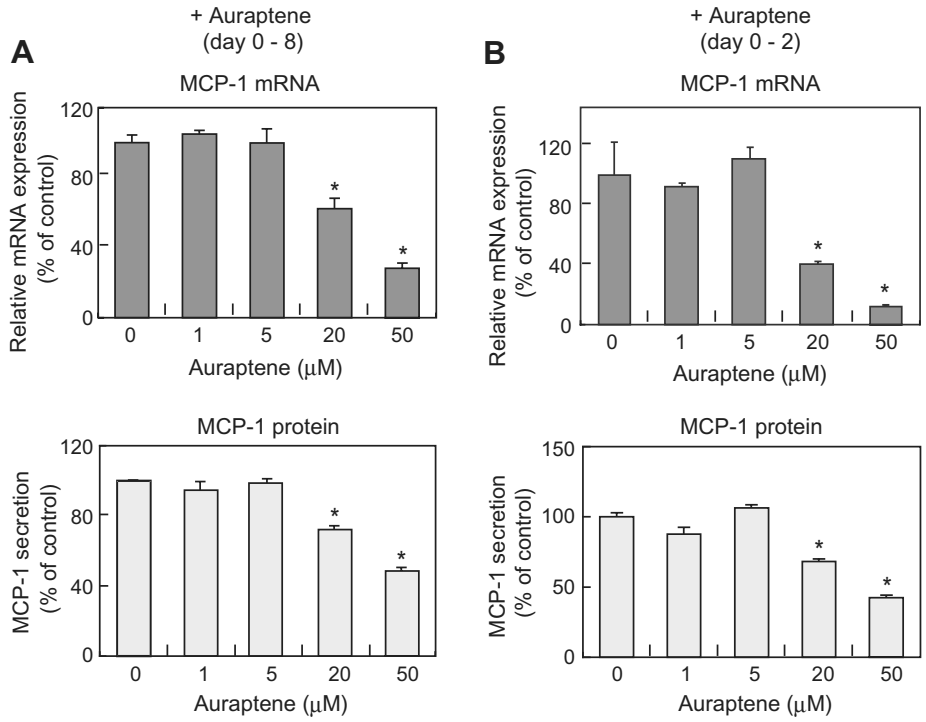

Fig. 3. The mRNA and protein expression levels of MCP-1 are inhibited by auraptene in 3T3-L1 adipocytes. 3T3-L1 cells were treated with 1, 5, 20, and 50 μM auraptene throughout a promotion and/or an induction period of differentiation. The mRNA expression and protein expression levels of MCP-1 with auraptene addition throughout both an induction and a promotion period of differentiation are shown in (A). Similar effects of auraptene during only differentiation induction are shown in (B). mRNAs were prepared 8 days after differentiation induction. MCP-1 secretion level was measured using aliquots collected from differentiated 3T3-L1 adipocyte culture medium 8 days after differentiation induction. The expression level of a vehicle control in each experiment was set at 100%, and relative mRNA or protein expression level was presented as fold induction relative to that of the vehicle control. The values are the means ± SEM of 3–4 tests. $^{*}p < 0.05$ compared with vehicle controls.

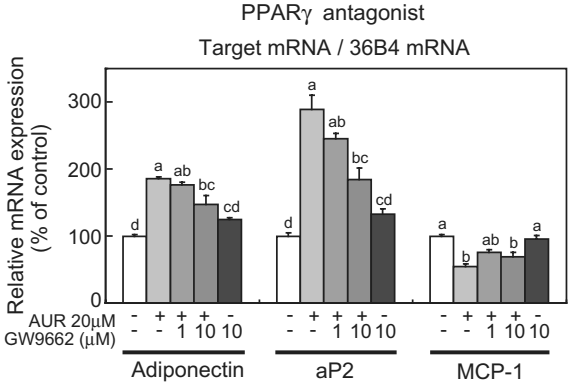

Fig. 4. Effects of auraptene on the expressions of PPAR-target genes are cancelled by the addition of PPAR antagonists in 3T3-L1 adipocytes. 3T3-L1 cells were treated with 20 μM auraptene and GW9662 at different concentrations during only differentiation induction. mRNAs were prepared 8 days after differentiation induction. The expression level of a vehicle control in each experiment was set at 100%, and relative expression level was presented as fold induction relative to that of the vehicle control. The values are the means ± SEM of 3–4 tests. Columns with different letters are significantly different ($p < 0.05$).

tion as ligands of PPARs and improve functions of adipocytes, macrophages and hepatocytes by increasing the expression levels of genes associated with glucose/lipid metabolism and inflammation [19,25]. Furthermore, isoprenols from herbal medicines act as dual agonists of both PPARα and PPARγ [18]. Taking these into consideration, we focused on natural compounds, particularly, citrus fruit compounds and determined whether these compounds actually affect PPARα and/or PPARγ activations. As results, among many citrus fruit compounds, auraptene dose-dependently induced PPARγ and PPARα activations using the luciferase ligand assay system. Furthermore, auraptene induced PPARγ target gene expression like LPL (data not shown) and aP2 at mRNA level during differentiation of 3T3-L1 adipocytes. This indicates that auraptene acts as a PPARγ ligand in intact cells. This is confirmed by the fact that effects of auraptene on regulation of adiponectin and MCP-1, PPARγ target genes, disappeared by the addition of GW9662, a PPARγ antagonist. In addition, several PPARα target genes like those encoding human liver carnitine palmitoyltransferase-1, acyl-CoA synthetase, and acyl-CoA oxidase, were also induced at the mRNA level in PPARα-expressing HepG2 hepatocytes by auraptene treatment (data not shown) suggesting that auraptene controls fatty acid β-oxidation. It is likely that auraptene regulates the expressions of both PPARα and PPARγ target genes as a dual agonist. However, it needs further investigations using other cells like hepatocytes and skeletal muscle cells to indicate physiological effects of auraptene via PPARα activation.

Until now, adipose tissues have been considered as an energy storage organ. However, it has been demonstrated that adipocytes actually express and secrete various bioactive factors called adipocytokine [2]. Among adipocytokines, we focused on adiponectin and MCP-1, which have opposite effects in obesity-related diseases. Adiponectin expression is exclusive to adipose tissues and the mRNA expression level of adiponectin is low in obese/diabetic mice and humans [26]. Furthermore, a functional PPAR-responsive element (PPRE) has been identified in human adiponectin promoters [27]. Therefore, the PPARγ agonist increases plasma adiponectin level by transcriptional induction in adipose tissues [26]. In our study, auraptene induced both mRNA and protein expression levels of adiponectin. Furthermore, auraptene treatment increased the ratio of the amount of HMW multimers to the total amount of adiponectin, an effect similar to that of pioglitazone. These results suggest that auraptene affects insulin resistance improvement in adipose tissues and functions as a PPARγ ligand that enhances adipocyte differentiation similarly to pioglitazone, although its extent of mediation might be weaker. Interestingly, the mRNA expression and secretion of adiponectin were induced more strongly when auraptene was added throughout both an induction and a promotion period of adipocyte differentiation than when it was added during only an induction period of the differentiation. It is suggested that the continuous intake of auraptene is more effective in terms of preventing and managing insulin resistance and other obesity-related diseases.

On the other hand, MCP-1 expression induces inflammatory reactions in adipose tissues and enhances insulin resistance and atherosclerosis [28]. Therefore, suppression of MCP-1 expression level in adipocytes is important for management of common diseases. It has been reported that thiazolidinediones inhibit MCP-1 expression in adipocytes [20]. In our study, the auraptene treatment throughout both an induction and a promotion of differentiation and only differentiation induction inhibited the mRNA and protein expression levels of MCP-1. These demonstrate that auraptene has anti-proinflammatory effects due to its ability to prevent the expression and secretion of MCP-1.

In conclusion, auraptene is a dual ligand for PPARα and PPARγ in luciferase ligand assays. The ligand activity of auraptene promotes adiponectin production and MCP-1 reduction in differentiated 3T3-L1 adipocytes through PPARγ activation. Our results suggest that the consumption of citrus fruits, which contain auraptene, contributes to the partial prevention and improvement of obesity and related metabolic syndrome.

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