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Effects of Citrus Auraptene (7-Geranyloxycoumarin) on Hepatic Lipid Metabolism *in Vitro* and *in Vivo*

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Recent reports have shown that citrus auraptene (7-geranyloxycoumarin) possesses valuable pharmacological properties, including anticarcinogenic, anti-inflammatory, antihelicobacter, antigenotoxic, and neuroprotective effects. In the present study, we investigated the effect of dietary auraptene on hepatic lipid metabolism both *in vitro* and *in vivo*. Results suggested that auraptene has the ability to normalize lipid abnormalities in HepG2 hepatocytes. After 4 weeks of auraptene feeding, abdominal white adipose tissue weight and hepatic triglyceride (TG) levels were dose-dependently lowered in Otsuka Long-Evans Tokushima fatty (OLETF) rats. The activities of carnitine palmitoyltransferase, a key enzyme in mitochondrial fatty acid β-oxidation, and peroxisomal β-oxidation were markedly and dose-dependently enhanced in OLETF rat livers by auraptene feeding. Additionally, hepatic expression of acyl-CoA oxidase, the initial enzyme of the peroxisomal β-oxidation system, was significantly and dose-dependently enhanced by auraptene administration. These results suggest that auraptene administration alleviates obesity and hepatic TG accumulation in part through lipolysis enhancement in the livers of obese OLETF rats.

KEYWORDS: Auraptene; HepG2 cells; OLETF rats; hepatic lipid metabolism

INTRODUCTION

In industrialized countries, lifestyle-related diseases, such as hyperlipidemia, arteriosclerosis, diabetes mellitus, and hypertension, are widespread and increasingly prevalent, thus, contributing to increases in cardiovascular morbidity and mortality (*1*, *2*). Accompanied by the rapid increase in the number of elderly people, this becomes important not only medically but also socioeconomically. A clustering of metabolic disorders in an individual, defined as metabolic syndrome, is known to increase cardiovascular morbidity and mortality. Although the pathogenesis of metabolic syndrome is complicated and the precise details of its underlying mechanisms are not known, lipid abnormality is now proposed as a feature of metabolic syndrome along with insulin resistance (*1*−*3*). Many studies suggested that natural compounds, such as phytochemicals in fruits and vegetables, can be important modulators in terms of the risks associated with this syndrome (*4*, *5*).

Citrus plants contain a number of phytochemicals, such as monoterpenes, limonoids, flavonoids, and coumarins (*6*). Auraptene (7-geranyloxycoumarin, **Figure 1**) is a simple coumarin that has a geranyloxyl side chain at C-7. Ogawa et al. evaluated auraptene content in 77 *Citrus* species, 5 *Fortunella* species, 1 *Poncirus* species, 27 interspecific *Citrus* hybirds, and 51 intergeneric hybrids between *Citrus* and *Poncirus* and detected large quantities in both the peel (up to 16.571 mg/g) and the juice sac (up to 10.321 mg/g) (*7*). They also reported that *Citrus* fruit products, such as grapefruit juice and marmalade, retain 0.11−0.38 mg/100 g of auraptene (*7*). It has been reported that auraptene possesses valuable pharmacological properties, including anticarcinogenic, anti-inflammatory, antihelicobacter, antigenotoxic, and neuroprotective effects (*8*−*15*). The effects of auraptene on lipid metabolism, however, have not been fully evaluated. Previously, Kuroyanagi et al. reported that auraptene acts as agonist for peroxisome proliferator-activated receptors (PPARs) in adipocytes (*16*), and Takahashi et al. showed that auraptene regulates gene expression as a PPARα agonist in hepatocytes (*17*).

In the present study, we investigated the effect of dietary auraptene on hepatic lipid metabolism both *in vitro* and *in vivo*. We used human hepatoma HepG2 cells, the most suitable and accessible human-derived cells that retain many of the biochemical functions of human liver parenchymal cells (*18*), for the *in vitro* study. For the *in vivo* study, Otsuka Long-Evans Tokushima fatty (OLETF) rats, which develop a syndrome with multiple metabolic and hormonal disorders that shares many features of human obesity, were used. OLETF rats have hyperphagia, owing to the lack of cholecystokinin receptors; as a result, they become obese and develop hyperlipidemia, fatty liver, and diabetes (*19*−*22*).

MATERIALS AND METHODS

Cell Culture. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 100 units/mL penicillin and 100 μg/mL

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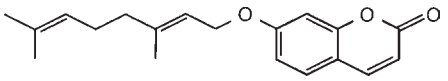

Figure 1. Chemical structure of auraptene.

Table 1. Effect of Auraptene on Lipid Metabolism in HepG2 Cells[a]

	Cont	FA	FA + AUR
apoB100 secretion (μg/mg of protein)	2.04 ± 0.11 a	2.13 ± 0.14 a	1.59 ± 0.14 b
TG synthesis (10^{-3} cpm/mg of protein)	206 ± 7 a	259 ± 10 b	266 ± 3 b

[a] Cont, control group; FA, fatty acid treatment group; FA + AUR, fatty acid plus auraptene treatment group. Values are expressed as means ± SE of five samples. Different letters show significant difference at $p < 0.05$.

streptomycin and supplemented with 10% fetal bovine serum at 37 °C in a humidified atmosphere of 5% CO_2. At approximately 70−80% confluence, the cells were preincubated with 1% bovine serum albumin (BSA)−DMEM with 0.5 mM oleic acid for 24 h. The fatty acid−BSA complex was prepared as described by Van Harken et al. (*23*). The cellular protein concentration was determined using the method by Lowry et al. (*24*) or the bicinchoninic acid method (*25*).

Measurement of Apolipoprotein B100 (apoB100) Secretion. To evaluate the effect of auraptene on apoB100 secretion, HepG2 cells were further incubated with experimental media [control group (Cont), 1% BSA−DMEM; fatty acid treatment group (FA), 1% BSA−DMEM with 0.5 mM oleic acid; and fatty acid plus aurapene treatment group (FA + AUR), 1% BSA−DMEM with 0.5 mM oleic acid plus 50 μM auraptene] for 24 h. At the end of the experiment, the media were harvested for the measurement of apoB100 levels and the cells were used for determination of cellular protein levels. apoB100 levels in the culture media were quantitated using the apoB Microwell enzyme-linked immunosorbent assay (ELISA) kit (AlerCHEK, Portland, ME).

Measurement of Cellular Triglyceride (TG) Synthesis. To evaluate the effect of auraptene on cellular TG synthesis, HepG2 cells were incubated with experimental medium [Cont, 1% BSA−DMEM; FA, 1% BSA−DMEM with 0.5 mM oleic acid; and FA + AUR, 1% BSA−DMEM with 0.5 mM oleic acid plus 50 μM auraptene] containing 18.5 KBq [1-^{14}C] acetic acid sodium salt ([1-^{14}C] acetate) (American Radiolabeled Chemicals, St. Louis, MO) for 24 h. After incubation, cells were washed once and collected in 2 mL of phosphate-buffered saline (PBS) using a rubber policeman. Cells were thawed and homogenized with a sonicator (Sonifier 250TM, Branson Ultrasonic Co., CT) before analysis. Total lipids in the cells were extracted and purified by the method of Bligh and Dyer (*26*) and then measured by a liquid scintillation counter (Wallic System 1410, Pharmacia, Uppsala, Sweden). The lipids were fractionated using thin-layer chromatography (TLC) in a solvent mixture of petroleum ether/diethyl ether/acetate (82:18:1, v/v/v). After separation with TLC, the radioactivities of the lipid fractions were measured with a bioimaging analyzer (BAS1000, Fuji Photo Film, Kanagawa, Japan).

Animals and Diets. All aspects of the animal experiment were conducted according to the guidelines provided by the ethical committee of experimental animal care at Saga University. Male OLETF rats aged 4 weeks were provided by Tokushima Research Institute (Otsuka Phamaceutical, Tokushima, Japan). The rats were housed individually in metal cages in a temperature-controlled room (24 °C) under a 12 h light/dark cycle. After a 1 week adaptation period, the rats were assigned to three groups (six rats each) and were fed one of the following three diets: (i) a semi-synthetic diet containing 20 wt % casein, 7 wt % corn oil, 15 wt % cornstarch, 1 wt % vitamin mixture (AIN-76), 3.5 wt % mineral mixture (AIN-76), 0.3 wt % D,L-methionine, 0.2 wt % choline bitartrate, 5 wt % cellulose, and 45 wt % sucrose (control group); (ii) a semi-synthetic diet supplemented with 0.05 wt % auraptene at the expense of sucrose (low-auraptene group, LA); and (iii) a semi-synthetic diet supplemented with 0.1 wt % auraptene at the expense of sucrose (high-auraptene group, HA). The basal semi-synthetic diets were prepared according to recommendations of the American Institute of Nutrition (AIN-76) (*27*). Auraptene was provided by Arkray, Inc. (Kyoto, Japan). The compositions of the semi-synthetic diets are provided in **Table 2**. Rats were pair-fed the assigned diets for 4 weeks.

Table 2. Composition of Experimental Diets[a]

ingredients	control (g/kg)	LA (g/kg)	HA (g/kg)
casein	200	200	200
corn starch	150	150	150
cellulose	50	50	50
mineral mixture (AIN 76)	35	35	35
vitamin mixture (AIN 76)	10	10	10
D,L-methionine	3	3	3
choline bitartrate	2	2	2
corn oil	70	70	70
auraptene	0	0.5	1
sucrose	480	479.5	479

[a] LA, low-auraptene group; HA, high-auraptene group.

Measurement of Body Fat and TG Levels in Serum and Liver. All rats were killed by aortic exsanguination under diethyl ether anesthesia. Liver and abdominal (perirenal, epididymal, and omental) white adipose tissues (WATs) were also excised for analysis. Serum was separated from the blood, and serum TG levels were measured using a commercial enzyme assay kit (Wako Pure Chemicals, Tokyo, Japan). Hepatic lipid was extracted according to the method by Folch et al. (*28*), and the TG concentration was measured by the method by Fletcher (*29*).

Preparation of Hepatic Subcellular Fractions. A piece of liver was homogenized in 6 volumes of a 0.25 M sucrose solution containing 1 mM ethylenediaminetetraacetic acid (EDTA) in a 10 mM Tris-HCl buffer (pH 7.4). After the nuclei fraction was precipitated, the supernatant was centrifuged at 10000*g* for 10 min at 4 °C to obtain mitochondrial fractions. The resulting supernatant was recentrifuged at 125000*g* for 60 min to precipitate microsomes, and the remaining supernatant was used as the cytosol fraction. The protein concentration was determined according to the method by Lowry et al. (*24*), with BSA used as the standard.

Assays of Hepatic Enzyme Activity. The enzyme activities of phosphatidate phosphohydrolase (PAP) (*30*), fatty acid synthase (FAS) (*31*), malic enzyme (*32*), glucose 6-phosphate dehydrogenase (G6PDH) (*33*), carnitine palmitoyltransferase (CPT) (*34*), and peroxisomal β-oxidation (*35*) were determined as described elsewhere.

Analysis of mRNA Expression. Total RNA was extracted from 50 mg of liver, using a RNeasy lipid tissue mini kit (Qiagen, Tokyo, Japan). A TaqMan Universal PCR master mix [Applied Biosystems, Tokyo, Japan; assay-on-demand, gene expression products: Rn00595644_m1 for acyl-CoA oxidase (ACO) 2, Rn00566193_m1 for PPARα, and Hs99999901_s1 for 18S RNA, Applied Biosystems] was used for quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis of ACO, PPARα, and 18S RNA expression in the liver. The amplification was performed with a real-time PCR system (ABI Prism 7000 sequence detection system, Applied Biosystems). Results were quantified with a comparative method and expressed as a relative value after normalization to the 18S RNA expression.

Statistical Analysis. All values are expressed as means ± standard error (SE). Data were analyzed by one-way analysis of variation (ANOVA), and all differences were inspected by the Tukey−Kramer posthoc test (Kaleida Graph, Synergy Software, Reading, PA). Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

The liver is the pivotal organ concerned with lipid metabolism and apoB100-containing lipoprotein assembly and secretion. It is known that the blood apoB100 level is positively correlated with the incidence of coronary heart disease and that enhanced secretion of apoB100 by the liver is a biomarker of hepatic lipid abnormality (*36*−*40*). Therefore, dietary components that control the rate of apoB100 secretion by the liver are of great interest (*41*−*44*). In the first part of the current study, we evaluated the effect of auraptene treatment on apoB100 secretion from HepG2 cells. In comparison to the control group, apoB100 levels tended to increase under fatty acid treatment, but auraptene induced a 25% reduction, despite containing the same concentration of fatty acid in the media (**Table 1**). apoB100, a 556 kDa hydrophobic

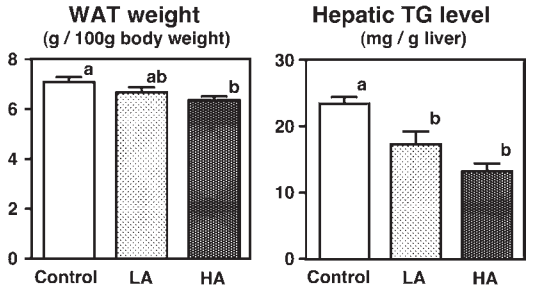

Figure 2. Effect of dietary auraptene on the abdominal WAT weight and hepatic TG level in OLETF rats. Values are expressed as means ± SE of six rats. See **Table 2** for composition of the diets. LA, low-auraptene group; HA, high-auraptene group. Different letters show significant difference at $p < 0.05$.

protein, is synthesized in the endoplasmic reticulum (*45*). After synthesis, apoB100 is stabilized by binding with lipids and then assembled and secreted as an apoB100-containing lipoprotein, such as very low-density lipoprotein (*45*). Because TG availability is a major factor in the regulation of assembly and secretion of apoB100-containing lipoprotein (*46*), we next examined the effect of auraptene on cellular TG synthesis in HepG2 cells. Incorporation of [1-^{14}C] acetate into the cellular TG fraction is shown in **Table 1**. Fatty acid treatment resulted in an increase in [1-^{14}C] acetate incorporation into newly synthesized cellular TG, which indicates enhanced TG synthesis, but this increase was not altered by auraptene treatment. The results from this study suggest that auraptene has some ability to normalize lipid abnormality in hepatocytes but that this ability does not occur through the suppression of hepatic TG synthesis. Previously, Takahashi et al. reported that auraptene regulates gene expression as an agonist of PPARα, a master regulator of lipolytic gene expression, in HepG2 hepatocytes (*17*). Therefore, these results suggest that auraptene may improve hepatic lipid abnormalities through the enhancement of lipolysis, even though auraptene does not suppress lipogenesis. In addition, auraptene absorption and metabolism were previously investigated, and stable localization of auraptene was observed in rat livers after a single gastric administration (*47*, *48*). Together, these findings led us to explore the effect of auraptene on lipid metabolism *in vivo* in the second part of the current study.

Obesity is defined as an increased mass of adipose tissue, and its prevalence and severity are markedly increasing in westernized countries. Although the pathogenesis of lifestyle-related diseases is complicated and the precise mechanisms have not been elucidated, obesity has emerged as one of the major cardiovascular risk factors according to epidemiologic studies (*1*−*3*). In the second part of the current study, we evaluated the effect of auraptene treatment on lipid metabolism in obese OLETF rats. After a 4 week feeding period, the amount of food intake (control, 659 ± 5 g; LA, 657 ± 5 g; HA, 654 ± 13 g), final body weight (control, 352 ± 4 g; LA, 355 ± 4 g; HA, 354 ± 4 g), and serum TG level (control, 156 ± 14 mg/dL; LA, 129 ± 11 mg/dL; HA, 141 ± 11 mg/dL) were not significantly altered by auraptene administration in OLETF rats. In contrast, abdominal WAT weight and hepatic TG levels were dose-dependently lowered by auraptene administration in OLETF rats (**Figure 2**). In agreement with the results from the *in vitro* study, these data suggest that auraptene administration can alleviate lipid abnormalities in obese OLETF rats.

To examine further the effect of dietary auraptene on the liver, hepatic enzymes related to TG metabolism were analyzed (**Table 3**). Activities of PAP and FAS, key enzymes in the regulation of TG and FA *de novo* synthesis, did not differ between groups. Additionally, the activities of G6PDH and malic enzyme, which provide

Table 3. Effect of Dietary Auraptene on Activities of Hepatic TG Metabolism-Related Enzymes in OLETF Rats[a]

	control (nmol min^{-1} mg of protein^{-1})	LA (nmol min^{-1} mg of protein^{-1})	HA (nmol min^{-1} mg of protein^{-1})
PAP	14.1 ± 0.5	13.4 ± 0.5	14.2 ± 0.5
FAS	11.8 ± 0.7	10.3 ± 0.11	10.8 ± 0.7
G6PDH	92.1 ± 8.4	83.2 ± 5.0	78.7 ± 4.0
malic enzyme	117 ± 8	123 ± 10	139 ± 8
CPT	3.69 ± 0.09 a	4.03 ± 0.17 a	4.46 ± 0.15 b
peroxisomal β-oxidation	4.37 ± 0.18 a	5.66 ± 0.45 b	6.44 ± 0.27 b

[a] LA, low-auraptene group; HA, high-auraptene group. Values are expressed as means ± SE of six rats. Different letters show significant difference at $p < 0.05$.

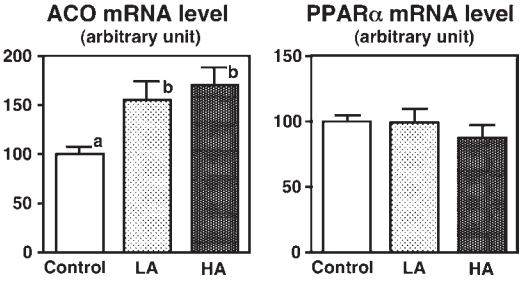

Figure 3. Effect of dietary auraptene on hepatic mRNA levels of ACO and PPARα in OLETF rats. Values are expressed as means ± SE of six rats. See **Table 2** for composition of the diets. LA, low-auraptene group; HA, high-auraptene group. Different letters show significant difference at $p < 0.05$.

nicotinamide adenine dinucleotide phosphate (NADPH) required for fatty acid synthesis, were not significantly altered by dietary auraptene. These data are consistent with the results from the *in vitro* study and suggest that auraptene administration does not alter hepatic lipogenesis in obese OLETF rats. However, the activity of CPT, a key enzyme of mitochondrial fatty acid β-oxidation, and the activity of peroxisomal β-oxidation were markedly and dose-dependently enhanced by auraptene feeding in OLETF rat livers. These results suggest that auraptene administration alleviates hepatic TG accumulation through lipolysis enhancement in the livers of obese OLETF rats.

To gain insight into the effect of dietary auraptene on the levels of mRNA related to lipid metabolism, we analyzed hepatic lipolytic gene expressions by quantitative real-time RT-PCR. In the present study, hepatic expression of ACO, the initial enzyme of the peroxisomal β-oxidation system, was significantly and dose-dependently enhanced by auraptene administration in OLETF rats (**Figure 3**), in accordance with a previous report showing that treatment with auraptene induced upregulation of ACO expression in HepG2 hepatocytes (*17*). There was, however, no significant difference in mRNA levels of PPARα among groups (**Figure 3**). Given the fact that auraptene regulates gene expressions as PPAR agonists *in vitro* (*16*, *17*), dietary auraptene might have enhanced lipolytic gene expression through the activation of PPARα as a ligand in the OLETF rats.

In conclusion, our *in vitro* and *in vivo* studies showed that auraptene has the potential to alleviate lipid abnormarities. Additionally, auraptene administration had an anti-obesity effect in obese OLETF rats. These effects were, at least in part, attributable to enhanced lipolysis in the liver. Further studies are necessary to evaluate the effect of dietary auraptene on lipid abnormalities in several animal disease models and to determine the lowest effective concentration.

ABBREVIATIONS USED

apoB100, apolipoprotein B100; ACO, acyl-CoA oxidase; BSA, bovine serum albumin; CPT, carnitine palmitoyltransferase;

DMEM, Dulbecco's modified Eagle's medium; FAS, fatty acid synthase; G6PDH, glucose 6-phosphate dehydrogenase; NADPH, nicotinamide adenine dinucleotide phosphate; OLETF, Otsuka Long-Evans Tokushima fatty; PAP, phosphatidate phosphohydrolase; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcription-polymerase chain reaction; TG, triglyceride; TLC, thin-layer chromatography; WAT, white adipose tissue.

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