Coffee Inhibits Adipocyte Differentiation *via* Inactivation of PPAR γ

Ryohei Aoyagi, Megumi Funakoshi-Tago, Yosuke Fujiwara, and Hiroomi Tamura*

Graduate School of Pharmaceutical Sciences, Keio University; 1–5–30 Shibakoen, Minato-ku, Tokyo 105–8512, Japan.

Received May 21, 2014; accepted August 19, 2014; advance publication released online September 11, 2014

Recent epidemiological studies showed that coffee consumption is associated with a lower risk of type 2 diabetes, presumably due to suppression of excess fat accumulation in adipocytes. However, the mechanism underlying the effect of coffee on adipocyte differentiation has not been well documented. To elucidate the mechanism, we investigated the effect of coffee on the differentiation of mouse preadipocyte 3T3-L1 cells. Coffee reduced the accumulation of lipids during adipocytic differentiation of 3T3-L1 cells. At 5% coffee, the accumulation of lipids decreased to half that of the control. Coffee also inhibited the expression of the peroxisome proliferator-activated receptor γ (PPAR γ), a transcription factor controlling the differentiation of adipocytes. Furthermore, coffee reduced the expression of other differentiation marker genes, aP2, adiponectin, CCAAT-enhancer-binding protein α (C/EBP α), glucose transporter 4 (GLUT4), and lipoprotein lipase (LPL), during adipocyte differentiation. Major bioactive constituents in coffee extracts, such as caffeine, trigonelline, chlorogenic acid, and caffeic acid, showed no effect on PPAR γ gene expression. The inhibitory activity was produced by the roasting of the coffee beans.

Key words 3T3-L1; adipocyte; coffee; differentiation; obesity; peroxisome proliferator-activated receptor γ (PPAR γ)

Obesity is associated with various chronic diseases, such as cardiovascular diseases, type 2 diabetes mellitus (T2DM), certain types of cancer, osteoarthritis, and asthma. Obesity is caused by excessive fat accumulation in the body. Adipocytes play a central role in maintaining lipid homeostasis and energy balance by storing triacylglycerols and releasing free fatty acids in response to changes in energy demands. Adipose tissue produces several proteins (adipocytokines), such as adiponectin and interleukin-6 (IL-6), that modulate insulin sensitivity and play an important role in the pathogenesis of insulin resistance and T2DM.¹⁾ Because increased adipose tissue mass can result from both hyperplasia and hypertrophy of adipocytes, adipocyte proliferation and differentiation have been the focus of obesity research.^{1,2)}

Coffee is a beverage consumed world-wide and a moderate intake of coffee has been linked to reduced risk of chronic diseases.³⁾ Coffee consumption has been associated with a lower risk of T2DM in prospective cohort studies.^{4,5)} Although weight loss attenuation of subclinical inflammation, reduction of oxidative stress, and ameliorating effects on liver function might be potential mechanisms underlying the risk association, the precise mechanisms of action remain unknown.⁶⁾ Several studies showed that coffee intake improves insulin sensitivity and glucose tolerance in diabetic mice by reducing adipocytokines in adipose tissue and fatty acid synthesis in the liver, both of which contributed to the anti-diabetic effect of coffee.^{7,8)} To elucidate further the mechanism underlying the effect of coffee on adipocyte differentiation, we investigated the effects of coffee on the differentiation of preadipocyte 3T3-L1 cells.

MATERIALS AND METHODS

Materials Mouse 3T3-L1 cells were purchased from DS Pharma Biomedical Co., Ltd. (Osaka, Japan). Dexamethasone

(DEX), 3-isobutyl-1-methylxanthine (IBMX), and insulin were purchased from Sigma (St. Louis, MO, U.S.A.).

Preparation of Coffee Extract Roasted coffee powder (Columbia Arabica) was obtained from Starbucks Coffee Japan (Tokyo, Japan). Coffee extracts were prepared by a common method, in which 8 g of powder was extracted with 140 mL hot water (95°C). The extract was then filtered, divided into small aliquots and stored at –80°C until used. Undiluted extract, with a dry weight of 8.4 mg/mL, was assigned a concentration of 100% (v/v). For roasting experiments, Columbian Arabica coffee green beans were purchased from Nakaya Coffee (Tokyo, Japan). Coffee green beans were roasted at 200–220°C for up to 20 min. Coffee extracts were prepared and stored as described above.

Cell Culture 3T3-L1 cells were cultured as described by Hemati *et al.*⁹⁾ Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum until the cells became confluent. To induce differentiation, 2-d post-confluence 3T3-L1 preadipocytes (D0) were stimulated with 0.5 mM IBMX, 1 μ M DEX, and 10 μ g/mL insulin in DMEM containing 10% fetal bovine serum (FBS) (MDI differentiation medium) for 2 d (D2). Cells were then maintained in a 10% FBS/DMEM medium with 10 μ g/mL insulin for another 2 d (D4) and then cultured in 10% FBS/DMEM medium for an additional 2 d (D6), at which time more than 90% of cells had become mature adipocytes with lipid-filled droplets. All media contained penicillin (100 U/mL) and streptomycin (100 μ g/mL). The cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Analysis of Gene Expression Total RNA was isolated from the cultured cells by guanidinium thiocyanate–phenol–chloroform extraction method using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's protocol. First strand cDNA was synthesized from 1 μ g of total RNA using 1 unit of Revertra Ace reverse transcriptase with random primers, according to the manufacturer's protocol (Toyobo Life Science, Inc., Tokyo, Japan). The primers used for the

The authors declare no conflict of interest.

*To whom correspondence should be addressed. e-mail: tamura-hr@pha.keio.ac.jp

amplification of cDNAs were designed based on the published sequences as follows (forward and reverse, 5' to 3'): *ppary*, AACTCTGGGAGATTCTCTGTTGA and TGGTAATTTCTTGTAAGTGTCTCATA; *c/ebpa*, AGGTGCTGGAGTGAACAGT and CAGCCTAGAGATCCAGCGAC; *ap2*, AGCATCATAACCCTAGATGGCG and CATAACACATTCCACCACCAGC; *lpl*, GCA GACGCGGGAAGAGATT and TGGCAGTTAGACACA GAGTCTGCTA; *adiponectin*, CTGGCCACTTTCCTCTCATTTTC and GGCATGACTGGG CAGGATTA; *pref-1*, TGTCAATGGAGTCTGCAAGG and CAAGCCCGAACGTCTATTTC; *glut4*, AGGTGCTGGAGTGAACAGT and CAGCCTAGAGATCCAGCGAC; *gr*, GTGGAAGGACAGCACAAATTACCT and CGCGGCAGGAAC TATTGTTT. Quantitative real-time polymerase chain reaction (PCR) was performed in an ABI 7300 thermal cycler using a SYBR green PCR core reagent kit (Applied Biosystems Inc., Warrington, U.K.). Samples were denatured at 94°C for 10min, and cDNA products were amplified using 40 cycles of denaturation at 94°C for 30s followed by annealing and extension at 60°C for 60s. The amount of target gene relative to a reference gene (18S RNA) was quantified using the cycle threshold (Ct).

Western Blot Analysis Cells were harvested in ice-cold phosphate buffered saline (PBS) and lysed in Nonidet P-40 lysis buffer (50mM Tris-HCl, pH 8.0, 120mM NaCl, 1mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.5% Nonidet P-40, 10mM β -glycerophosphate, 2.5mM NaF, 0.1mM Na_3VO_4) supplemented with protease inhibitors. Protein concentrations were measured using Bradford reagents (Pierce; Rockford,

IL, U.S.A.). Protein samples (15 μ g each) were resolved by polyacrylamide-sodium dodecyl sulfate (SDS) gel electrophoresis and then electrotransferred to a polyvinylidene difluoride membrane (Millipore; Billerica, MA, U.S.A.). The membrane was incubated overnight at 4°C in blocking buffer consisting of TBS (20mM Tris-HCl (pH 7.6), 137mM NaCl) containing 1% skim milk. After one wash with 0.1% Tween 20 in TBS (TBS-Tween), the membrane was incubated for 1h at 37°C with anti-PPAR γ (Santa Cruz Biotechnology Inc., Santa Cruz, CA, U.S.A.) diluted in Can Get Signal Immunoreaction Enhancer Solution (Toyobo, Tokyo, Japan). After three additional washes with TBS-Tween, the membrane was incubated for 1h at 37°C with anti-rabbit immunoglobulin G (IgG) linked to horseradish peroxidase (GE Healthcare Bio-Sciences; Little Chalfont, U.K.). Immunoreactive proteins were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce; Rockford, IL, U.S.A.) and detected with a VersaDoc 5000 and quantified by Quantity One Software (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Oil Red O Staining Coffee (1.25, 2.5 and 5%) or a vehicle was added to the MDI differentiation medium at D0 and cells were cultured for 6d. On D6, cells were fixed in fresh 10% formalin (pH 7.4) and stained with Oil Red O dye (Sigma, St. Louis, MO, U.S.A.). Oil Red O-stained intracellular lipids were then extracted by isopropanol and quantified by measuring the optical density at 490nm.

Cytotoxicity Assay Cytotoxic effects on cell metabolism were assayed using a tetrazolium family compound (WST-1). Preadipocytic 3T3-L1 cells (1×10^4) were seeded in 96-well

Fig. 1. Effects of Coffee on the Differentiation of 3T3-L1 Adipocytes

(A) Preadipocytic 3T3-L1 cells (1×10^4) were seeded in 96-well plates and grown for 2d at 37°C. After incubation with coffee at various concentrations for 24h, cytotoxicity was measured by WST assay. (B) 3T3-L1 adipocytes that were untreated (control, 0%), or were treated with 1.25, 2.5, or 5% (v/v) coffee were stained by Oil Red O on D6 after induction of differentiation and photographed. (C) Oil Red O-stained intracellular lipids were then extracted using isopropanol and quantified by measuring the optical density at 490nm (C). Asterisks denote the significance levels when compared to the controls. * $p < 0.05$, ** $p < 0.01$ ($n = 3$).

plates and grown for 2 d at 37°C. After incubation with coffee at various concentrations for 24 h, the cells were washed with PBS and subjected to the WST assay according to the manufacturer's protocol (Dojin Chemicals, Tokyo, Japan). After a 2 h incubation with the WST-1 reagent, the absorbance was measured at 450 nm.

Statistical Analysis Student's *t*-test was used for statistical analysis and $p < 0.05$ was considered significant.

RESULTS

Effects of Coffee on Adipocyte Differentiation To investigate whether coffee affects adipocyte differentiation, we measured the effect of coffee on lipid accumulation during the differentiation of mouse preadipocyte 3T3-L1 cells. Cells were induced to differentiate to adipocytes with the addition of IBMX, DEX, and insulin. Simultaneously, coffee was added to the medium, and the cells were cultured until day 6 with medium changes at day 2 and day 4. No cytotoxicity towards preadipocytic 3T3-L1 cells was detected up to 5% coffee, as judged by the WST assay (Fig. 1A). Cells were stained with Oil Red O and photographed (Fig. 1B). Accumulation of lipid in the cells was reduced by increasing the coffee concentration. This result was further supported by the quantitative analysis shown in Fig. 1C.

Coffee Reduced the Induction of Peroxisome Proliferator-Activated Receptor γ (PPAR γ) Gene Expression upon Adipocyte Differentiation of Preadipocyte 3T3 L1 Cells

Fig. 2. Coffee Reduces PPAR γ Gene Expression in 3T3-L1 Adipocytes

(A) Expression of the PPAR γ gene was monitored by quantitative RT-PCR during adipocytic differentiation of 3T3-L1 cells. Light gray bars, control (no coffee); dark gray bars, treated with 2.5% (v/v) coffee. (B) Cells were treated with MDI medium to induce differentiation in the absence or presence of coffee at the indicated concentrations (v/v). On D6, total RNA was extracted and subjected to quantitative RT-PCR analyses for PPAR γ gene expression. (C) On D6, whole cell lysates were prepared and proteins (15 μ g each) were subjected to immunoblot analysis of PPAR γ as described in Materials and Methods. Immunoblotting of β -actin was used as a loading control. (D) Densitometric analysis was performed on the data in (C). The ratio of PPAR γ / β -actin was quantified and is expressed relative to control. Asterisks denote the significance levels when compared to the controls. * $p < 0.05$, $n = 3$.

It is well established that PPAR γ plays a critical role in adipocyte differentiation, most importantly to maintain the adipocyte phenotype.^{10,11)} To address the question of whether coffee-mediated inhibition of adipogenesis is associated with PPAR γ levels, we determined the expression level of PPAR γ in coffee-treated differentiating 3T3-L1 cells. The expression of PPAR γ was significantly reduced by D6, while no significant effect was observed until on D4 (Fig. 2A). The coffee-mediated reduction of PPAR γ gene expression on D6 was coffee-dose dependent (Fig. 2B). With 5% coffee, PPAR γ expression was reduced to 25% of the control level on D6. Western blot analysis also revealed that 2.5% coffee reduced the amount of PPAR γ protein in 3T3-L1 cells to 50% of the control level on D6, comparable to the gene expression levels (Fig. 2D).

Coffee Alters the Expression of Adipocyte Marker Genes during Differentiation PPAR γ is recognized as a master regulator of adipocyte differentiation and regulates the expression of adipocyte markers, such as aP2, adiponectin, glucose transporter 4 (GLUT4), and lipoprotein lipase (LPL). As shown in Fig. 3, coffee reduced the expression of the marker genes during adipocytic differentiation. By contrast, no effect was observed on the expression of non-marker genes, such as the glucocorticoid receptor (GR) and Pref-1 genes (Fig. 3).

Characterization of Active Coffee Compounds There are several major constituents in coffee extracts, which exhibit specific physiological activities. These include caffeine, chlorogenic acid, caffeic acid, and trigonelline. As shown in

Fig. 3. Effects of Coffee on the Expression of Differentiation-Related Marker Genes in 3T3-L1 Cells

Cells were treated with MDI to induce differentiation in the absence or presence of coffee at the indicated concentrations (v/v). On D6, total RNA was extracted and subjected to quantitative RT-PCR analyses for the differentiation-related genes aP2, adiponectin, C/EBPα, GLUT4, and LPL, and for two genes not related to adipocyte differentiation, GR and Pref-1. Asterisks denote the significance levels when compared to the controls. * $p < 0.05$, $n = 3$.

Fig. 4. Analysis of Coffee Extract Components Responsible for the Reduction of PPARγ Gene Expression during Adipogenesis of 3T3-L1 Cells

(A) Cells were treated with MDI to induce differentiation in the absence or presence of each major component (caffeine, trigonelline, chlorogenic acid, or caffeic acid) at 100 μM. On D6, total RNA was extracted and subjected to quantitative RT-PCR analyses for PPARγ gene expression. (B) Arabica coffee beans were roasted at 220°C for 5, 10, or 20 min and photographed. (C) The beans were pulverized and extracted with boiling water as described in Materials and Methods. Extracts were added to the culture medium at a concentration of 2.5% (v/v). The effects of the extracts on the expression of PPARγ was monitored as in (A). Asterisks denote the significance levels when compared to the controls. * $p < 0.05$, $n = 3$.

Fig. 4A, these compounds at 100 μ M did not show any effect on PPAR γ gene expression by D6 in the differentiated 3T3-L1 cells. In addition, no effect of these compounds was observed on lipid accumulation in 3T3-L1 cells by D6 (data not shown). The concentration (100 μ M) of each compound is roughly equivalent to that in 10–50% coffee.

To examine the possibility that active components are formed during the roasting process, we assayed the activity of extracts of green coffee beans that had undergone varying degrees of roasting before brewing. We extracted beans roasted for 0–20 min (Fig. 4B) and used the extracts in the gene expression assays. Increasing the degree of roasting resulted in greater reductions in the PPAR γ gene expression. Significant inhibitory activity was detected after the beans had been roasted for 20 min (Fig. 4C).

DISCUSSION

Recent epidemiological studies showed that coffee consumption is associated with a lower risk of type 2 diabetes, presumably due to the suppression of excess fat accumulation in adipocytes.⁴⁾ However, the mechanism underlying the effect of coffee on adipocyte differentiation has not yet been well documented. Adipocyte differentiation has been extensively studied using mouse 3T3-L1 preadipocytes, which differentiate into adipocytes in response to stimulation with a combination of IBMX, DEX, and insulin (MDI). On exposure to MDI differentiation medium, 3T3-L1 cells differentiate into adipocytes under the control of a transcription factor network.^{12,13)} Among these transcription factors, PPAR γ is thought to be a master regulator of adipogenesis. In this study, we demonstrated that coffee reduced the accumulation of lipids in the adipose differentiation of mouse 3T3-L1 cells (Fig. 1), and that the reduction was correlated with a decrease in PPAR γ gene expression (Fig. 2). C/EBP α gene expression, as well as other adipocyte marker genes (aP2, LPL, adiponectin, and GLUT4), was also downregulated during adipocyte differentiation in the coffee-treated cells (Fig. 3). Although the precise mechanism of the coffee-mediated reduction in PPAR γ gene expression is unclear at the moment, our preliminary data indicated that exposure to coffee at an early stage of adipose differentiation (<2 d) is necessary for the reduction of PPAR γ gene expression (Aoyagi *et al.*, unpublished data). Further investigations should be carried out to clarify the mechanism by which coffee affects adipose differentiation.

The component(s) in coffee responsible for the reduction in PPAR γ expression is unclear at the moment. It is not certain whether the active component(s) in coffee is absorbed and transferred to preadipocytes in the body, although studies showed that more than half of the major components in coffee such as caffeine, chlorogenic acid, caffeic acid, or trigonelline are rapidly absorbed in the stomach and small intestine and distributed to all tissues.³⁾ Our preliminary data showed that the inhibitory activity in coffee was extractable with ethyl acetate, in which the major constituents are recovered. However, these major components did not show any activity (Fig. 4A). It has been reported that caffeine suppresses the intracellular lipid accumulation of 3T3-L1 cells after complete differentiation.¹⁴⁾ A part of the anti-obesity effect of coffee may be attributed to caffeine in a different mechanism from our study.

The active component(s) seem likely to be generated by the

roasting process (Fig. 4B). After roasting for 20 min at 220°C, the inhibitory activity appeared. Many phenolic constituents and Maillard reaction products are known to form during the coffee roasting process and extractable in ethyl acetate.^{15,16)} These compounds are reported to possess various types of antioxidant and pro-oxidant activity, which often modulate antioxidant transcription factors such as nuclear factor κ B (NF- κ B) and nuclear factor-E₂-related factor 2 (Nrf2).^{17–19)} Indeed, we have observed coffee-induced NF- κ B-mediated activation of the BCRP gene in Caco-2 cells.²⁰⁾ Recently, several studies reported the involvement of Nrf2 in differential regulation of PPAR γ expression in other cell types, although in these cases Nrf2 activates PPAR γ expression. Furthermore, intersection of the PPAR γ and NF- κ B signaling pathways has been reported.^{21–23)}

A very recent study reported that coffee intake down-regulates the hepatic gene expression of PPAR γ in mice fed a high fat diet.²⁴⁾ The study showed that in addition to roasted coffee, unroasted green coffee also exhibited the inhibitory activity. The difference in roasting dependency for the activity between ours and their results might be explained by the tissue difference, hepatocytes and adipocytes, and/or the difference of experimental models, *in vivo* and *in vitro*. The active component(s) in roasted coffee beans need to be identified to elucidate the mechanism of the effect of coffee on adipocyte differentiation.

Acknowledgments We are grateful to Dr. K. Oka for his encouragement. This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan, and by a Grant from the MEXT—Supported Program for the Strategic Research Foundation at Private Universities.

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