Coffee Inhibits Adipocyte Differentiation via Inactivation of PPARγ

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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 (PPARγ), 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/EBPα), glucose transporter 4 (GLUT4), and lipoprotein lipase (LPL), during adipocytic differentiation. Major bioactive constituents in coffee extracts, such as caffeine, trigonelline, chlorogenic acid, and caffeic acid, showed no effect on PPARγ 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 (PPARγ)

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. 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.

Coffee is a beverage consumed world-wide and a moderate intake of coffee has been linked to reduced risk of chronic diseases. Coffee consumption has been associated with a lower risk of T2DM in prospective cohort studies. 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. 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. To elucidate further the mechanism underlying the effect of coffee on adipocytic 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 Naka 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. 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 μM DEX, and 10 mg/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 μg/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 μg/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 μg 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
amplification of cDNAs were designed based on the published sequences as follows (forward and reverse, 5' to 3'): **pparg**, AAC TCT GGG AGA TTC TCC TGT TGA and TGG TAA TTT CTT GTG AAG TGC TCA TA; **c/ebpα**, AGG TGC TGG AGT TGA CCA GT and CAG CCT AGA GAT CCA GCG AC; **ap2**, AGC ATC ATA ACC CTA GAT GCC G and CAT AACA CAC CAG C; **lpl**, GCA GAC GCG GGA AGA GAT T and TGG CAG TTA GAC ACA GAG TCT GCT A; **adiponectin**, CTG GCC ACT TTC TCC TCA TTT C and GGC ATG ACT GGC CAG GAT TA; **pref-1**, TGT CAA TGG AGT CTG CAA GG and CAA GCC CGA ACG TCT ATT TC; **glut4**, AGG TGC TGG AGT TGA CCA GT and CAG CCT AGA GAT CCA GCG AC; **gr**, GTG GAA GGA CAG CAC AAT TAC CT and CGC GGC AGG AAC TAT TGT TT. 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 10 min, and cDNA products were amplified using 40 cycles of denaturation at 94°C for 30 s followed by annealing and extension at 60°C for 60 s. 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 (50 mM Tris–HCl, pH 8.0, 120 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA) pH 8.0, 0.5% Nonidet P-40, 10 mM β-glycerophosphate, 2.5 mM NaF, 0.1 mM Na3VO4) supplemented with protease inhibitors. Protein concentrations were measured using Bradford reagents (Pierce; Rockford, IL, U.S.A.). Protein samples (15 µg each) were resolved by 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 10 min, and cDNA products were amplified using 40 cycles of denaturation at 94°C for 30 s followed by annealing and extension at 60°C for 60 s. The amount of target gene relative to a reference gene (18S RNA) was quantified using the cycle threshold (Ct).

**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 6 d. 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 490 nm.

**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
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 (PPARγ) Gene Expression upon Adipocyte Differentiation of Preadipocyte 3T3 L1 Cells**

It is well established that PPARγ plays a critical role in adipocyte differentiation, most importantly to maintain the adipocyte phenotype.

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

**Coffee Alters the Expression of Adipocyte Marker Genes during Differentiation**  PPARγ 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

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**Fig. 2. Coffee Reduces PPARγ Gene Expression in 3T3-L1 Adipocytes**

(A) Expression of the PPARγ 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 PPARγ gene expression (C) On D6, whole cell lysates were prepared and proteins (15 µg each) were subjected to immunoblot analysis of PPARγ 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 PPARγ/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.
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 µL. 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 µM did not show any effect on PPARγ 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 µM) 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 PPARγ 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. 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. Among these transcription factors, PPARγ 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 PPARγ gene expression (Fig. 2). C/EBPα gene expression, as well as other adipocyte marker genes (p2, 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 PPARγ 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 PPARα gene expression (Aoyagi et al., unpublished data). Further investigations should be carried out to clarify the mechanism by which coffee affects adipocyte differentiation.

The component(s) in coffee responsible for the reduction in PPARγ 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. Our preliminary data showed that the inhibitory activity in coffee was extractable with ethyl acetate. The concentration (0.1 M) of each compound is roughly equivalent to that in 10–50% coffee.

REFERENCES


