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Avicularin, a Plant Flavonoid, Suppresses Lipid Accumulation through Repression of C/EBPα-Activated GLUT4-Mediated Glucose Uptake in 3T3-L1 Cells

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ABSTRACT: Avicularin (quercetin-3-*O*-α-L-arabinofuranoside) is a plant flavonoid and a quercetin glycoside. In this study, we found that avicularin suppressed the accumulation of intracellular lipids through repression of glucose transporter 4 (GLUT4)-mediated glucose uptake in mouse adipocytic 3T3-L1 cells. Avicularin was highly purified (purity of more than at least 99%) from *Taxillus kaempferi* (DC.) Danser (Loranthaceae) by high-performance liquid chromatography, and its structure was determined by nuclear magnetic resonance and mass spectrometry. Avicularin decreased the intracellular triglyceride level along with a reduction in the expression of adipogenic genes such as peroxisome proliferator-activated receptor γ, CCAAT/enhancer-binding protein (C/EBP) α, and aP2 (fatty acid-binding protein 4). In contrast, avicularin did not affect the expression of lipogenic and lipolytic genes. Interestingly, the expression of the GLUT4 gene was significantly suppressed in an avicularin-concentration-dependent manner. Moreover, the binding of C/EBPα to the promoter region of the GLUT4 gene was repressed by adding avicularin to the medium in 3T3-L1 cells, as demonstrated by the results of a chromatin immunoprecipitation assay. These results indicate that avicularin inhibited the accumulation of the intracellular lipids by decreasing C/EBPα-activated GLUT4-mediated glucose uptake in adipocytes.

KEYWORDS: *avicularin, flavonoid, HPLC, GLUT4, C/EBPα, adipocytes*

INTRODUCTION

Adipocytes play an important role in energy homeostasis. Adipose tissue stores energy in the form of lipids and releases fatty acids in response to nutritional signals or energy insufficiency.[1] Adipose tissue, especially adipocytes, plays central roles in the development of obesity, which is characterized by an increase in the number or size of adipocytes or both. The accumulation of an excess amount of fat causes obesity, which is closely associated with various human diseases such as type 2 diabetes mellitus, hypertension, cardiovascular disease, and certain cancers.[2,3] Adipocytes are also known to be endocrine cells that secrete various hormones, called adipocytokines,[4−6] which affect the metabolism and function of many organs and tissues.[7,8]

Adipogenesis is regulated through a complex process including coordinated changes in hormone sensitivity and gene expression, whose regulation involves various transcription factors.[9,10] Three families of transcription factors, i.e., peroxisome proliferator-activated receptor (PPAR), CCAAT/enhancer-binding proteins (C/EBPs), and sterol regulatory-element binding proteins (SREBPs), are most important in the regulation of adipogenesis,[10] and they control the expression of a variety of genes involved in the maturation process of adipocytes. Therefore, suppression of this process is critical for achieving an antiobesity effect, and the search for agents that inhibit this process has been extensively undertaken.[11]

The insulin-responsive glucose transporter, GLUT, is expressed in heart, skeletal muscle, and adipose tissues, and is regulated at both transcriptional[12] and post-translational[13] levels. Glucose transport is the rate-limiting step in glucose uptake,[14] and its function is fully dependent upon the insulin-signaling that causes translocation of the glucose transporter from the cytosol to the cell membrane so that it can act as a transporter.[13] GLUT4 is a major glucose transporter in adipocytes, and changes in the GLUT4 level directly affect glucose homeostasis.[15] The incorporated glucose is then utilized for the synthesis of ATP and triglycerides. Uptake of an excess amount of glucose causes an increase in the intracellular lipid level. Thus, the regulation of glucose transport is important in the control of the levels of intracellular lipids.

Natural compounds that regulate adipogenesis have been extensively studied.[16,17] Flavonoids, a well-known group of natural compounds, are polyphenolic compounds present in vegetables, fruits, and certain beverages.[18] Flavonoids have a variety of biological activities, including inhibition of cell proliferation in cell cultures and induction of apoptosis, and additionally, they cause changes in the activity of certain intracellular enzymes and also function as antioxidants.[19−21] Avicularin, which is quercetin 3-*O*-α-L-arabinofuranoside (Figure 1A), is a quercetin derivative[22] and is known to be included in *Taxillus kaempferi*,[23] *Polygonum aviculare*,[24] and fruits such as apple[25] and mango.[26] However, its function and the regulatory mechanism of avicularin action toward adipocytes have never been identified. In this study, we highly purified this plant flavonoid by HPLC, confirmed its structure

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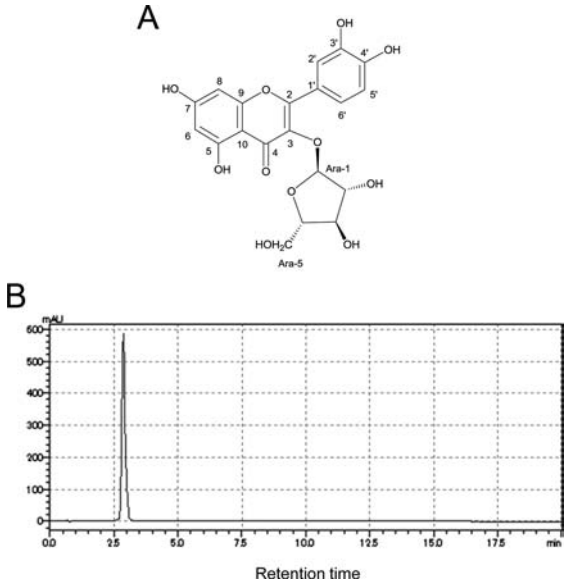

Figure 1. Purification of avicularin. (A) Chemical structure of avicularin. (B) Typical chromatogram of avicularin highly purified from *Taxillus kaempferi* by HPLC.

by nuclear magnetic resonance (NMR) and mass spectrometry (MS), and found that avicularin repressed the accumulation of intracellular lipids in mouse adipocytic 3T3-L1 cells by suppressing C/EBPα-activated GLUT4-mediated glucose uptake in these cells. Therefore, avicularin has the potential for use as a lipid-lower agent to repress the accumulation of body fat and prevent the gain in body weight.

MATERIALS AND METHODS

Extraction and Isolation of Avicularin. The dried stems and leaves of *Taxillus kaempferi* (DC.) Danser (Loranthaceae; 200 g, collected in Osaka, Japan) were cut finely and then extracted with methanol. The methanol extracts was concentrated in vacuo, after which the residue (18 g) was subjected to silica gel column chromatography using a gradient of mixtures of CH_2Cl_2−methanol. The fraction containing avicularin was rechromatographed on a preparative HPLC apparatus (conditions: column, Cosmosil 5C-18 AR-II, 10 mm i.d. × 250 mm [Nacalai Tesque, Kyoto, Japan]; mobile phase, 15% acetonitrile; flow rate, 2 mL/min; and detection, UV 254 nm).

The purity of avicularin was checked by the use of ultrahigh-performance liquid chromatography (UHPLC; Nexera, Shimadzu, Kyoto, Japan) with diode-array detection. A Shim-pack XR-ODS III column (150 × 2.0 mm i.d.; 2.2 μm; Shimadzu) was used for UHPLC analysis. The mobile phase consisted of water (1% acetic acid) and acetonitrile at a ratio of 85:15 (v/v). The flow rate was 0.4 mL/min, the column oven temperature was 40 °C, and detection was made at 400−200 nm.

Spectral Identification of Avicularin by NMR and MS. ^{1}H- and ^{13}C NMR, COSY, HMQC, and HMBC spectra were recorded on an Agilent VNMRS-600 spectrometer (Santa Clara, CA, USA), operating at 600 MHz for proton and 150 MHz for carbon, with tetramethylsilane as an internal standard. FAB-MS spectra were obtained by using a JEOL-MS700V mass spectrometer (Tokyo, Japan).

Avicularin, pale yellow powder; FAB-MS, m/z 435 $(M+H)^+$; ^{1}H NMR (pyridine-d_5, 600 MHz) δ 4.09 (Ara-5, dd, J = 11.5, 4.7 Hz), 4.16 (Ara-5, dd, J = 11.5, 5.0 Hz), 4.81 (Ara-4, br.q, J = 4.7 Hz), 4.89 (Ara-3, dd, J = 4.7, 3.0 Hz), 5.18 (Ara-2, br.d, J = 3.0 Hz), 6.51 (Ara-1, br.s), 6.66 (H-8, d, J = 2.0 Hz), 6.72 (H-6, d, J = 2.0 Hz), 7.35 (H-5′, d, J = 8.2 Hz), 7.98 (H-6′, dd, J = 8.2, 2.0 Hz), 8.28 (H-2′, d, J = 2.0 Hz), 13.29 (5-OH, br,s); ^{13}C NMR (pyridine-d_5, 150 MHz) δ 158.0 (C-2), 134.7 (C-3), 179.3 (C-4), 162.8 (C-5), 99.8 (C-6), 166.0 (C-7), 94.6 (C-8), 157.7 (C-9), 105.2 (C-10), 122.39 (C-1′), 117.1 (C-2′), 147.1 (C-3′), 150.7 (C-4′), 116.7 (C-5′), 122.37 (C-6′), 109.8 (Ara-1), 83.5 (Ara-2), 78.9 (Ara-3), 88.6 (Ara-4), 62.5 (Ara-5). The δ values of ^{1}H NMR were described by two decimal point digits. However, those of ^{13}C NMR were shown by one decimal point digit except for two values, 122.39 (C-1′) and 122.37 (C-6′), to distinguish two signals.

Cell Culture. Mouse adipocytic 3T3-L1 cells (Human Science Research Resources Bank, Osaka, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum and antibiotics, and maintained in a humidified atmosphere of 5% CO_2 at 37 °C.

Adipocyte differentiation of 3T3-L1 cells was initiated by incubation for 2 days in DMEM containing insulin (10 μg/mL; Sigma, St. Louis, MO, USA), 1 μM dexamethasone (Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (Nacalai Tesque). On day 2, the medium was replaced with DMEM containing insulin (10 μg/mL) alone and changed every 2 days.

Intracellular neutral lipids were stained with Bodipy 493/503 (Invitrogen, Carlsbad, CA, USA). Cells were viewed with a fluorescence microscope (CKX41FL; Olympus, Tokyo, Japan).

Cell Toxicity Assay. 3T3-L1 cells were seeded in 96-well plates and allowed to attach overnight at 37 °C. They were then incubated with various concentrations of avicularin (0−100 μM) for 6 days. Cell toxicity was measured by the use of Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the protocols prescribed by the manufacturer. Absorbance was measured at 450 nm by using a Microplatereader Model 680 (Bio-Rad Laboratories; Hercules, CA, USA).

Measurement of Intracellular Triglyceride Level. 3T3-L1 cells were caused to differentiate into adipocytes for 6 days in the presence or absence of avicularin (50 μM). The intracellular triglyceride levels were measured by using a WAKO LabAssay Triglyceride Kit (Wako Pure Chemical, Osaka, Japan) according to the manufacturer's

Table 1. Primers Used in This Study

gene	acc. no.	forward	reverse
PPARγ	NM_011146	5′-CAAGAATACCAAAGTGCGATCAA-3′	5′-GAGCTGGGTCTTTTCAGAATAATAAG-3′
C/EBPα	NM_007678	5′-CTGGAAAGAAGGCCACCTC-3′	5′-AAGAGAAGGAAGCGGTCCA-3′
aP2	NM_024406	5′-GCCAGACACCCCTGCTA-3′	5′-GTTCTGGGCGTCACTCC-3′
FAS	NM_007988	5′-GTTGGGGGTGTCTTCAACC-3′	5′-GAAGAGCTCTGGGGTCTGG-3′
ACC1	NM_133360	5′-GCGTCGGGTAGATCCAGTT-3′	5′-CTCAGTGGGGCTTAGCTCTG-3′
SCD1	NM_009127	5′-TTCCCTCCTGCAAGCTCTAC-3′	5′-CAGAGCGCTGGTCATGTAGT-3′
SREBP-1c	NM_011480	5′-GGAGCCATGGATTGCACATT-3′	5′-GCTTCCAGAGAGGAGGCCAG-3′
ATGL	NM_001163689	5′-TGACCATCTGCCTTCCAGA-3′	5′-TGTAGGTGGCGCAAGACA-3′
HSL	NM_010719	5′-GCACTGTGACCTGCTTGGT-3′	5′-CTGGCACCCTCACTCCATA-3′
MGL	NM_011844	5′-TCGGAACAAGTCGGAGGT-3′	5′-TCAGCAGCTGTATGCCAAAG-3′
TBP	NM_013684	5′-GTGATGTGAAGTTCCCCATAAG-3′	5′-CTACTGAACTGCTGGTGGGTCA-3′

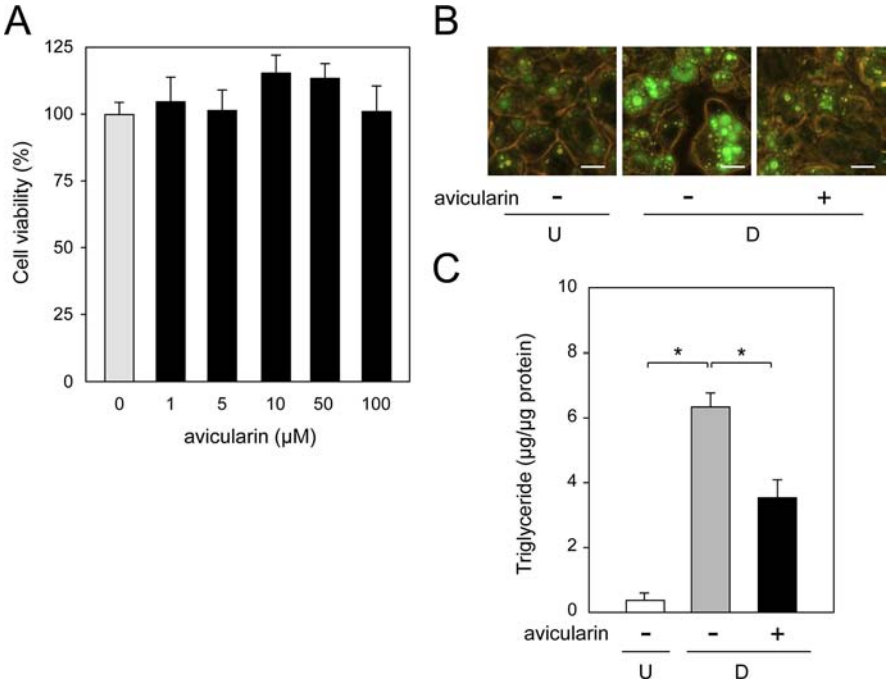

Figure 2. Repression of the accumulation of intracellular lipids by avicularin. (A) Cell toxicity of avicularin toward 3T3-L1 cells. Cells were incubated with various concentrations of avicularin (0−100 μM) for 6 days, and cell toxicity was then measured in terms of cell viability. Data are the mean ± SD from 3 independent experiments. (B) Staining of intracellular lipids in 3T3-L1 cells with Bodipy 493/503. The cells (undifferentiated cells, U) were cause to differentiate into adipocytes (D) for 6 days in medium with or without avicularin (50 μM). Intracellular lipid droplets were stained with Bodipy 493/503. Bar = 50 μm. (C) Avicularin-mediated suppression of the intracellular triglyceride level in 3T3-L1 cells. The cells (undifferentiated cells, U, white column) were caused to differentiate (D) into adipocytes for 6 days in the absence (gray column) or presence of avicularin (50 μM; black column). Data are presented as the mean ± SD from 3 independent experiments. *$p < 0.01$, as indicated by the brackets.

instructions. Protein concentrations were measured with Pierce BCA Protein Assay Reagent (Thermo Scientific, Rockford, IL, USA).

Preparation of RNA and Quantification of mRNA Levels. Total RNA was prepared with TriPure Isolation Reagent (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions. First-strand cDNAs were synthesized from 1 μg of total RNA with random hexamer (Takara-Bio, Kyoto, Japan) and ReverTra Ace Reverse Transcriptase (Toyobo, Osaka, Japan) at 42 °C for 60 min after initial denaturation at 72 °C for 3 min, followed by heat-denaturation of the enzyme at 99 °C for 5 min. The cDNAs were diluted and further used as the templates for quantitative PCR analysis.

Expression levels of the desired genes were quantified by using a LightCycler system (Roche Diagnostics) with THUNDERBIRD SYBR qPCR Mix (Toyobo) and primer sets (Table 1). The expression levels of the target genes were normalized to that level of the TATA-binding protein (TBP).

Western Blot Analysis. Cells were lysed in RIPA buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.1% (w/v) SDS, 0.5% (w/v) sodium deoxycholate, 1%(v/v) Nonidet P-40, and 1% (v/v) Triton X-100 along with a Protease Inhibitor Cocktail (Nacalai Tesque) and phosphatase inhibitors (50 μM Na_2MoO_4, 1 mM NaF, and 1 mM Na_3VO_4). After sonication, crude cell extracts were prepared by centrifugation for 20 min at 17,000g at 4 °C to remove the cell debris. Protein concentrations were measured as described above. The proteins were separated on SDS−PAGE gels and then transferred onto Immobilon PVDF membranes (Millipore, Bedford, MA, USA) for Western blot analysis. Blots were first incubated with primary antibodies, i.e., anti-PPARγ (1:1,000; H-100; Santa Cruz Biotech., Santa Cruz, CA, USA), anti-C/EBPα (1:1,000; C-18; Santa Cruz Biotech.), anti-GLUT4 (1:1,000; Sigma) polyclonal antibody, antifatty acid-binding protein 4 (aP2; 1:1,000; Epitomics, Burlingame, CA, USA), or antiactin (1:2,000, AC-15; Sigma) monoclonal antibody, followed by incubation with the appropriate secondary antibody, i.e., antirabbit, antigoat, or antimouse IgG antibody conjugated with horseradish peroxidase (Santa Cruz Biotech.). Immunoreactive signals were detected by the use of Pierce Western Blotting Substrate (Thermo Scientific) and an LAS-3000 Image Analyzer (Fujifilm, Tokyo, Japan), and analyzed with Multi Gauge software (Fujifilm).

Chromatin Immunoprecipitation (ChIP) Assay. ChIP assays were performed as described previously.[27,28] Briefly, 3T3-L1 cells were caused to differentiate into adipocytes in DMEM containing avicularin (50 μM) or not for 6 days. The cells were cross-linked with 1%(v/v) formaldehyde for 10 min and then collected and lysed in SDS lysis buffer containing 50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 1%(w/v) SDS, and Protease Inhibitor Cocktail (Nacalai Tesque). Chromatin was sonicated on ice and then incubated with a polyclonal antibody against C/EBPα (C-18; Santa Cruz Biotech.) or rabbit normal IgG (Cell Signaling Technology, Danvers, MA, USA) at 4 °C overnight, followed by further incubation with Pureproteome Protein G Magnetic beads system (Millipore) at 4 °C for 1 h. The beads were washed, and the immunoprecipitated DNA−protein complexes were eluted and then reverse-cross-linked for 4 h at 65 °C. The DNA fragments were purified with a MinElute PCR Purification Kit (Qiagen, Hilden, Germany). The purified DNA fragments were used for PCR analysis with the following specific primer set: 5′-GCAGGCGGGAACCTT-AGGGGCG-3′ and 5′-CCAAGGCTCTCCGGGATCTAGTG-3′. PCR was conducted with ExTaq DNA Polymerase (Takara-Bio) under the following conditions: initial denaturation at 95 °C for 3 min, followed by 35 cycles of 94 °C for 10 s, 55 °C for 20 s, and 74 °C for 20 s. The resultant PCR products (expected size of amplicons was 202-bp) were analyzed by performing 2% (w/v) agarose gel electrophoresis. Band intensity was measured with LAS-3000 Image Analyzer and analyzed with Multi Gauge software.

2-Deoxyglucose Uptake Assay. 3T3-L1 cells were caused to differentiate into adipocytes in DMEM in the presence or absence of avicularin (50 μM) for 6 days. The medium was then removed, fresh serum-free DMEM was added, and then cells were cultured for 6 more hours. Thereafter, the cells were washed 3 times with Krebs Ringer Phosphate Hepes (KRPH) buffer containing 1.2 mM KH_2PO_4, 1.2

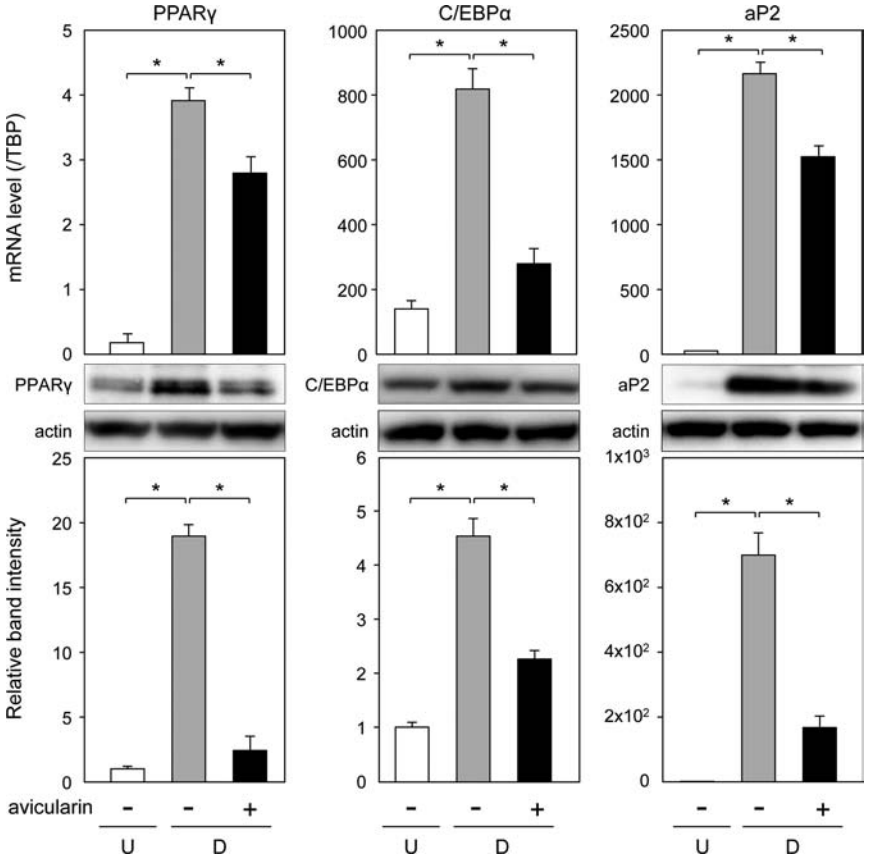

Figure 3. Suppression of expression of adipogenic genes in avicularin-treated 3T3-L1 cells. (A) Expression levels of adipogenic genes in avicularin-treated cells. 3T3-L1 cells (undifferentiated cells, U; white columns) were caused to differentiate (D) into adipocytes for 6 days in the absence (gray columns) or presence of avicularin (50 μM; black columns). Data are the mean ± SD from 3 independent experiments. *$p < 0.01$, as indicated by the brackets. Crude cell extracts were prepared and used for Western blot analysis (20 μg/lane). Band intensities were measured by using Multi Gauge software. *$p < 0.01$, as indicated by the brackets.

mM $MgSO_4$, 1.3 mM $CaCl_2$, 118 mM NaCl, 5 mM KCl, and 30 mM HEPES-KOH at pH 7.5. Then, the cells were cultured in KRPH buffer containing 2% (w/v) BSA (fatty acid-free, globulin-free; Sigma) and insulin (1 μM), to which was added 2-deoxy-D-glucose (1 mM). The total culture period was 20 min at 37 °C. The buffer was then discarded, and the cells were washed 3 times with ice-cold PBS containing Cytochalasin B (200 μM; Wako Pure Chemical). The cells were next suspended in 10 mM Tris-Cl at pH 8.0; disrupted by sonication; and heat-denatured at 80 °C for 15 min. Following centrifugation to remove the cell debris, the resulting supernatant was taken and used to measure the uptake of 2-deoxy-D-glucose with 2-Deoxyglucose Uptake Measurement Kit (Cosmo Bio, Tokyo, Japan).[29] The 2-deoxy-D-glucose uptake level was normalized against the protein concentration in each sample and expressed as the degree of increase compared with the uptake by the untreated cells.

Statistical Analysis. The data were expressed as the mean ± SD. Comparison of 2 groups was analyzed by using Student's t -test. For comparison of more than 2 groups with comparable variances, one-way ANOVA and Tukey's posthoc test were carried out. $p < 0.05$ was considered significant.

RESULTS

Extraction, Purification, and Structural Identification of Avicularin. The stems and leaves of *Taxillus kaempferi* were extracted with methanol, and the flavonol glycoside constituents were concentrated as described in Materials and Methods. Avicularin was isolated by preparative HPLC of the concentrated flavonol glycoside mixture.

We further checked the purity of the isolated avicularin by UHPLC. The resulting data showed high purity of more than at least 99% (Figure 1B). Furthermore, the chemical structure of purified avicularin (Figure 1A) was confirmed on the basis of spectral analyses (^{1}H NMR, ^{13}C NMR, two-dimensional NMR, and MS) and by comparison of their spectral data with those reported previously in the literature.[25]

Effects of Avicularin on Adipocyte Differentiation of 3T3-L1 Cells. At first, we examined the cell toxicity of avicularin toward mouse adipocytic 3T3-L1 cells. Cells were incubated for 6 days in DMEM with various concentrations of avicularin (0−100 μM), and cell toxicity was then measured. No significant effect on cell viability was observed up to 100 μM avicularin (Figure 2A).

Next, we investigated the effects of avicularin on adipocyte differentiation. 3T3-L1 cells were caused to differentiate into adipocytes for 6 days in the presence or absence of avicularin, and the accumulated intracellular neutral lipids were stained with Bodipy fluorescent dye. The number of lipid droplets in the differentiated cells was increased compared with that in the undifferentiated cells (Figure 2B). On the contrary, the number of intracellular lipid droplets in the differentiated cells was decreased when avicularin was present (Figure 2B).

Subsequently, we measured the level of intracellular triglycerides. 3T3-L1 cells were caused to differentiate into adipocytes for 6 days in DMEM with or without avicularin (50 μM). The intracellular triglyceride level in the differentiated

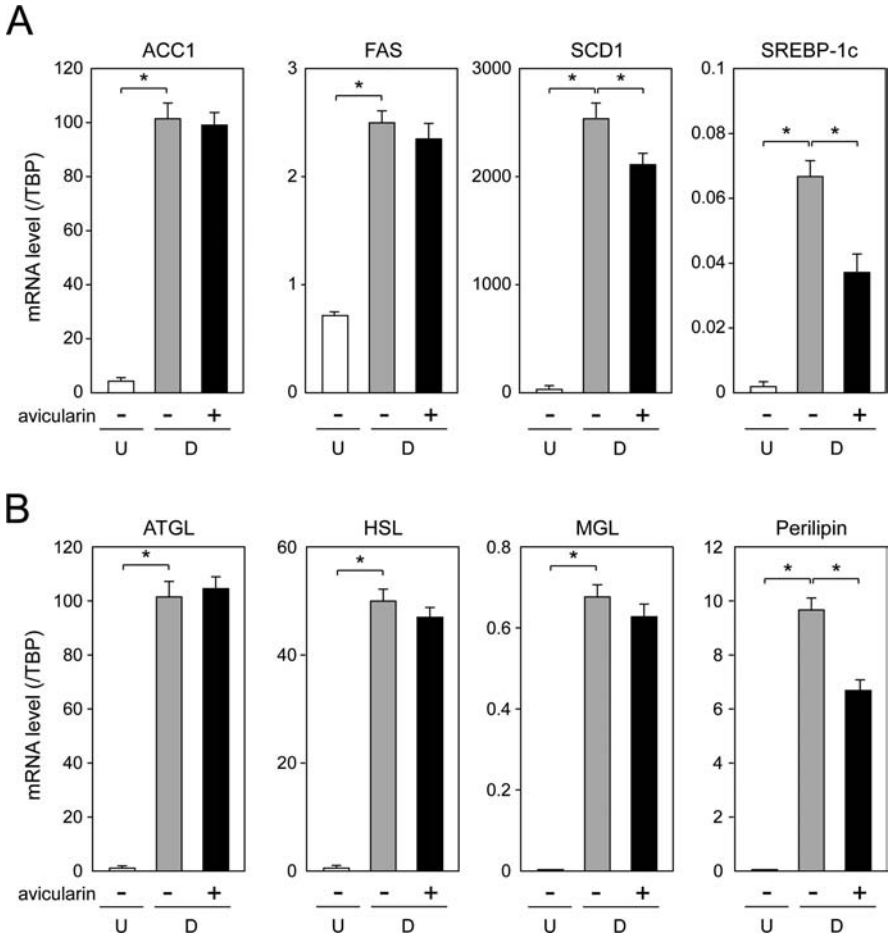

Figure 4. Expression of lipogenic and lipolytic genes in avicularin-treated 3T3-L1 cells. (A) Expression in avicularin-treated 3T3-L1 cells of ACC1, FAS, SCD1, and SREBP-1c of lipogenic genes and ATGL, HSL, MGL, and perilipin genes involved in the lipolysis. The cells (undifferentiated cells, U; white columns) were caused to differentiate (D) into adipocytes for 4 days in medium without (gray columns) or with (black columns) avicularin (50 μM). Transcription levels were measured by quantitative PCR analysis. Data are presented as the mean ± SD from 3 independent experiments. *$p < 0.01$, as indicated by the brackets.

cells was increased approximately 18.5-fold as compared with that in the undifferentiated cells (Figure 2C). However, when the cells were caused to differentiate into adipocytes in the presence of avicularin, the intracellular triglyceride level was decreased to about 60.5% of that in the differentiated cells (Figure 2C). These results thus indicate that avicularin suppressed the accumulation of intracellular lipids in 3T3-L1 cells.

Repression of Adipogenic Gene Expression by Avicularin. When we investigated the effects of avicularin on the expression of the adipogenic genes such as PPARγ, C/EBPα, and aP2 by quantitative PCR, the results indicated that the expression levels of PPARγ, C/EBPα, and aP2 genes was enhanced approximately 18.6-, 4.8-, and 128-fold, respectively, during adipogenesis, as compared with those in the undifferentiated cells (Figure 3). In the avicularin (50 μM)-treated cells, however, PPARγ, C/EBPα, and aP2 mRNA levels were decreased approximately 28.7, 69.5, and 18.3%, respectively, as compared with those in the vehicle-treated differentiated cells (Figure 3). Almost the same results were obtained by Western blot analysis (Figure 3).

Avicularin Did Not Affect Lipogenesis or Lipolysis in 3T3-L1 Cells. We examined the transcription level of the genes involved in lipogenesis and lipolysis in the avicularin (50 μM)-treated 3T3-L1 cells. When the cells were caused to differentiate into adipocytes for 6 days in the presence or absence of avicularin, the expression levels of the lipogenic genes such as acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD)-1, and SREBP-1c were enhanced about 16.8-, 3.3-, 39.1-, and 28.3-fold, respectively, as compared with those of the undifferentiated cells (Figure 4A). In contrast, when the cells were cultured with avicularin, the mRNA levels of SCD1 and SREBP-1c were reduced approximately 18.6% and 46.3%, respectively, compared with those of the differentiated cells (Figure 4A). However, the transcription levels of ACC1 and FAS genes were not altered even in the presence of avicularin (Figure 4A).

Then, we examined the expression level of the genes involved in the lipolysis, i.e., adipocyte triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), monoacyl glyceride lipase (MGL), and perilipin, a lipid droplet-associated protein. The expression levels of the ATGL, HSL, MGL, and perilipin genes were up-regulated during adipogenesis, approximately 48.6-, 43.3-, 88.3-, and 57.6-fold, respectively, as compared with those of the undifferentiated cells (Figure 4B). Moreover, when the cells were caused to differentiate into adipocytes in the presence of avicularin (50 μM), the transcription levels of the ATGL, HSL, and MGL genes were not altered, whereas the level of the perilipin gene was decreased to about 64.6% of that

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of the differentiated cells (Figure 4B). These results, all taken together, indicate that lipogenesis and lipolysis were not involved in the avicularin-mediated suppression of the accumulation of the intracellular lipids in the adipocytes.

Avicularin Suppresses GLUT4-Mediated Glucose Uptake in Adipocytes. Next, we investigated the expression of the glucose transporter GLUT4 and glucose uptake in the avicularin-treated 3T3-L1 cells. The transcription level of the GLUT4 gene was up-regulated approximately 3.9-fold during adipogenesis, as compared with that for the undifferentiated cells (Figure 5A). When the cells were caused to differentiate into adipocytes in the presence of avicularin (50 μM), the mRNA level of the GLUT4 gene was decreased to about 34.6% of that for the differentiated cells without the flavonoid (Figure 5A).

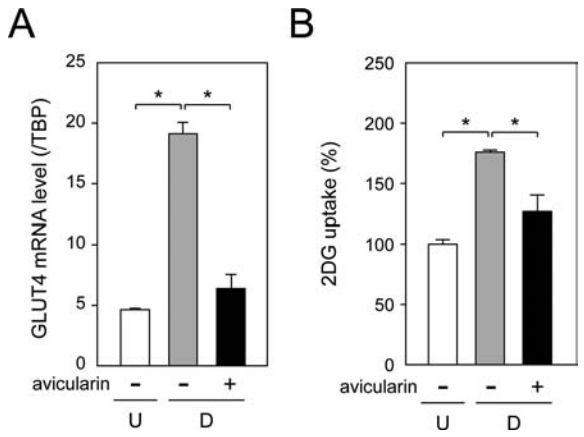

Figure 5. Repression of glucose uptake by avicularin in 3T3-L1 cells. (A) Decrease in GLUT4 expression in avicularin-treated cells. 3T3-L1 cells (undifferentiated cells, U; white column) were caused to differentiate (D) into adipocytes for 6 days in DMEM without (gray column) or with (black column) avicularin (50 μM). The transcription level of GLUT4 was examined by quantitative PCR analysis. Data are presented as the mean ± SD from 3 independent experiments. *p < 0.01, as indicated by the brackets. (B) Suppression of glucose uptake by avicularin. 3T3-L1 cells were caused to differentiate into adipocytes for 6 days in DMEM without (gray column) or with (black column) avicularin (50 μM), and glucose uptake was then measured. The data are represented as the value relative to that of the undifferentiated cells. Data are presented as the mean ± SD from 3 independent experiments. *p < 0.01, as indicated by the brackets.

Then, we measured glucose uptake in the avicularin-treated cells. Glucose uptake was enhanced about 1.6-fold during the differentiation of 3T3-L1 cells (Figure 5B). In contrast, when the cells were caused to differentiate into adipocytes in the presence of avicularin (50 μM), glucose uptake was suppressed to approximately 72.7% of that by the differentiated cells. These results reveal that GLUT4 expression in and glucose uptake by 3T3-L1 cells were reduced by the treatment with avicularin.

Avicularin Represses C/EBPα-Mediated GLUT4 Expression in Adipocytes. To further elucidate the molecular mechanism of the suppressive effect of avicularin on adipogenesis, we examined the transcriptional regulatory mechanism of the GLUT4 gene in the avicularin-treated cells. The expression of the GLUT4 gene in 3T3-L1 cells is known to be activated by C/EBP.[30] Thus, we examined the C/EBPα-mediated expression of the GLUT4 gene in the cells. To confirm this expression, we investigated the expression levels of GLUT4 and C/EBPα in avicularin-treated cells at the protein level. Both GLUT4 and C/EBPα protein levels were elevated about 2.9- and 2.6-fold, respectively, during adipogenesis, as compared with those in the undifferentiated cells (Figure 6A). When the cells were caused to differentiate into adipocyte in medium containing avicularin (0−50 μM), the level of each protein was decreased in a concentration-dependent manner (Figure 6A).

Next, we performed a ChIP assay to investigate the binding of C/EBPα to the C/EBP element of the GLUT4 promoter in 3T3-L1 cells. The expected size (202-bp; Figure 6B) of an amplicon containing the C/EBP element at −92 was detected in the formaldehyde-fixed DNA−protein complexes immunoprecipitated with anti-C/EBPα antibody (Figure 6B). The efficiency of binding of C/EBPα to the C/EBP element in the differentiated 3T3-L1 cells was enhanced approximately 3.8-fold, as compared with that for the undifferentiated cells (Figure 6B). In contrast, when the cells underwent differentiation into adipocytes in the presence of avicularin (50 μM), the band intensity was decreased to about 61.4% of that for the differentiated cells (Figure 6B). There was no detectable signal when rabbit normal IgG was added. The input control was positive in all samples. These results, taken together, indicate that C/EBPα bound to the C/EBP element of the proximal promoter region of the GLUT4 gene and that its binding was repressed by the treatment of 3T3-L1 cells with avicularin.

DISCUSSION

Obesity is a risk factor for various diseases such as hypertension, cardiovascular disease, diabetes mellitus, and hyperlipidemia.[2,3] Thus, the observed increase in obesity is a major public health problem worldwide. Obesity is a consequence of an imbalance between intake and expenditure of energy, and results in excessive fat deposition.[1] Excess adipose tissue is the consequence of both an increased number (hyperplasia) and enlarged size (hypertrophy) of adipose cells. Thus, treatments that regulate either hyperplasia or hypertrophy or both may provide a novel therapeutic strategy for obesity.

Flavonoids are naturally occurring plant polyphenols found in abundance in fruits and vegetables,[18] and they are considered as a promising source for drug development, as they have been shown to have impressive biological effects such as anticancer, antioxidant, and anti-inflammatory.[19−21] However, flavonoid-derived drugs have never been used in the clinical field because of their unexpected side effects. We supposed that one of the reasons for these problems is the purity of the flavonoid used in the studies. In fact, as the purities of most commercial available flavonoids are not so high (less than 98%), trace contaminants in their preparations may show unexpected side effects that have been studied in vitro and in vivo, e.g., toxic flavonoid−drug interactions, antithyroid activity, and estrogen-like actions.[31,32]

Most flavonoids show suppressive effects on adipogenesis.[33] Quercetin suppresses adipogenesis through AMP-activated protein kinase (AMPK)-mediated inhibition of ACC activity.[34] Moreover, apigenin, which has a structure similar to that of quercetin, also suppresses adipogenesis through activation of AMPK.[16] However, avicularin did not activate AMPK in adipocytes (data not shown). Moreover, avicularin, but not quercetin and apigenin, suppresses the accumulation of the intracellular lipids through repression of C/EBPα-activated GLUT4 expression. This difference might be derived from the existence of a five-membered ring with the flavonoid structure

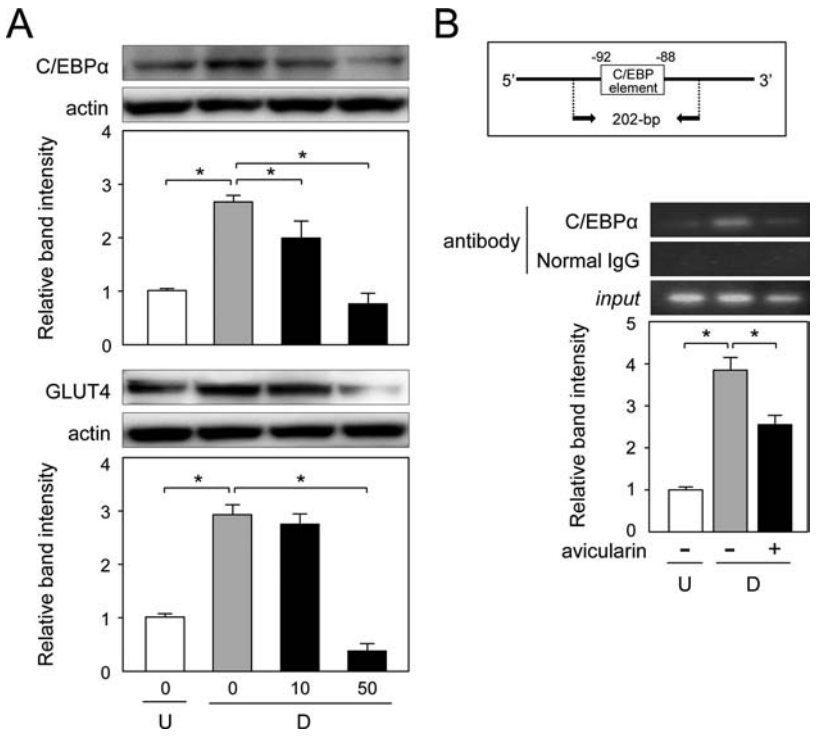

Figure 6. Suppression of C/EBPα-activated GLUT4 expression in avicularin-treated 3T3-L1 cells. (A) Suppression of C/EBPα and GLUT4 expression in avicularin-treated cells. 3T3-L1 cells (undifferentiated cells, U; white columns) were caused to differentiate (D) into adipocytes for 6 days in medium containing various concentrations of avicularin (0−50 μM; gray or black columns). Crude cell extracts were prepared and used for Western blot analysis (20 μg/lane). Band intensities were measured by using Multi Gauge software and normalized to those of actin. *$p < 0.01$, as indicated by the brackets. (B) ChIP assay of the C/EBP element of mouse GLUT4 promoter in 3T3-L1 cells. The scheme for the ChIP assay of the mouse GLUT4 promoter is shown at the top. The cells (undifferentiated cells, U; white column) were caused to differentiate (D) into adipocytes (gray column) or with (black column) avicularin (50 μM) for 6 days, and the ChIP assay was then performed. The profile of the amplicon is shown, and the input control (input) means that a small diluted aliquot before immunoprecipitation was used for PCR amplification. The data are representative of 3 independent experiments. *$p < 0.01$, as indicated by the brackets.

in avicularin. Thus, the precise structure−activity relationship of flavonoids in the regulation of adipogenesis should be further examined. In this study, we highly purified natural avicularin by HPLC (more than at least 99%, Figure 1) and examined its suppressive effect on adipogenesis.

Avicularin is a plant flavonoid and quercetin derivative.[22] However, until now, there have been few reports about the biological activities of avicularin, although quercetin has a variety of beneficial effects such as suppression of neuronal apoptosis[35] and adipogenesis.[36] The regulatory mechanism of avicularin-mediated suppression of adipogenesis has never been identified. In this study, we demonstrated that avicularin-mediated suppression of adipogenesis occurred through suppression of C/EBPα-activated GLUT4-mediated glucose uptake in 3T3-L1 cells. The amount of adipose tissue mass can be decreased by deletion of adipose cells via their apoptosis as well as by inhibition of adipogenesis. However, avicularin showed no apparent toxicity toward 3T3-L1 cells, at least up to the 100 μM concentration tested (Figure 2A), thus indicating that avicularin had no apoptotic activity toward adipocytes.

Glucose uptake is mediated by the GLUT family of glucose transporter proteins in mammals.[37] The GLUT4 transporter is expressed in insulin target tissues such as adipocytes and muscle, and GLUT4-mediated glucose uptake is responsible for glucose metabolism and is dependent upon stimulation by insulin.[15] The increased intrinsic activity of GLUT4 can potentially induce enhanced glucose uptake in 3T3-L1 adipocytes.[38] It is known that GLUT4 is involved in the activation of glucose uptake in adipocytes. The transcription level of the GLUT4 gene in 3T3-L1 cells was decreased by treatment with avicularin. The level of intracellular lipids is reduced in GLUT4 siRNA-transfected cells.[39] Moreover, our present study demonstrated that C/EBPα bound to the GLUT4 promoter, as demonstrated by the results of the ChIP assay (Figure 6B). Furthermore, avicularin significantly suppressed the expression of the GLUT4 and C/EBPα genes along with the reduction in glucose uptake. In addition, it has been reported that GLUT4 expression can be up-regulated by C/EBP,[30] indicating that avicularin repressed C/EBPα-mediated GLUT4 expression closely associated with glucose uptake. In fact, glucose uptake is regulated by flavonoids such as apigenin, luteolin, kaempferol, quercetin, fisetin, genistein, silybin, and epigallocatechin gallate in adipose cells.[40] Thus, avicularin may act as an antidietary flavonoid and prevent obesity.

Our current data also showed that avicularin acted as a repressor of the expression of the C/EBPα gene. C/EBPα is a key transcription factor in adipogenesis and its expression is activated by C/EBPβ, C/EBPδ, and PPARγ in adipocytes.[41] However, avicularin did not affect the expression of C/EBPβ and C/EBPδ in 3T3-L1 cells (data not shown). Moreover, although some dietary products are valuable components having antiobesity effects through the modulation of the activity of PPARγ,[42] avicularin had no antagonistic activity toward PPARγ, as demonstrated by a mammalian two-hybrid assay (data not shown). As the mechanism underlying avicularin-

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mediated suppression of C/EBPα expression is still unknown, it should be further elucidated.

In summary, we showed that avicularin, a plant flavone, suppressed the accumulation of intracellular lipids by suppressing C/EBPα-mediated activation of GLUT4 expression in 3T3-L1 cells. In a future study, the action of avicularin as an antiobesity agent should be evaluated in vivo, and the molecular mechanism of the activation of C/EBPα by avicularin should be investigated to understand the whole regulatory mechanism of avicularin-mediated suppression of adipogenesis.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

NMR, nuclear magnetic resonance; MS, mass spectrometry; PPAR, peroxisome proliferator-activated receptor; C/EBP, CCAAT/Enhancer binding proteins; SREBP, sterol regulatory-element binding protein; GLUT, glucose transporter; DMEM, Dulbecco's modified Eagle's medium; aP2, fatty acid-binding protein 4; TBP, TATA-binding protein; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; SCD, stearoyl-CoA desaturase; ATGL, adipocyte triglyceride lipase; HSL, hormone-sensitive lipase; MGL, monoacyl glyceride lipase; AMPK, AMP-activated protein kinase

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