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Artemisinic Acid is a Regulator of Adipocyte Differentiation and C/EBP δ Expression

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ABSTRACT

Adipocyte dysfunction is associated with the development of obesity. In this study, artemisinic acid, which was isolated from *Artemisia annua L.*, inhibited adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells (hAMSCs) and its mechanism of action was determined. The mRNA levels of peroxidase proliferation-activated receptor (PPAR) γ and CCAAT/enhancer binding protein (C/EBP) α, late adipogenic factors, were reduced by artemisinic acid. Moreover, the mRNA levels of the PPAR γ target genes lipoprotein lipase, CD36, adipocyte protein, and liver X receptor were down-regulated by artemisinic acid. Artemisinic acid reduced expression of the C/EBP δ gene without impacting C/EBP β. In addition, attempts to elucidate a possible mechanism underlying the artemisinic acid-mediated effects revealed that reduced expression of the C/EBP δ gene was mediated by inhibiting Jun N-terminal kinase (JNK). Additionally, artemisinic acid also reduced the expression of the adipogenesis-associated genes glucose transporter-4 and vascular endothelial growth factor. In addition to the interference of artemisinic acid with adipogenesis, artemisinic acid significantly attenuated tumor necrosis factor-α-induced secretion of interleukin-6 by undifferentiated hAMSCs, thus influencing insulin resistance and the inflammatory state characterizing obesity. Taken together, these findings indicate that inhibiting adipogenic differentiation of hAMSCs by artemisinic acid occurs primarily through reduced expression of C/EBP δ, which is mediated by the inhibition of JNK and suggest that aremisinic acid may be used as a complementary treatment option for obesity associated with metabolic syndrome. J. Cell. Biochem. 113: 2488–2499, 2012.

KEY WORDS: ADIPOGENESIS; C/EBP Δ; JNK; ARTEMISINIC ACID

Obesity is characterized by excess adipose tissue and has been recognized as an important risk factor in a broad range of diseases, such as metabolic syndrome, insulin resistance, diabetes, hypertension, cardiovascular diseases, and cancer [Mokdad et al., 2003; Xu et al., 2003; Schwartz and Porte, 2005; Ramachandran et al., 2006; American Diabetes Association, 2007]. Adipose tissue serves as energy storage in the form of triglycerides and exerts secretory/endocrine gland functions [Kahn and Flier, 2000]. It has been estimated that 20–30% of genes expressed in adipose tissue encode secreted proteins, including pro-inflammatory cytokines, complementary and related proteins, fibrinolytic proteins, and other hormone-like proteins [Matsuzawa, 2006]. These bioactive molecules, collectively termed adipokines, regulate the biological function of adipocytes via autocrine, paracrine, and endocrine mechanisms; thus, adipokines play an important role in inflammation, energy metabolism, and insulin sensitivity [Drevon, 2005].

Adipogenesis is the process whereby hormonal stimuli induce the differentiation of fibroblasts or mesenchymal cells into adipocytes. Adipogenesis requires organized and controlled expression of a cascade of transcription factors and modification of the chromatin within preadipocytes [Rangwala and Lazar, 2000; Rosen, 2000; Evans et al., 2004]. The factors involved in adipocyte differentiation include the nuclear receptor peroxisome proliferation-activated receptor γ (PPAR γ) and a group of CCAAT/enhancer-binding

Abbreviations: hAMSCs, human adipose tissue-derived mesenchymal stem cells; C/EBP, CCAAT/enhancer binding protein; PPAR γ, peroxisome proliferation-activated receptor γ; JNK, Jun N-terminal kinase.

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proteins (C/EBPs) [Morrison and Farmer, 2000; Rosen et al., 2000]. Rapid and transient induction of C/EBP β and δ expression is one of the earliest events in adipogenesis [Rosen, 2000]. These transcription factors bind to specific sequences in the C/EBP α and the PPAR γ2 gene promoter regions, induce their expression, which, in turn, activates the full gene expression program [Christy et al., 1991; Lin et al., 1993; Wu et al., 1995].

Adipocytes are the major cellular component in adipose tissue and their excessive growth, differentiation, and hypertrophy are fundamental processes involved in obesity. Maturation of adipocytes can occur among cells from a pre-existing pool of adipocyte progenitor cells that are present, irrespective of age [Gregore et al., 1998]. Therefore, both the proliferation and differentiation of preadipocytes into mature adipocytes remain important issues from a pathophysiological point of view.

Most intensive clinical interventions for obesity and related diseases have been primarily directed at decreasing excessive amounts of adipose tissue by changing the balance between intake and expenditure of energy [Yamauchi et al., 2003]. Such changes are typically induced via daily exercise and diet control [Spiegelman and Flier, 2001]. Mechanical stimuli such as stretching and rubbing of fat and skeletal muscle also decrease obesity [Tanabe et al., 2004]. Despite these traditional approaches to treat obesity, there is still an urgent need to develop more safe and effective anti-obesity drugs. For this reason, the effects of a wide variety of natural materials on obesity treatment have been examined. In this study, triglyceride and glycerol-3-phosphate dehydrogenase (GPDH) activity assays were used as a screening tool with human adipose tissue-derived mesenchymal stem cells (hAMSCs) to evaluate the anti-adipogenesis effects of plants. During the screening, *Artemisia annua L.* was selected as a candidate, and artemisinic acid was isolated and tested as a potential anti-adipogenesis agent.

We investigated the effects of artemisinic acid on adipogenesis and the signaling pathways involved in adipogenic differentiation as well as the effect of artemisinic acid on IL-6 production induced by pro-inflammatory stimuli in undifferentiated hAMSCs.

MATERIALS AND METHODS

GENERAL ANALYTICAL METHODS

Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on a Varian Unity Inova 500NB instrument at 500 MHz for ^1H and 125 MHz for ^{13}C using standard pulse sequence programs. All chemical shifts were recorded with respect to trimethoxysilane (TMS), which was used as an internal standard. All chemical shifts are reported in δ (ppm) units relative to the TMS signal and coupling constants (J) in Hz. Gas chromatography–mass spectrometry (GC–MS) and GC-selected ion monitoring were conducted using a 5972 Plus mass spectrometer (electron impact ionization, 70 electron volt, Hewlett-Packard, Fullerton, CA) connected to a 5890 GC fitted with a fused silica capillary column (HP-5, 0.25 × 30 m, 0.25 μm film thickness, Hewlett-Packard). The GC conditions were as follows: On-column injection mode, He 1 ml/min; oven temperature thermal gradient, 10°C/min from 45 to 280°C. Silica gel 60 F254s thin layer chromatography (TLC) plates (0.5 mm, Merck, Darmstadt, Germany) were used for the TLC analysis. Medium pressure liquid chromatography (MPLC) was conducted using a Combiflash Companion instrument with an UV/Vis detector (Teledyne ISCO, Inc., Lincoln, NE). Preparative high-pressure liquid chromatography (HPLC) was conducted using a Prep LC 2000 and a 2487 Dual λ Absorbance detector (Waters Corp., Billerica, MA). A silica gel (230–400 mesh, Merck) column was used for column chromatography. All HPLC-grade organic solvents and bulk organic solvents were purchased from J.T. Baker (Phillipsburg, NJ) and Duksan Co. (Republic of Korea).

PLANT MATERIAL

Naturally grown *A. annua* were collected from Jeju island, Korea, from June to September 2010. A voucher sample was deposited at the Jeju Biodiversity Research Institute of the Jeju Hi-Tech Industry Development Institute.

EXTRACTION AND ISOLATION

Whole bodies of *A. annua* (300 g dry wt.) were homogenized and extracted with *n*-hexane (3 L × 3). The extracts were then concentrated in vacuo, after which they were re-extracted with 6% methanol in chloroform (400 ml). After completely drying the extracts in vacuo, they (3.38 g) were then subjected to MPLC (RediSep, silica 120 g, 3.5 × 200 mm; detection, UV at 254 nm; flow rate, 85 ml/min). The elution buffer contained 20% ethylacetate in hexane, which was run for 35 s and produced 50 subfractions. Fractions 10–18 were further separated by preparative TLC on a Si gel using a 6% ethylacetate in $CHCl_3$ solvent system to produce 940 mg of a mixture. This mixture was subjected to reversed-phase preparative HPLC (Phenomenex Luna C18(2), 21.2 × 250 mm, 5 μm, 60% acetonitrile in water) to yield artemisinic acid (480 mg). Artemisinic acid was analyzed by GC-MS and ^1H- and ^{13}C-NMR.

Artemisinic acid. ^1H-NMR (500 MHz, $CDCl_3$): δ 6.45 (1H, s, H-13), 5.55 (1H, s, H-13), 4.98 (1H, s, H-5), 2.69 (1H, m, J = 3.8 Hz, H-7), 2.61 (1H, br s, H-6), 1.92 (2H, d, J = 1.2 Hz, H-2), 1.78 (2H, d, J = 3.5 Hz, H-3), 1.59 (3H, s, H-15), 1.41 (1H, m, J = 5.3 Hz, H-1), 1.39 (1H, m, J = 2.2 Hz, H-10), 1.35 (2H, m, J = 3.4 Hz, H-8), 1.07 (2H, m, J = 3.8 Hz, H-9), 0.90 (3H, d, J = 4.1 Hz, H-14); ^{13}C-NMR (500 MHz, $CDCl_3$): δ 172.9 (C-12), 142.8 (C-11), 135.1 (C-4), 126.8 (C-13), 120.3(C-5), 42.2 (C-7), 41.6 (C-1), 38.1 (C-6), 35.4 (C-9), 27.8 (C-10), 26.6 (C-3), 26.1 (C-8), 25.7 (C-2), 23.8 (C-15), 19.5 (C-14).

MATERIALS

Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-β-actin monoclonal antibody, isobutylmethylxanthine, dexamethasone, and insulin were purchased from Sigma-Aldrich (St. Louis, MO). TRIzol reagent, random primers, and Moloney murine leukemia virus reverse transcriptase were obtained from Invitrogen (Carlsbad, CA). Anti-C/EBP β antibody, anti-C/EBP δ antibody, phospho-p38 MAPK (Thr180/Tyr182) (28B10), phospho-SAPK/JNK (Thr183/Tyr185) (G9), phospho-p42/44 MAPK (Thr202/Tyr204) (E10), p38 MAPK, SAPK/JNK, and p42/44 MAPK antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Artemisinic acid (99%) was isolated and purified from *A. annua L.*

HUMAN ADIPOSE TISSUE-DERIVED MESENCHYMAL STEM CELL CILTURE AND STIMULATION

Three kinds of hAMSCs were used in this study and purchased from Invitrogen, ATCC (Manassas, VA), and Thermo Fisher Scientific, Inc. (Waltham, MA). hAMSCs were seeded in 6-cm diameter dishes at a density of 15×10^4 cells/well. Cells were grown in MesenPro RS^{TM} media (Invitrogen) at 37°C under 5% CO_2. To induce differentiation, 2-day post confluent hAMSCs (day 0) were incubated for 15 days with STEM PRO adipocyte differentiation media (Invitrogen). To examine the effects of artemisinic acid on adipocyte differentiation of hAMSCs, 2-day post confluent hAMSCs were treated with the indicated concentrations of artemisinic acid and then stimulated with STEM PRO adipocyte differentiation media for 3 days. The medium was replaced with STEM PRO adipocyte differentiation media every 3 days until the end of the experiment on day 15.

OIL RED O STAINING

hAMSCs that had been treated as described above were washed with PBS and then fixed with 10% formalin for 30 min. The cells were washed twice with distilled water and stained for at least 1 h at room temperature in freshly diluted Oil Red O solution (six parts Oil Red O stock solution and four parts H_2O; Oil Red O stock solution is 0.5% Oil Red O in isopropanol). Stained cells were air dried overnight and then dissolved in 1-butanol for OD detection at 520 nm. The results were confirmed by three independent experiments.

TRIGLYCERIDE ASSAY

hAMSCs that had been treated as described above were washed with PBS and harvested in 25 mM Tris buffer (pH 7.5) containing 1 mM EDTA. The samples were then sonicated three times for 15 s each using a UP50H instrument with a MS7 (Hielscher Ultrasonics GmbH, Teltow, Germany) to homogenize the cell suspension, after which the total triglyceride content was evaluated using a triglyceride assay kit (Cayman Chemical, Ann Arbor, MI). The protein content in an aliquot of the homogenate was also determined using a protein assay kit (Pierce, Rockford, IL). Results were confirmed by three independent experiments.

GLYCEROL-3-PHOSPHATE DEHYDROGENASE (GPDH) ACTIVITY

hAMSCs that had been treated as described above were washed twice with PBS and harvested in 25 mM Tris buffer (pH 7.5) containing 1 mM EDTA and 1 mM DTT. The cells were then disrupted by sonication, after which they were centrifuged at 12,000*g* for 20 min at 4°C. Next, the GPDH activity in the supernatant was evaluated using GPDH activity assay kits (Takara Bio Inc., Ohtsu, Japan) following the method described by Kozak and Jensen, [1974]. The results were confirmed by four independent experiments.

CELL VIABILITY ASSAY

The general viability of cultured cells that were treated as described above was determined by the reduction of WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Dojindo Laboratories, Tokyo, Japan) to a highly water-soluble formazan dye. This assay was performed following an incubation of hAMSCs under the conditions described above. Next, 10 μl of WST-8 solution was added to each well, after which the samples were incubated at 37°C for 3 h, and the absorbance was measured at 450 nm using a spectrophotometer (Power Wave, Bio-tek Inc, Winooski, VT). The results were confirmed by four independent experiments.

GLYCEROL RELEASE ASSAY

Differentiated hAMSCs were treated with MesenPro RS media containing artemisinic acid for 2 days. The free glycerol content was determined using free glycerol reagent (Sigma-Aldrich). First, 0.8 ml of the warmed free glycerol reagent and 10 μl of cell medium in a test tube were incubated for 5 min at 37°C. After 5 min, the glycerol content was measured at an absorbance of 540 nm using a model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA).

RNA PREPARATION AND REAL-TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) (TAQMAN) ANALYSIS

Total cellular RNA was extracted from the hAMSCs 1 or 15 days after inducing differentiation with TRIzol reagent according to the manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA in a 20 μl reaction mix using random primers and Moloney murine leukemia virus reverse transcriptase. Real-time RT-PCR analysis was performed using an ABI7900HT machine (Applied Biosystems, Foster City, CA). All TaqMan RT-PCR reagents, including primers and probes, were purchased from Applied Biosystems. The TaqMan analysis was performed using predesigned and optimized Assays on Demand (Applied Biosystems). The following assays were used: CD36 (ID: Hs00169627_m1), PPAR γ (ID: Hs01115729_m1), C/EBP α (ID: Hs00269972_s1), C/EBP β (ID: Hs00942496_s1), C/EBP δ (ID: Hs00997180_s1), LPL (ID: Hs00173425_m1), aP2 (ID: Hs02559763_s1), CD36 (ID: Hs01567191_m1), glucose transporter 4 (GLUT4) (ID: Hs00168966_m1), vascular endothelial growth factor (VEGF) (ID: Hs00900055_m1), matrix metalloproteinase 2 (MMP2) (ID: Hs01548727_m1), MMP9 (ID: Hs00957555_m1), GAPDH (ID: Hs00266705_g1), liver X receptor (LXR) α (ID: Hs00172885_m1), hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Hs02800695_m1), 18S (Hs03003631_g1). The reaction parameters were as follows: 2 min at 50°C hold, 30 min at 60°C hold, and 5 min at 95°C hold, followed by 45 cycles of 20 s at 94°C for melting and 1 min at 60°C for annealing/extension. All measurements were performed in duplicate or triplicate, and the results were analyzed using the ABI Sequence Detector software version 2.0 (Applied Biosystems). Relative quantitation was performed as described previously [Langmann et al., 2003]. Results were first normalized to endogenous GAPDH expression level. Selected samples were tested against two additional housekeeping genes, 18S and HPRT, and the results were not different from the results obtained using GAPDH. The final results were then normalized to the controls.

PLASMIDS

Activator protein (AP)-1 Luc and nuclear factor (NF)-κB-Luc reporter plasmids were purchased from Stratagene (La Jolla, CA). The *Renilla* luciferase expression vector driven by a thymidine kinase promoter was purchased from Promega (Madison, WI). The pMEV-2HA expression vector, pMEVHA-JNK1-wt, pMEVHA-MEKK1-KD (constitutively active), and pMEVHA-JNKK2-wt were

purchased from Biomyx Technology (San Diego, CA). pMEVHA-C/EBP δ-wt and pMEVHA-C/EBP β-wt were obtained from Bioclone Inc. (San Diego, CA).

LUCIFERASE REPORTER ASSAY

To assay for AP-1 and NF-κB luciferase activities, hAMSCs were transfected with AP-1 (Stratagene) and NF-κB (Stratagene) along with 1 μg of the *Renilla* luciferase expression vector, which was driven by a thymidine kinase promoter (Promega) (internal standard), using the DharmFECT Duo transfection reagent (Thermo Fisher Scientific) according to the manufacturer's protocols. After a 24-h incubation, the cells were incubated with DMEM or MesenPro RS media in the presence or absence of the indicated concentrations of artemisinic acid for 20 h, after which luciferase activity was assayed using the Luciferase Assay System (Promega). The cells were then harvested, lysed, and centrifuged. The luciferase activity in the supernatant was measured using the Dual Luciferase Assay System (Promega) and a LB953 luminometer (Berthold, Bad Wildbad, Germany). Luciferase activity was expressed as the ratio of the AP-1 or NF-κB-dependent firefly luciferase activity to the control thymidine kinase *Renilla* luciferase activity (% control). Results were confirmed by eight independent transfections.

IMMUNOBLOTTING

Two-days post confluency, hAMSCs were treated with the indicated concentrations of artemisinic acid and then stimulated with STEM PRO® adipocyte differentiation media (Invitrogen, Carlsbad, CA) for 1 day. On day 1, the cells were washed twice with cold PBS, after which they were lysed in 150 μl of sample buffer (100 mM Tris-HCl, pH 6.8, 10% glycerol, 4% sodium dodecyl sulfate [SDS], 1% bromophenol blue, 10% β-mercaptoethanol). The samples were then resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon-P PVDF membranes (Millipore Corporation, Bedford, MA). The membranes were then incubated overnight at 4°C with anti-C/EBP β, anti-C/EBP δ, and anti-β-actin antibodies. The membranes were then washed three times with Tris-buffered saline containing Tween-20 (Sigma) (TBST), probed with horseradish peroxidase-conjugated secondary antibody, and developed using an ECL (enhanced chemiluminescence) western blotting detection system (Amersham Biosciences).

ELISA

The concentrations of IL-6 in the culture supernatant were measured using ELISA kits (Genzyme, Minneapolis, MN). Culture supernatants were added to 96-well plates and then diluted biotinylated IL-6 was added to the sample wells. The samples were incubated at room temperature for 3 h, and then the sample wells were washed. Streptavidin-HRP was added to the sample wells and the plate was incubated for 30 min at room temperature. The wells were then washed, and 3, 3′, 5, 5′-tetramethylbenzidine substrate solution was added. Finally, the samples were incubated in the dark, after which the absorbance at 450 nm was measured according to the manufacturer's instructions. The levels of phospho-SAPK/JNK(Thr183/Tyr185), phospho-p38 MAPK (Thr180/Tyr182), JNK, and p38 MAPK were measured using a PathScan Inflammation Multi-Target Sandwich enzyme linked immunosorbent assay (ELISA) Kit (Cell Signaling Technology) according to the manufacturer's instructions. The levels of phospho-p42/44 MAPK (Thr202/Tyr204) and p42/44 MAPK expression were also determined using a PathScan Cell Growth Multi-Target Sandwich ELISA Kit (Cell Signaling Technology) according to the manufacturer's instructions.

STATISTICAL ANALYSIS

All data are expressed as means ± SDs. Differences between the control and the treated group were evaluated by a Student's *t*-test using Statview software (Abacus Concepts, Piscataway, NJ). A $P < 0.05$ was considered statistically significant.

RESULTS

ARTEMISINIC ACID INHIBITS ADIPOGENIC DIFFERENTIATION

A. annua L. was selected as the candidate in the screen for anti-obesity agents from natural plants. Separation of the active compounds was performed using a series of silica gel column chromatographic steps, with final purification performed by HPLC to yield an active compound, which was determined to be artemisinic acid by GC–MS analysis (Fig. 1A). Two-days post confluency, hAMSCs (day 0) were treated with the indicated concentrations of artemisinic acid and then stimulated with adipocyte differentiation media for 3 days. The media were then replaced with adipocyte differentiation media, and the cells were treated with artemisinic acid every 3 days until the end of the experiment (day 15). After the preadipocytes differentiated into adipocytes, morphological alterations were observed due to the accumulation of lipid droplets in the cytoplasm. In particular, the cellular triglyceride content and GPDH enzyme activity assays revealed that artemisinic acid reduced triglyceride accumulation ($P < 0.05$) (Fig. 1B) and GPDH activity ($P < 0.05$) (Fig. 1C) on day 15 after full differentiation had occurred. A cell viability assay was conducted to exclude the possibility that these artemisinic acid effects were simply a consequence of cytotoxicity. As shown in Figure 1D, the tested concentrations of artemisinic acid induced no cytotoxic effects in hAMSCs. Additionally, lipid accumulation in artemisinic acid-treated cells was significantly lower than in the control cells, consistent with the observed reduction in triglyceride accumulation and GPDH activity (Fig. 1E). Taken together, these results indicate that artemisinic acid exerted an anti-adipogenic effect on hAMSCs.

EFFECTS OF ARTEMISINIC ACID ON THE EXPRESSION OF THE ADIPOGENIC TRANSCRIPTION FACTORS C/EBPα AND PPAR γ, AND THE PPAR γ TARGET GENES

Adipogenesis is driven by a complex transcriptional cascade that involves the sequential activation of CCAAT/enhancer binding protein (C/EBP)s and PPAR γ [Alessi et al., 2004], which are rapidly and transiently expressed after hormonal induction of differentiation. Time-course expression patterns of these adipogenic factors during differentiation were also confirmed in hAMSCs (Fig. 2A). These factors act synergistically to induce the expression of C/EBP α and PPAR γ, the master adipogenic transcription regulators, which, in turn, promote terminal differentiation by activating the

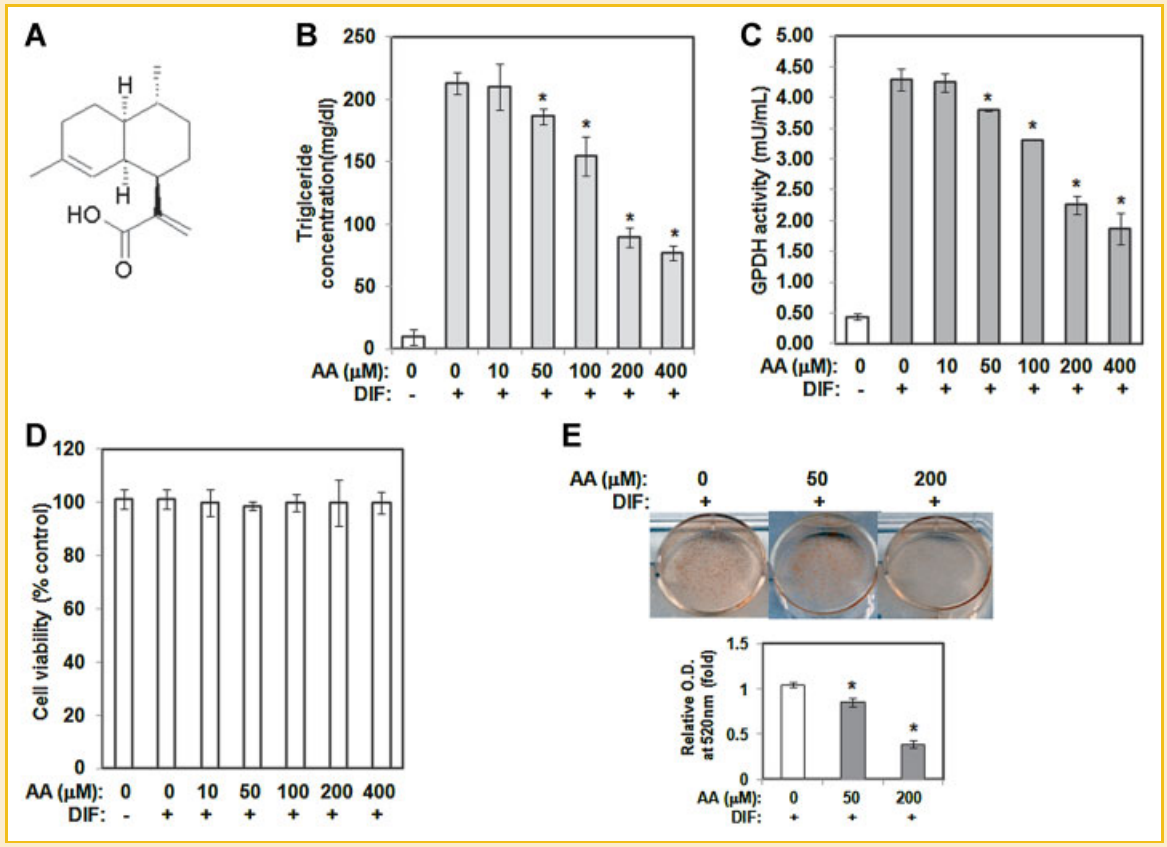

Fig. 1. Artemisinic acid inhibits adipocyte differentiation. Two-days post confluency, human adipose tissue-derived mesenchymal stem cells (hAMSCs) (day 0) were treated with the indicated concentrations of artemisinic acid and then stimulated with STEM PRO adipocyte differentiation media for 3 days. The medium was then replaced with STEM PRO adipocyte differentiation media every 3 days until the end of the experiment (day 15). Assays were performed on fully differentiated adipocytes (day 15). A: Chemical structure of artemisinic acid. B: Triglyceride content was measured using a triglyceride assay kit. Data are expressed as means ± SDs. * $P < 0.05$ versus controls. Results were verified by three repetitions of the experiments, each of which was conducted in triplicate. C: Glycerol-3-phosphate dehydrogenase (GPDH) activity was measured using GPDH activity assay kits. Data are expressed as means ± SDs. * $P < 0.05$ versus controls. Results were verified by repeating the experiment four times. D: The general viability of cultured cells was determined by the reduction of WST-8 to a highly water-soluble formazan dye. Data are expressed as means ± SDs. * $P < 0.05$ versus controls. Results were verified by repeating the experiment four times. E: Intracellular lipids were stained with Oil Red O. The results were confirmed by three independent experiments, which were each conducted in duplicate. DIF, differentiation media; AA, artemisinic acid.

transcription of genes involved in the creation and maintenance of the adipocyte phenotype. Loss-of-function studies have shown that PPAR γ is necessary and sufficient to promote adipogenesis, and that C/EBP α plays a role maintaining PPAR γ expression. Therefore, we examined the effects of artemisinic acid on the expression of adipokines including PPAR γ. In these experiments, we first evaluated the effects of artemisinic acid on PPAR γ and C/EBP α expression. As shown in Figure 2B, the PPAR γ mRNA level was significantly lower in cells treated with artemisinic acid during adipocyte differentiation than that in control cells. Additionally, C/EBP α mRNA levels decreased significantly following artemisinic acid treatment. Taken together, these findings indicate that PPAR γ and C/EBP α are involved in the anti-adipogenesis effects of artemisinic acid.

Activation of PPAR γ induces the expression of genes that control adipocyte fatty acid metabolism, including lipoprotein lipase (LPL), fatty acid translocase (CD36), and LXR α. In the present study, we demonstrated that artemisinic acid reduced PPAR γ expression during adipogenesis. Therefore, we evaluated this down-regulation to determine if it was related to the decrease in the mRNA levels of PPAR γ target genes. We found that aP2, LPL, CD36, and LXR α mRNA levels were significantly lower during adipocyte differentiation following artemisinic acid treatment (Fig. 2C,D). Collectively, these results indicate that PPAR γ expression plays an important role in the regulation of adipocyte differentiation by artemisinic acid.

ARTEMISINIC ACID DOWNREGULATES C/EBP δ EXPRESSION WITHOUT IMPACTING C/EBP β DURING ADIPOCYTE DIFFERENTIATION

The rapid and transient induction of C/EBP β and δ expression is one of the earliest events in adipogenesis [Spiegelman and Flier, 2001]. These transcription factors bind to specific sequences in the C/EBP α and PPAR γ2 gene promoters and induce their expression [Tyrrell and Reeve, 2006; Potapovich et al., 2009; Zhong et al., 2010]. Therefore, we investigated the effects of artemisinic acid on C/EBP β and δ expression, which are upstream molecules of C/EBP α and PPAR γ. As shown in Figure 2E, hAMSCs treated with artemisinic acid had significantly lower levels of C/EBP δ mRNA during

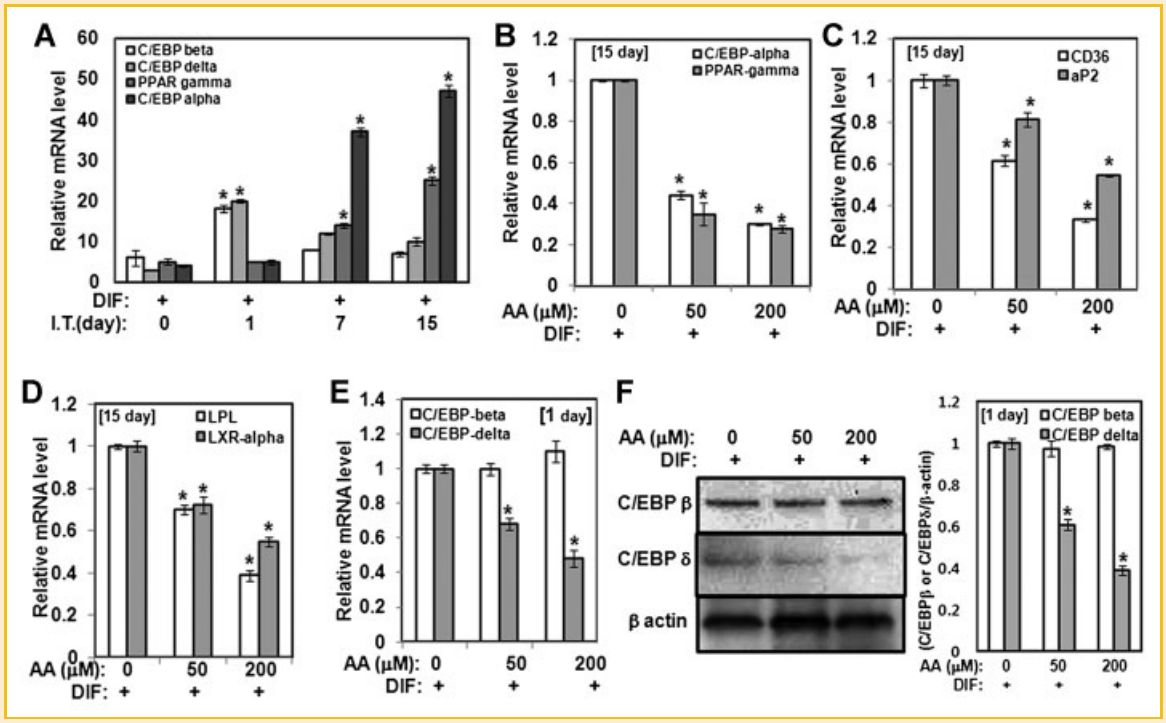

Fig. 2. Effects of artemisinic acid on the expression of the adipogenic transcription factors. Human adipose tissue-derived mesenchymal stem cells (hAMSCs) (day 0) were treated with the indicated concentrations of artemisinic acid and then stimulated with STEM PRO® adipocyte differentiation media for 3 days. The medium was replaced with STEM PRO® adipocyte differentiation media every 3 days until the end of the experiment (day 15). A: Time-course expression patterns of adipogenic factors during the adipogenesis. PPAR γ, C/EBP α, C/EBP β, and C/EBP δ gene mRNA levels were measured by real-time quantitative RT-PCR. Results are expressed relative to untreated cells after normalization against GAPDH. Data are expressed as the means ± SDs. * $P < 0.05$ versus controls. Results were verified by repeating the experiment four times, each of which was conducted in triplicate. B,C,D: Total RNA was isolated 15 days after the induction of differentiation, and the mRNA levels of the PPAR γ (B), C/EBP α (B), aP2 (C), LPL (D), CD36 (C), and LXR α (D) genes were measured by real-time quantitative RT-PCR. Results are expressed relative to untreated cells after normalization against GAPDH. Data are expressed as means ± SDs. * $P < 0.05$ versus controls. Results were verified by repeating the experiment four times, each of which was conducted in triplicate. E: At 1 day after the induction of differentiation, total RNA was isolated and the C/EBP β and C/EBP δ gene mRNA levels were measured by real-time quantitative RT-PCR. Results are expressed relative to untreated cells after normalization against the GAPDH. Data are expressed as means ± SDs. * $P < 0.05$ versus controls. Results were verified by repeating the experiment four times, each of which was conducted in triplicate. F: Cell lysates were analyzed by western blot analysis using the indicated antibodies at day 1 after the induction of differentiation. Results were verified by repeating the experiments three times, each of which was conducted in duplicate. I.T. (day), incubation time with the DIF; DIF, differentiation media; AA, artemisinic acid.

adipocyte differentiation than those in control cells. However, C/EBP β mRNA levels were not affected by artemisinic acid. Consistent with these findings, artemisinic acid reduced C/EBP δ protein levels without affecting C/EBP β protein levels (Fig. 2F). These findings suggest that artemisinic acid exerted its anti-adipogenic effects by downregulating the C/EBP δgene. To confirm this, we investigated the effects of overexpressed C/EBP δ on artemisinic acid-induced effects. As shown in Figure 3A,B, the artemisinic acid-induced reduction in triglyceride content and GPDH activity was significantly attenuated by C/EBP δ overexpression. In addition, expression of the PPAR γ gene was increased by overexpressing C/EBP δ (Fig. 3C). However, overexpressed C/EBP β showed no effects. These results indicate that artemisinic acid inhibited adipogenesis in hAMSCs by regulating the C/EBP δ gene.

REDUCED EXPRESSION OF C/EBP δ BY ARTEMISINIC ACID IS MEDIATED THROUGH THE INHIBITION OF JNK ACTIVATION

C/EBP δ gene expression is regulated by AP-1 and NF-κB (Fig. 4A) [Liu et al., 2007]. Therefore, AP-1 and NF-κB luciferase reporter assays were conducted to determine if the inhibitory effect of artemisinic acid on C/EBP δ gene expression was mediated by the inhibition of NF-κB or AP-1. While artemisinic had no effect on NF-κB activity, AP-1 activity decreased following artemisinic acid treatment, suggesting that the effects of artemisinic acid involve AP-1 (Fig. 4B,C). In addition, phorbol myristate acetate-induced AP-1 activation was not affected by artemisinic acid, suggesting that the inhibitory effects of artemisinic acid on AP-1 activation are not dependent on protein kinase C. An ELISA for MAPKs was conducted to further confirm these findings. While artemisinic acid had no effect on phosphorylated p42/44 MAPK and p38 MAPK, the level of phosphorylated JNK was reduced by artemisinic acid in a concentration-dependent manner (Fig. 4D). These findings suggest that the decrease in C/EBP δ expression in response to artemisinic acid treatment may be dependent on JNK inhibition. Additionally, the AP-1 luciferase reporter assay and real-time PCR assay for C/EBP δ were employed with hAMSCs to elucidate a possible mechanism underlying the artemisinic acid-mediated JNK inactivation signaling. In these experiments, several molecules such as constitutively active MEKK-1-KD, JNKK-2, and JNK1 which are involved in AP-1

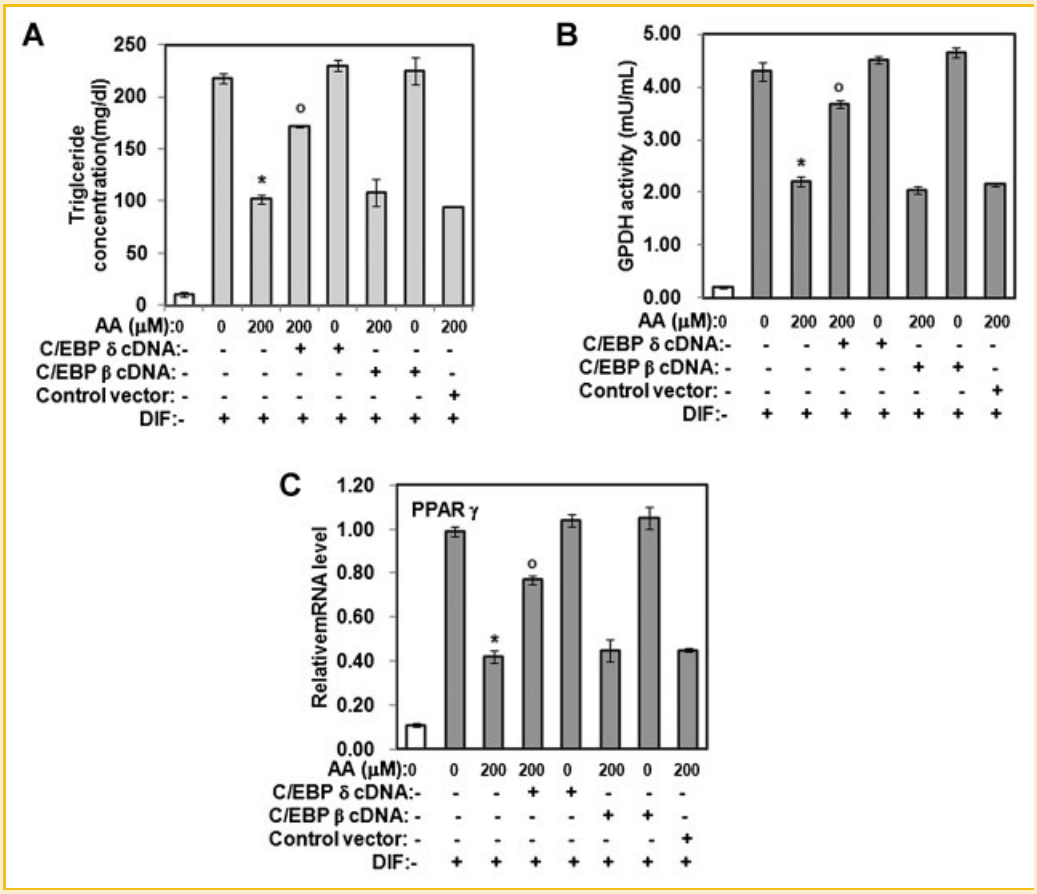

Fig. 3. Artemisinic acid inhibits adipogenesis by downregulating the C/EBP δ gene. Human adipose tissue-derived mesenchymal stem cells (hAMSCs) (day 0) were transfected with expression plasmid for the C/EBP δ, and C/EBP β genes using the DharmFECT Duo transfection reagent. After a 2-day incubation, cells were treated with artemisinic acid (200 μM) and then stimulated with STEM PRO adipocyte differentiation media for 3 days. A: After a 3-day incubation, triglyceride content was measured using a triglyceride assay kit. Data are expressed as means ± SDs. * $P < 0.05$ versus untreated controls; ° $P < 0.05$ versus artemisinic acid-treated controls. Results were verified by three repetitions of the experiments, each of which was conducted in triplicate. B: After a 3-day incubation, glycerol-3-phosphate dehydrogenase (GPDH) activity was measured using GPDH activity assay kits. Data are expressed as means ± SDs. * $P < 0.05$ versus untreated controls; ° $P < 0.05$ versus artemisinic acid-treated controls. Results were verified by repeating the experiment four times. C: After a 3-day incubation, total RNA was isolated and PPAR γ gene mRNA levels of were measured by real-time quantitative RT-PCR. Data are expressed as the means ± SDs. * $P < 0.05$ versus untreated controls; ° $P < 0.05$ versus artemisinic acid-treated controls. Results were verified by repeating the experiment four times, each of which was conducted in triplicate.

activation, were also introduced. As shown in Figure 5A, MEKK-1-KD-induced reporter activation of AP-1 was attenuated by artemisinic acid. AP-1 activation induced by MEKK-1-KD-JNKK-2 and MEKK-1-KD–JNK1, respectively, was also reduced by artemisinic acid. Consistent with these results, artemisinic acid inhibited the expression of C/EBP δ induced by MEKK-1-KD, MEKK-1-KD-JNKK-2, and MEKK-1-KD-JNK1, respectively. These results indicate that artemisinic acid operates downstream of MEKK-1. Additionally, the involvement of JNK in the anti-adipogenic effects of artemisinic acid was demonstrated in Figure 5C. SP600125, a JNK inhibitor, reduced adipogenic differentiation of hAMSCs.

ARTEMISINIC ACID EXERTS ITS INHIBITORY EFFECTS ON AN EARLY STEP OF ADIPOGENESIS

The results described above demonstrated that C/EBP δ plays an important role in the anti-adipogenesis effect of artemisinic acid and suggest that artemisinic acid functions during an early step of adipogenesis. The cellular triglyceride content and GPDH enzyme activity were measured and Oil Red O staining assays were performed to better understand the mechanism of action of artemisinic acid during adipogenesis. Artemisinic acid was added to differentiating cells on days 0, 3, 6, and 9. As shown in Figure 6B–D, the results revealed that artemisinic acid treatment (days 3, 6, and 9) during the relatively late stages of adipocyte differentiation did not alter adipogenesis, suggesting that artemisinic acid had no effect on late adipocyte differentiation. However, artemisinic acid treatment on day 0 significantly inhibited adipogenesis. These findings indicate that artemisinic acid inhibits an early event, specifically C/EBP δ gene expression during adipocyte differentiation.

ARTEMISINIC ACID ATTENUATES hAMSC DIFFERENTIATION-DEPENDENT EXPRESSION OF GLUT4 AND VEGF AND IMPAIRS GELATINASE MMP-2 EXPRESSION

hAMSCs were differentiated using the differentiation cocktail in combination with artemisinic acid to study the effects of artemisinic

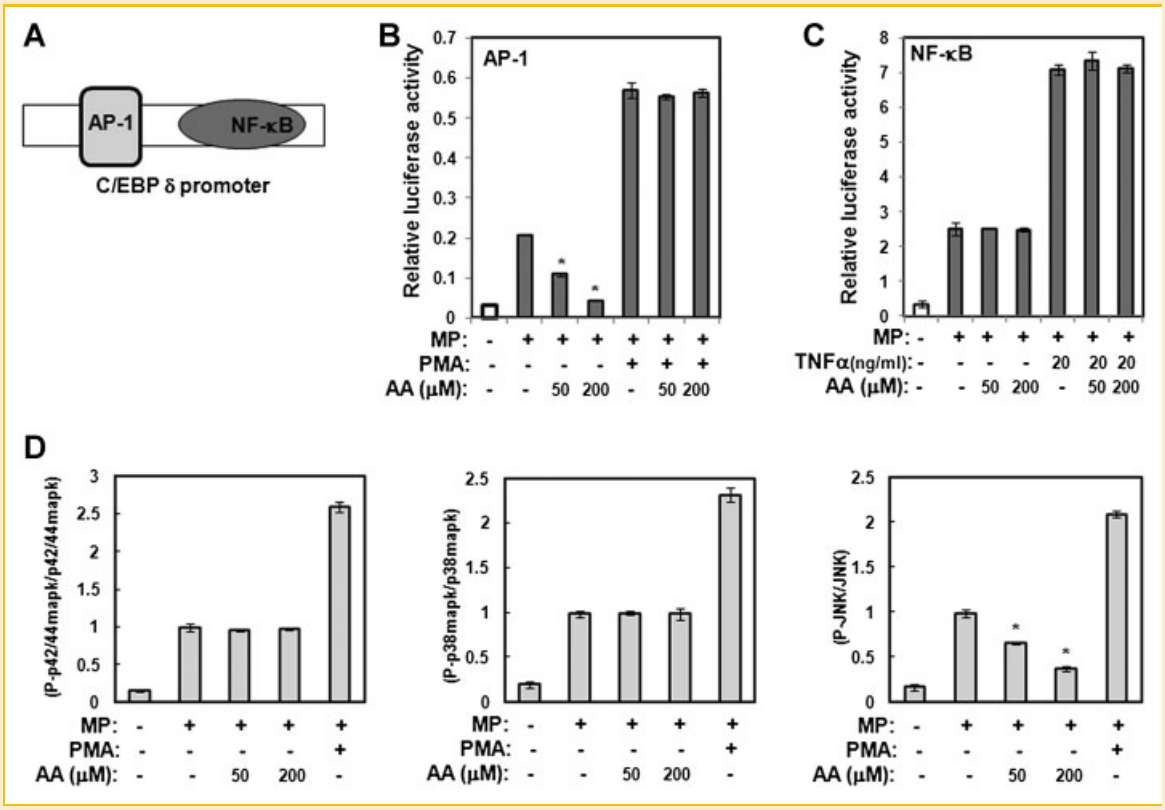

Fig. 4. Reduced expression of C/EBP δ by artemisinic acid is mediated through the inhibition of JNK activation. A: Structure of the C/EBP δ gene promoter. B,C: Human adipose tissue-derived mesenchymal stem cells (hAMSCs) were cotransfected with luciferase constructs that contained AP-Luc reporter and NF-κB-Luc reporter plasmids. After 16 h, the transfected cells were incubated for 20 h with the indicated concentrations of artemisinic acid and then further incubated with MesenPro RS media for 1 day. One day after the incubation in the presence of MesenPro RS media, the cells were harvested and lysed. Luciferase activity was expressed as the ratio of AP-1 or NF-κB promoter-dependent firefly luciferase activity divided by the control thymidine kinase *Renilla* luciferase activity (relative luciferase units). Data are expressed as the means ± SDs. * $P < 0.05$ versus controls. The experiments were repeated eight times, with each experiment being conducted in triplicate. PMA, phorbol myristate acetate. D: Total lysates were analyzed using a Multi-Target Sandwich ELISA Kit 40 min after incubation with the indicated samples in the presence or absence of MesenPro RS™ media. Results were verified by repeating the experiments three times. Data are expressed as means ± SDs. * $P < 0.05$ versus controls. MP, MesenPro RS media; AA, artemisinic acid.

acid on the differentiation-associated expression of GLUT4 and VEGF. As shown by the real-time PCR analysis, both marker genes, GLUT4 and VEGF, decreased significantly following artemisinic acid treatment (Fig. 7A), indicating that artemisinic acid attenuated adipogenesis.

The proteolytic activity of gelatinase is important in the development of adipose tissue [Croissandeau et al., 2002]. Therefore, we tested the effects of artemisinic acid on MMP-2 and MMP-9 expression by hAMSCs and adipocyte differentiation. As shown in Figure 7A, gelatinase MMP-2 expression was reduced by artemisinic acid. However, MMP-9 gene expression was not detected (data not shown).

ARTEMISINIC ACID INHIBITS IL-6 PRODUCTION BY UNDIFFERENTIATED hAMSCs

Obesity has been linked to chronic inflammation and pro-inflammatory cytokine secretion by preadipocytes, which contributes to insulin resistance. Therefore, we investigated the impact of artemisinic acid on the production of IL-6 by undifferentiated hAMSCs. As shown in Figure 7B, artemisinic acid alone had no effect on IL-6 production in undifferentiated hAMSCs. Undifferentiated hAMSCs released high amounts of IL-6 when treated with 20 ng/ml TNF α. Combined artemisinic acid and TNF α led to significantly lower IL-6 concentrations in the supernatants of undifferentiated hAMSCs when compared to those in the control. Taken together, these results indicate that artemisinic acid not only has direct anti-adipogenic activity but also additional anti-inflammatory activities.

ARTEMISINIC ACID IS NOT INVOLVED IN LIPOLYSIS OF DIFFERENTIATED hAMSCs

We found that artemisinic acid regulates adipogenic differentiation of hAMSCs. However, the effects of artemisinic acid on differentiated adipocytes are unknown. Therefore, we also examined the effects of artemisinic acid on lipolysis in differentiated hAMSCs. As shown in Figure 7C, artemisinic acid had no effect on lipolysis in differentiated hAMSCs. However, IBMX which was introduced as a positive control for lipolysis, increased glycerol release. These results indicate that the effects of artemisinic acid were limited to preadipocytes and differentiating cells.

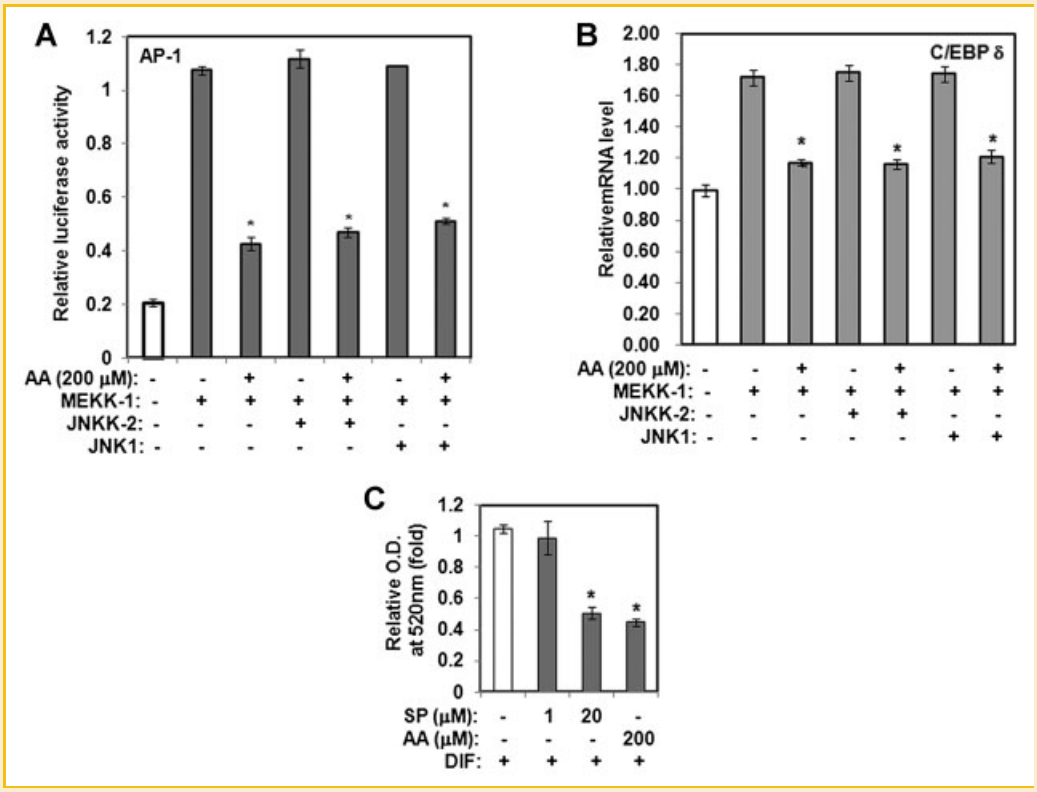

Fig. 5. Artemisinic acid operates downstream of MEKK-1 in JNK activation signaling. A: Human adipose tissue-derived mesenchymal stem cells (hAMSCs) were cotransfected with AP-Luc and expression plasmids for constitutively active MEKK-1-KD, JNKK-2, and JNK-1, along with the *Renilla* luciferase expression vector driven by a thymidine kinase promoter using the DharmFECT Duo transfection reagent. After a 48-h incubation, cells were stimulated with the indicated concentrations of artemisinic acid for 14 h. These cells were then harvested, lysed, and assayed. Results were confirmed by three independent transfections. Data are expressed as means ± SDs. $^{*}P < 0.05$ compared to the MEKK-1-KD, MEKK-1-KD/JNKK-2, or MEKK-1-KD/JNK-1-transfect controls. B: hAMSCs were transfected with expression plasmids for MEKK-1-KD, MEKK-1-KD/JNKK-2, and MEKK-1-KD/JNK-1 using the DharmFECT Duo transfection reagent. After a 48-h incubation, the cells were stimulated with artemisinic acid (200 μM) and then stimulated with STEM PRO® adipocyte differentiation media for 3 days. Three days after inducing differentiation, total RNA was isolated and C/EBP δ mRNA levels were measured by real-time quantitative RT-PCR. Results are expressed relative to untreated cells after normalization against GAPDH. Data are expressed as means ± SDs. $^{*}P < 0.05$ compared to the MEKK-1-KD, MEKK-1-KD/JNKK-2, or MEKK-1-KD/JNK-1-transfect controls. Results were verified by repeating the experiment four times, each of which was conducted in triplicate. C: Intracellular lipids were stained with Oil Red O. Two-days post confluency, hAMSCs (day 0) were treated with the indicated concentrations of SP600125 and 200 μM artemisinic acid and then stimulated with STEM PRO adipocyte differentiation media for 3 days. The medium was then replaced with STEM PRO adipocyte differentiation media every 3 days until the end of the experiment (day 15). Assays were performed on fully differentiated adipocytes (day 15). The results were confirmed by three independent experiments, which were each conducted in duplicate. DIF, differentiation media; AA, artemisinic acid; SP, SP600125.

DISCUSSION

The results of this study provide direct evidence of the effects of artemisinic acid on adipocyte differentiation, as well as its inhibitory mechanisms on adipogenesis in hAMSCs. The findings demonstrated that artemisinic acid downregulated C/EBP δ gene expression by inhibiting JNK, after which it reduced adipogenic gene expression, consequently inhibiting adipocyte differentiation. The cytosolic enzyme GPDH plays a central role in the triglyceride synthesis pathway and is linked to the characteristic changes that occur during adipose conversion [Kozak and Jensen, 1974]. Here, we demonstrated that artemisinic acid treatment led to significantly lower GPDH activity and triglyceride content in adipocytes.

Members of the C/EBP family of transcription factors are involved in the regulation of various aspects of cellular differentiation and inflammation [Schrem et al., 2004]. There are at least six C/EBP family members (α, β, δ, γ, ε, ζ) that form homo- or heterodimers with C/EBPs or other leucine zipper proteins and bind to DNA to activate or repress transcription [Ramji and Foka, 2002]. Studies targeting C/EBP δ in mice have shown that it plays an essential role in the control of mammary epithelial cell growth in the mouse nulliparous mammary gland [Gigliotti et al., 2003]. Loss of C/EBP δ causes genome instability and centrosome amplification in primary embryonic fibroblasts derived from 129S1 mice [Huang et al., 2004]. C/EBP β and C/EBP δ play a synergistic role in terminal adipocyte differentiation in vivo [Tanaka et al., 1997]. Consistent with these findings, C/EBP δ gene expression was inhibited by artemisinic acid treatment in this study. However, C/EBP β expression was not affected by artemisinic acid. Moreover, the mRNA levels of PPAR γ and C/EBP α, downstream C/EBP β and C/EBP δ molecules, were reduced by artemisinic acid. The results of this study may have important implications for adipocyte biology, as well as for the role of C/EBP δ in artemisinic acid-induced anti-adipogenesis.

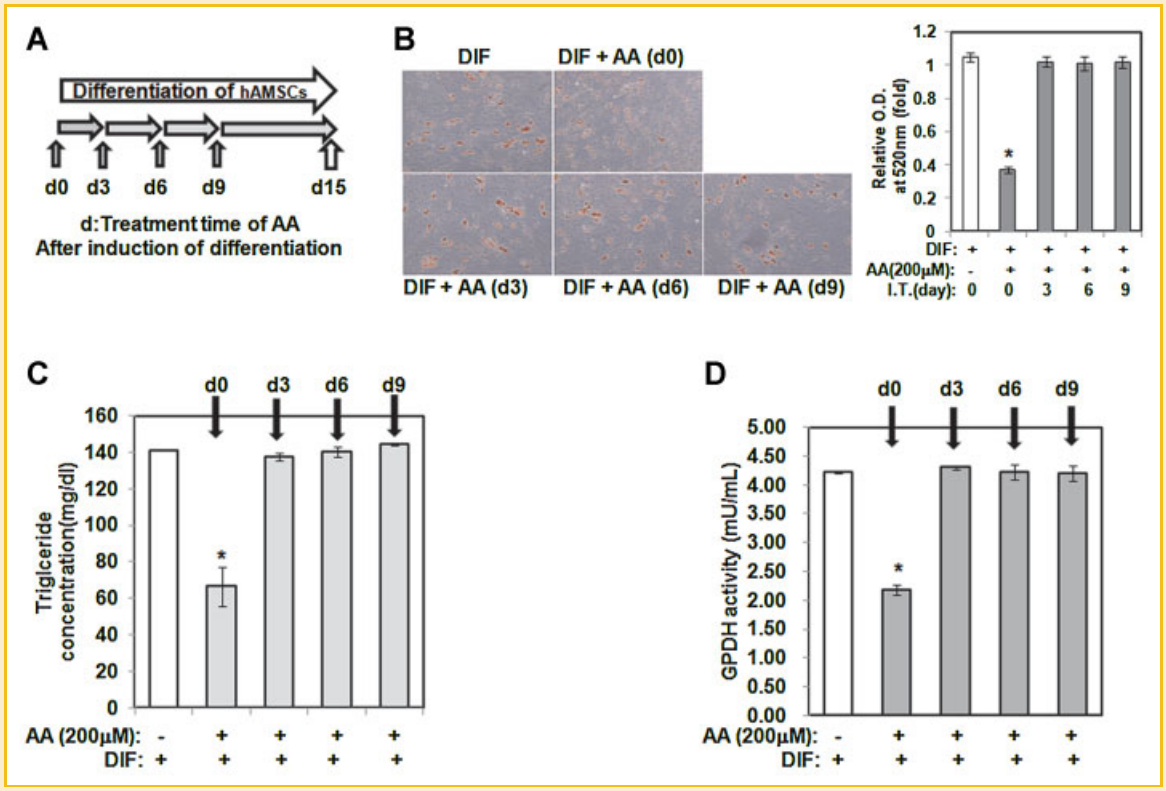

Fig. 6. Artemisinic acid exerts its inhibitory effects on an early step of adipogenesis. Two-days post confluency, human adipose tissue-derived mesenchymal stem cells (hAMSCs) (day 0) were treated with 200 μM artemisinic acid at the indicated times (day 3, 6, or 9) and then stimulated with STEM PRO adipocyte differentiation media for 3 days. The medium was replaced with STEM PRO adipocyte differentiation media every 3 days until the end of the experiment (day 15). The assays were performed on fully differentiated adipocytes (day 15). A: Experimental scheme. AA, artemisinic acid; d: artemisinic acid treatment day. B: Intracellular lipids were stained with Oil Red O. Results were confirmed by three independent experiments, which were each conducted in duplicate. DIF, differentiation media; AA, artemisinic acid. C: Triglyceride content was measured using a triglyceride assay kit. Data are expressed as means ± SDs. * $P < 0.05$ versus controls. Results were verified by repeating the experiments three times, each of which was conducted in triplicate. D: GPDH activity was measured using GPDH activity assay kits. Data are expressed as means ± SDs. * $P < 0.05$ versus controls. Results were verified by four repetitions of the experiments.

Various natural compounds have been hypothesized to exert anti-obesity effects by inhibiting preadipocyte differentiation [Lin et al., 2005; Yang et al., 2006, 2007; Rayalam et al., 2007, 2008]. In our study, artemisinic acid inhibited the generation of intracellular lipids during hormone-induced adipocyte differentiation. Further evidence of impaired adipogenesis was observed in the real-time PCR analysis of GLUT4, which is a marker gene of late-stage preadipocyte differentiation [Rea and James, 1997; Fernyhough et al., 2007], and VEGF, which is abundantly expressed in mature adipocytes and in minor amounts in preadipocytes [Miyazawa-Hoshimoto et al., 2005]. The presence of artemisinic acid during hAMSC differentiation reduced GLUT4 and VEGF expression, confirming that artemisinic acid has an anti-adipogenic effect. Additionally, gelatinases (MMP-2 and MMP-9), produced from (pre)adipocytes, are required for the initiation of adipogenesis [Bouloumie et al., 2001; Croissandeau et al., 2002; Bourlier et al., 2005]. The overall reduced expression of MMP-2 in hAMSCs, due to the presence of artemisinic acid in the hormone cocktail treatment, confirmed that artemisinic acid impaired adipogenic differentiation of hAMSCs.

Recent studies have closely linked obesity and metabolic syndrome to inflammation [Rotter et al., 2003; Wellen and Hotamisligil, 2003; Sopasakis et al., 2004]. Proinflammatory stimuli such as leptin, TNF α, and IL-6, which are overproduced during obesity, contribute to the pathogenesis of insulin resistance [Hotamisligil et al., 1993; Bastard et al., 2006]. Therefore, our finding that artemisinic acid significantly reduced IL-6 release from undifferentiated hAMSCs treated with TNF α suggests that artemisinic acid may also have an additional anti-inflammatory effect.

Many attempts have been made to correct the metabolic disparity that occurs in obesity using reagents such as sibutramine (appetite suppressor), orlistat (gastrointestinal lipid inhibitor), and fibrates (PPAR α agonist) [Chapman, 2003; Padwal and Majumdar, 2007]. However, administering these drugs frequently causes undesirable side effects such as a dry mouth, anorexia, constipation, insomnia, dizziness, and nausea [Bray, 2001]. Therefore, there is a high demand for therapeutically potent yet safe anti-obesity reagents. Our in vitro experimental data indicate that artemisinic acid inhibited adipogenesis and the expression of adipokines through the PPAR γ pathway by downregulating the C/EBP δ gene through the inhibition of JNK, implying that artemisinic acid may be beneficial for reducing diet-induced obesity via regulation of adipocyte differentiation.

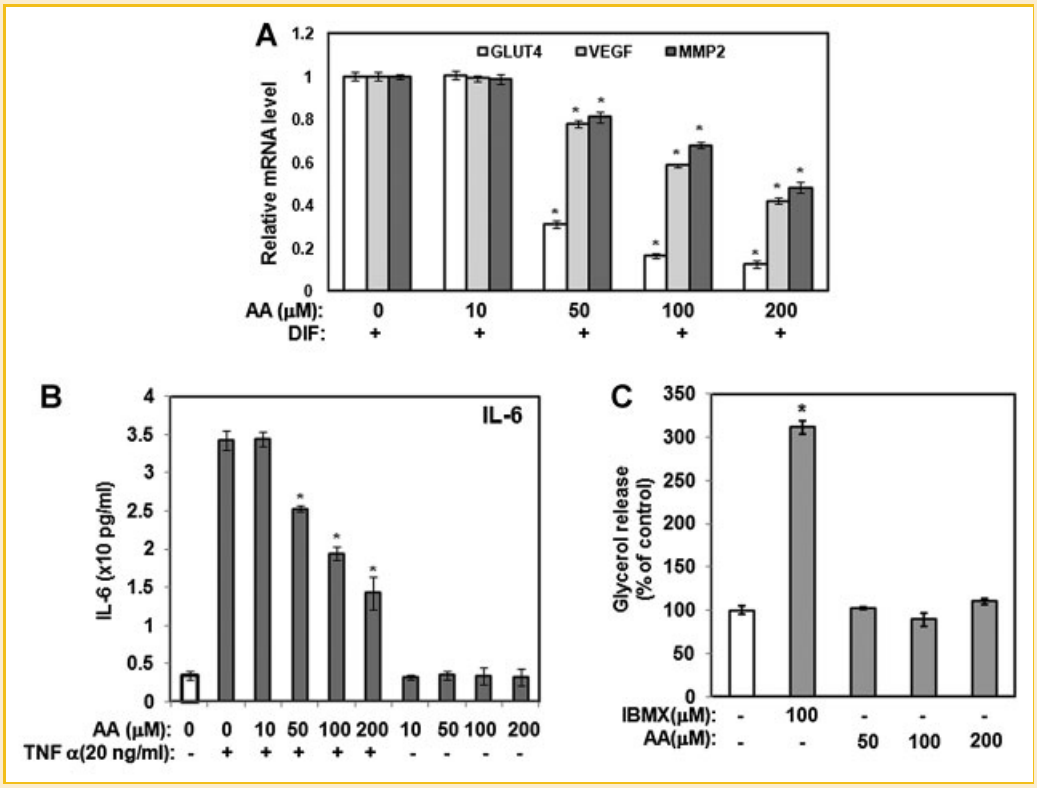

Fig. 7. Artemisinic acid attenuates the human adipose tissue-derived mesenchymal stem cell (hAMSC) differentiation-dependent expression of glucose transporter 4 (GLUT4) and vascular endothelial growth factor (VEGF), impairs expression of gelatinase matrix metalloproteinase (MMP)-2 and exerts no effects on lipolysis. A: hAMSCs (day 0) were treated with the indicated concentrations of artemisinic acid and then stimulated with STEM PRO adipocyte differentiation media for 3 days. The medium was replaced with STEM PRO adipocyte differentiation media every 3 days until the end of the experiment (day 15). Total RNA was isolated and the GLUT4, VEGF, and MMP2gene mRNA levels were measured by real-time quantitative RT-PCR 15 days after the induction of differentiation. Results are expressed relative to untreated cells after normalization against GAPDH. Data are expressed as means ± SDs. * $P < 0.05$ versus controls. Results were verified by repeating the experiment four times, each of which was conducted in triplicate. DIF, differentiation media; AA, artemisinic acid. B: hAMSCs (day 0) were treated with the indicated concentrations of artemisinic acid and then stimulated with tumor necrosis factor (TNF) α (20 ng/ml) for 2 days. Two days after induction, the supernatants were harvested for IL-6 measurement. Data are expressed as means ± SDs. * $P < 0.05$ versus controls. Results were verified by repeating the experiment three times, each of which was conducted in duplicate. AA, artemisinic acid. (C) Differentiated hAMSCs were treated with MesenPro RS™ media containing the indicated concentrations of artemisinic acid for 2 days. Glycerol content was measured using a glycerol assay kit. Data are expressed as means ± SDs. * $P < 0.05$ versus controls. Results were verified by four independent experiments. IBMX, 1-methyl-3-(2-methylpropyl)-7H-purine-2,6-dione; AA, artemisinic acid.

Taken together, the results of our study demonstrate that artemisinic acid inhibited adipogenic differentiation of hAMSCs by reducing the C/EBP δ mRNA level. These results show that the anti-adipogenesis induced by artemisinic acid was mediated by reduced expression of C/EBP δ, which was mediated by the inhibition of JNK.

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