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Artepillin C, as a PPARγ ligand, enhances adipocyte differentiation and glucose uptake in 3T3-L1 cells

Sun-Sil Choi [a], Byung-Yoon Cha [a], Kagami Iida [a], Young-Sil Lee [a], Takayuki Yonezawa [a], Toshiaki Teruya [a], Kazuo Nagai [a,b], Je-Tae Woo [a,b,c,*]

[a] Research Institute for Biological Functions, Chubu University, 1200 Matsumoto, Kasugai, Aichi 487-8501, Japan
[b] Department of Biological Chemistry, Chubu University, 1200 Matsumoto, Kasugai, Aichi 487-8501, Japan
[c] Department of Research and Development, Erina Co., Inc., 1-9-2 Higashi-Shinbashi, Minato-ku, Tokyo 105-0021, Japan

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ABSTRACT

The nuclear receptor peroxisome proliferator-activated receptor (PPAR) γ plays an important role in adipocyte differentiation. Its ligands, including thiazolidinediones, improve insulin sensitivity in type 2 diabetes. We investigated the effects of artepillin C, an ingredient of *Baccharis dracunculifolia*, on adipogenesis and glucose uptake using 3T3-L1 cells. In PPARγ ligand-binding assays, artepillin C exhibited binding affinity toward PPARγ. Artepillin C dose-dependently enhanced adipocyte differentiation of 3T3-L1 cells. As a result of the artepillin C-induced adipocyte differentiation, the gene expression of PPARγ and its target genes, such as aP2, adiponectin and glucose transporter (GLUT) 4, was increased. These increases were abolished by cotreatment with GW9662, a PPARγ antagonist. In mature 3T3-L1 adipocytes, artepillin C significantly enhanced the basal and insulin-stimulated glucose uptake. These effects were decreased by cotreatment with a PI3K inhibitor. Although artepillin C had no effects on the insulin signaling cascade, artepillin C enhanced the expression and plasma membrane translocation of GLUT1 and GLUT4 in mature adipocytes. In conclusion, these findings suggest that artepillin C promotes adipocyte differentiation and glucose uptake in part by direct binding to PPARγ, which could be the basis of the pharmacological benefits of green propolis intake in reducing the risk of type 2 diabetes.

1. Introduction

Peroxisome proliferator-activated receptor (PPAR) γ is a transcription factor that belongs to a subfamily of nuclear hormone receptors. It is predominantly expressed in adipose tissue and plays a central role in adipose tissue functions [1]. Heterodimers of PPARγ with retinoid X receptor (RXR) are activated by binding to ligands, which results in a conformational change of PPARγ and the regulation of gene transcription. PPARγ is the master regulator of adipocyte differentiation, and enhances the numbers of insulin-sensitive small adipocytes [1]. In addition, PPARγ activation in mature adipocytes regulates several genes involved in the insulin signaling cascade and glucose and lipid metabolism [2,3]. PPARγ ligands such as thiazolidinediones (TZDs) are known to have potent antihyperglycemic activity *in vivo* by enhancing insulin sensitivity in peripheral tissues, thus providing an effective therapy for the treatment of type 2 diabetes mellitus [4]. In recent studies, compounds derived from natural sources have been reported to enhance insulin sensitivity, and to promote adipocyte differentiation by activating PPARγ [5–9]. Among these bioactive compounds, emodin [6], magnolol [8] and luteolin [10] have been shown to exhibit PPARγ ligand activity and enhance glucose uptake in adipocytes.

Glucose uptake in insulin-responsive tissues such as skeletal muscle and adipose tissue is essential for the maintenance of whole-body glucose homeostasis. It is mediated by some members of a family of facilitated carrier proteins, glucose transporter (GLUT) 1–12 [11], which have different affinities for their substrates, tissue-specific expressions and regulatory processes. Among them, GLUT1 and GLUT4, the two main glucose transporter isoforms expressed in adipose tissue, play central roles in peripheral glucose disposal and have been extensively studied. GLUT1 is a constitutive glucose transporter that is present on the plasma membrane (PM) and intracellular membranes, while GLUT4 is an insulin-sensitive glucose transporter that is sequestered in intracellular vesicles in the absence of insulin [12,13]. Insulin triggers GLUT4 translocation to the plasma membrane (PM) by the phosphatidylinositol 3′ kinase (PI3K)/Akt pathway [12]. In several studies, overexpression of

* Corresponding author at: Department of Biological Chemistry, Chubu University, 1200 Matsumoto, Kasugai, Aichi 487-8501, Japan.
Tel.: +81 568 51 6189; fax: +81 568 52 6594.
E-mail address: jwoo@isc.chubu.ac.jp (J.-T. Woo).

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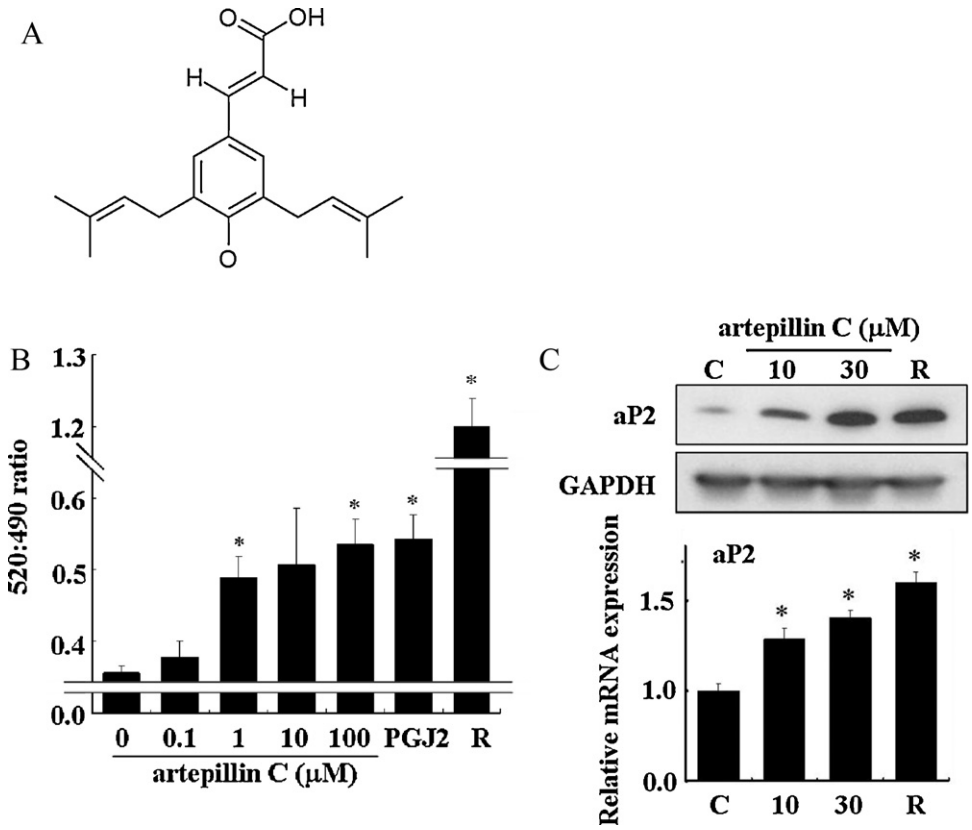

Fig. 1. Chemical structure and ligand-binding activity of artepillin C. (A) Chemical structure of artepillin C. (B) PPARγ ligand-binding activity of artepillin C. The binding affinity of artepillin C at the indicated concentrations toward the PPARγ-LBD was measured using the Lanthascreen™ TR-FRET Peroxisome Proliferator Activated Receptor γ Coactivator Assay. All values are presented as means ± SD ($n = 3$). The data shown are representative of two independent experiments. *$p < 0.05$ *vs.* the untreated group. PGJ2, 10 μM 15-deoxy-Δ12,14-prostaglandin J2; R, 1 μM rosiglitazone. (C) Artepillin C increases the expression of aP2. Post-confluent 3T3-L1 preadipocytes were incubated in differentiation medium for 2 days and the medium was then changed to DMEM containing insulin. The medium was then replaced with DMEM after another 2 days. Fully differentiated adipocytes were treated with 10 μM artepillin C for 48 h, and the total RNA and proteins were extracted. The expression of aP2 was analyzed by real-time PCR and immunoblotting. All values are presented as means ± SE ($n = 3$). *$p < 0.05$ *vs.* the control group. C, control (vehicle); R, 1 μM rosiglitazone. The data shown are representative of three independent experiments.

GLUT1 or GLUT4 was found to cause hypoglycemia while down-regulation of GLUT4 led to insulin resistance [13]. So far, increased expression and PM translocation of GLUT1 and GLUT4 have been found to lower blood glucose and enhance glucose transport and utilization [14].

Artepillin C (Fig. 1A) is one of the principal phenolic compounds found in Brazilian green propolis. In particular, *Baccharis dracunculifolia* DC is believed to be the major botanical origin of Brazilian green propolis and contains a large amount of artepillin C [15–17]. Although biological effects of artepillin C, such as antimicrobial [18], antioxidant [19,20] and antitumor [21–23] activities, have been reported, its effects on glucose metabolism in adipocytes are unknown. In the present study, we investigated the effects of artepillin C on adipocyte differentiation and glucose regulation in 3T3-L1 adipocytes. Our data revealed that artepillin C stimulates adipocyte differentiation and enhances glucose transport in adipocytes by inducing the expression and translocation of GLUT1 and GLUT4 vesicles in 3T3-L1 adipocytes as a PPARγ ligand.

2. Materials and methods

2.1. Materials

Artepillin C [3-{4-hydroxy-3,5-di(3-methyl-2-butenyl)phenyl}-2(E)-propenoic acid], 5′-diallyl-2,2′-biphenyldiol, 3-isobutylmethylxanthine (IBMX), dexamethasone (DEX), SDS, isopropanol, EDTA and PMSF were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Rosiglitazone was purchased from Alexis Biochemicals (San Diego, CA). GW9662, insulin and Oil Red O were purchased from Sigma–Aldrich (St. Louis, MO). SYBR Green reaction buffer was purchased from Roche (Mannheim, Germany). Polyclonal antibodies against GLUT4, PPARγ, GLUT1, GAPDH, aP2, IRS-1, phospho-IRS-1 (Tyr612), phospho-Akt (Ser473/Thr308) and Akt were purchased from Cell Signaling Technology Inc. (Beverly, MA). HRP-linked anti-rabbit IgG and polyvinylidene difluoride membrane were purchased from Amersham (Buckinghamshire, UK).

2.2. Cell culture and adipocyte differentiation

Mouse 3T3-L1 preadipocytes were obtained from Japanese Cancer Research Resources Bank (Osaka, Japan) and cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco BRL, Carlsbad, CA) supplemented with 10% bovine calf serum (Gibco BRL) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; Gibco BRL) at 37 °C under a humidified 5% CO_2 atmosphere. For 3T3-L1 preadipocyte differentiation, cells at 2 days after reaching confluency (defined as day 0) were cultured in differentiation medium comprising DMEM supplemented with 0.5 mM IBMX, 10 μg/ml insulin, 0.25 μM DEX and 10% fetal bovine serum (FBS) (Biosource Inc., Camarillo, CA). After 2 days, the culture medium was changed to DMEM containing 10 μg/ml insulin and 10% FBS. The medium was replaced again with fresh DMEM containing 10% FBS after another 2 days. The adipocytes were used at 6–8 days after the initiation of differentiation. In adipogenesis studies, 3T3-L1 preadipocytes cultured in DMEM supplemented with 10%

bovine calf serum (day 0) were treated with insulin (1 μg/ml) with or without artepillin C and GW9662 at various concentrations in DMEM containing 10% FBS for 9 days. Fresh medium containing insulin (1 μg/ml) and 10% FBS with or without artepillin C and GW9662 was replenished every 3 days.

2.3. Oil Red O staining

After differentiation, the cells were fixed with 10% formalin in phosphate-buffered saline (PBS) for 1 h at room temperature, washed three times with PBS and stained with filtered Oil Red O (0.5% in 60% isopropanol) for 1 h. After three washes with distilled water, the cells were photographed under a microscope. Lipid and Oil Red O were extracted using isopropanol, and the absorbances were measured at a wavelength of 520 nm using a spectrophotometer.

2.4. Glucose consumption assay

Fully differentiated 3T3-L1 preadipocytes in 96-well plates were incubated with various concentrations of artepillin C for 48 h. The cells were then washed twice and incubated with serum-free DMEM. After 3 h, the cells were incubated with or without 100 nM insulin in KHH buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM Na_2HPO_4, 1.2 mM KH_2PO_4, 0.1% BSA, 1 mM glucose, 10 mM Hepes, 2 mM sodium pyruvate, pH 7.4) with various concentrations of artepillin C for a further 1 h. The glucose concentrations in the buffer were determined by the Glucose CII Test (Wako Pure Chemical Industries Ltd.). The intracellular glucose concentrations were calculated by subtracting the glucose in the medium from the total glucose in blank wells.

2.5. RNA preparation and quantitative real-time PCR

Total RNA was isolated from cells using the Isogen reagent (Nippon Gene, Tokyo, Japan) and 1 μg of total RNA from each sample was reverse-transcribed to cDNA using random primers and a reverse transcription system (Promega, Piscataway, NJ) according to the manufacturer's protocol. After cDNA synthesis, quantitative real-time PCR was performed using an ABI Prism 7300 System (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. The PCR conditions were 1 cycle of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primer sequences used for the PCR amplifications were as follows: aP2, 5′-CAACCTGTGTGATGC CTTTGTG-3′ and 5′-CTCTTCCTTTGGCTCATGCC-3′; β-actin, 5′-TGT TACCAACTGGGACGACA-3′ and 5′-CTCTCAGCTGTGGTGGTGAA-3′; PPARγ2, 5′-GCTGTTATGGGTGAAACTCTG-3′ and 5′-ATAAGGTGGA-GATGCAGGTTC-3′; adiponectin, 5′-GTTGCAAGCTCTCCTGTTCC-3′ and 5′-CTTGCCAGTGCTGTTGTCAT-3′; GLUT4, 5′-CCCCGATACCTC-TACATCATC-3′ and 5′-GCATCAGACACATCAGCCCAG-3′; GLUT1, 5′-GAGGAGCTCTTCCACCCTCT-3′ and 5′-TCTGGAGCCATCAAAGTCCT-3′. All quantifications were performed in duplicate and the experiments were independently repeated three times.

2.6. PPARγ ligand-binding assay

For measurement of the PPARγ ligand-binding activity, we used the Lanthascreen™ TR-FRET Peroxisome Proliferator Activated Receptor γ Coactivator Assay (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The Lanthascreen™ TR-FRET-based nuclear receptor coactivator recruitment assay uses a terbium (Tb)-labeled anti-glutathione-S-transferase (GST) antibody, a fluorescein-labeled coactivator peptide and a PPARγ ligand-binding domain (PPARγ-LBD) tagged with GST. The Tb-anti-GST antibody indirectly labels the PPARγ-LBD by binding to the GST tag. Binding of an agonist to the PPARγ-LBD causes a conformational change that results in an increase in the affinity of PPARγ for the coactivator peptide. The close proximity of the fluorescently tagged coactivator peptide to the Tb-labeled antibody causes an increase in the TR-FRET signal. The TR-FRET ratio of 520/495 nm was calculated using measurements obtained with an EnVision™ multi-label reader (Perkin-Elmer Inc., Wellesley, MA) with an excitation wavelength of 340 nm and emission wavelengths of 520 and 495 nm.

2.7. Protein extraction and immunoblotting

Cells were washed with ice-cold PBS, and subjected to subcellular fractionation and total protein collection. Briefly, cells were harvested in buffer A (0.25 M sucrose, 10 mM Tris–HCl pH 7.4, 2 mM EDTA, 1 mM PMSF). The lysates were centrifuged at 760 × *g* for 10 min to remove nuclei and unbroken cells. The supernatants were centrifuged at 12,000 × *g* for 20 min to isolate the crude plasma membrane fraction as the pellets. The supernatants were collected as the cytosolic fraction and the crude plasma membrane pellets were suspended in buffer A. All samples were frozen at −80 °C until analysis. To prepare whole cell lysates, cells were washed with ice-cold PBS and harvested in a lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF). The lysates were centrifuged at 12,000 rpm for 20 min and the supernatants were collected. The proteins in the extracts were separated by 10% SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes. The membranes were blocked with blocking buffer (Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dry milk) and incubated with the primary antibodies and appropriate secondary antibodies according to the manufacturers' instructions. Antigen–antibody complexes were detected by chemiluminescence (ECL; Amersham).

2.8. Statistical analysis

All qualitative data are representative of at least three independent experiments. Quantitative data are presented as means ± SE and were compared by ANOVA using the Origin 6.0 software (Microcal Software Inc., Northampton, MA). Values of $p < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Artepillin C is a PPARγ ligand

To investigate whether artepillin C serves as a PPARγ ligand, we examined its PPARγ ligand-binding activity. As shown in Fig. 1B, 0.1 μM rosiglitazone and 10 μM 15-deoxy-delta-12,14-prostaglandin J2 (15d-PGJ2) had PPARγ ligand-binding activities ($p < 0.05$ for each). Artepillin C at 1 and 100 μM exhibited weak affinities for PPARγ ($p < 0.05$ for each). In addition, artepillin C significantly upregulated the expression of aP2, a PPARγ target gene, at the mRNA and protein levels (Fig. 1C).

3.2. Artepillin C enhances adipocyte differentiation of 3T3-L1 cells

Activation of PPARγ by its ligands is a key process in adipocyte differentiation and some natural products have been reported to act as PPARγ ligands [24,25]. Since artepillin C showed PPARγ ligand-binding activity, we examined whether it enhanced adipogenesis. To investigate the effects of artepillin C on adipocyte differentiation, 3T3-L1 preadipocytes were treated with insulin in the presence of artepillin C. At 9 days after the initiation of differentiation, accumulated lipid droplets were detected by staining with Oil Red O. Treatment of 3T3-L1 preadipocytes with

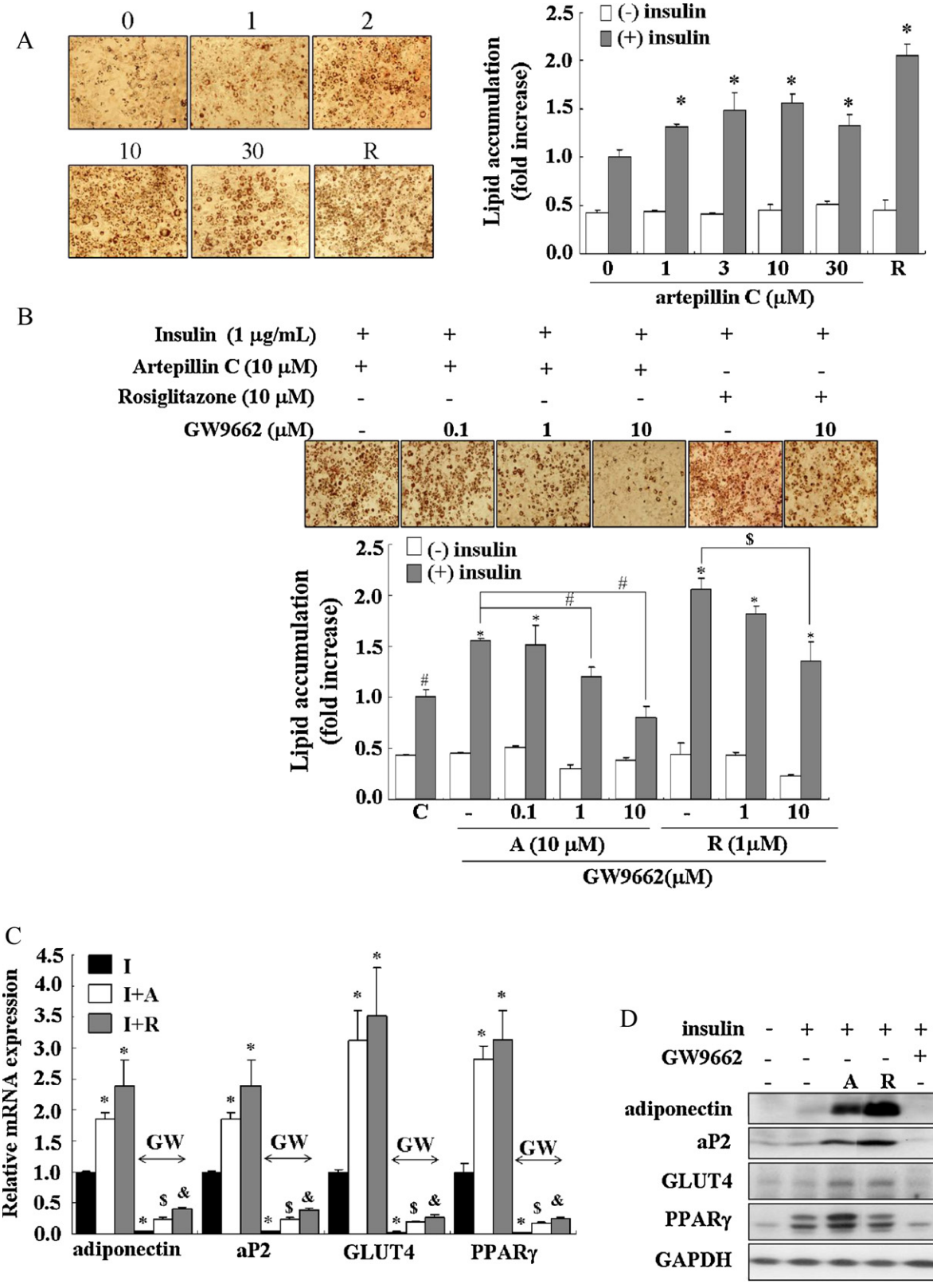

Fig. 2. Effects of artepillin C on adipocyte differentiation of 3T3-L1 preadipocytes as a PPARγ ligand. 3T3-L1 preadipocytes were incubated in medium containing insulin (1 μg/ml) for 9 days with or without the indicated concentrations of artepillin C or GW9662. The medium was replaced every 3 days. At day 9, the cells were fixed and stained with Oil Red O (A and B). The Oil Red O-stained adipocytes were photographed at magnification 100×. Next, the Oil Red O was eluted and quantified at 520 nm. All values are presented as means ± SE ($n = 3$). In addition, total RNA (C) and total protein (D) were extracted and the expressions of adipocyte-specific genes were analyzed by real-time PCR and immunoblotting, respectively. $^{*}p < 0.05$ *vs.* the insulin-treated group; $^{\$}p < 0.05$ *vs.* the 10 μM artepillin C-treated group; $^{\&}p < 0.05$ *vs.* the 1 μM rosiglitazone-treated group. C, control (vehicle); I, insulin; A, 10 μM artepillin C; R; 1 μM rosiglitazone; GW, 10 μM GW9662. The data shown are representative of three independent experiments.

artepillin C significantly enhanced the adipocyte differentiation in a dose-dependent manner. The optical densities of the Oil Red O eluted solutions were increased by 1.3- and 1.4-fold in 3T3-L1 preadipocytes treated with 3 and 10 μM artepillin C, respectively (Fig. 2A). Rosiglitazone, a PPARγ agonist, at a concentration of 1 μM also significantly promoted adipocyte differentiation of 3T3-L1 preadipocytes ($p < 0.05$). To further characterize the effects of artepillin C on adipocyte differentiation, we used quantitative real-time PCR to examine the expressions of PPARγ2 as well as aP2 and adiponectin, which are PPARγ target genes involved in the induction of adipocyte differentiation. At 9 days after treatment initiation, the 10 μM artepillin C-treated cells exhibited significant upregulation of the mRNA expressions of aP2, adiponectin, GLUT4 and PPARγ, respectively (Fig. 2C). At the same time, the protein expressions of aP2, GLUT4 and PPARγ were upregulated in artepillin C-treated cells (Fig. 2D). These results suggest that artepillin C promotes adipocyte differentiation of 3T3-L1 pre-adipocytes. To confirm the activity of artepillin C as a PPARγ ligand, we examined the effects of a PPARγ antagonist, GW9662, on artepillin C-induced differentiation. 3T3-L1 preadipocytes were induced to differentiate for 9 days with 10 μM artepillin C and 1 μg/ml insulin in the absence or presence of the indicated concentrations of GW9662. As shown in Fig. 2B, treatment with GW9662 resulted in significantly reduced lipid accumulation. The mRNA and protein expressions of adipocyte-specific genes in the cells treated with artepillin C and GW9662 were also significantly decreased (Fig. 2C and D). Taken together, these findings suggest that artepillin C enhances adipocyte differentiation as a PPARγ agonist through direct binding to the PPARγ ligand-binding domain.

3.3. Artepillin C blocks the inhibitory effects of tumor necrosis factor (TNF)-α on adipocyte differentiation

Impaired regulation of adipocyte differentiation may contribute to the pathogenesis of obesity-associated conditions including insulin resistance. Previous studies have demonstrated that TNF-α inhibits adipocyte differentiation, and that PPARγ ligands such as TZDs block the inhibitory effects of TNF-α on the differentiation of 3T3-L1 cells [26,27]. Therefore, we investigated the effects of TNF-α and artepillin C on the conversion of 3T3-L1 preadipocytes to adipocytes. At 5 ng/ml, TNF-α caused a marked reduction in adipocyte differentiation (Fig. 3A). However, when artepillin C (10 or 30 μM) was present together with TNF-α during differentiation, the inhibitory effects of TNF-α were blocked and the cells were able to differentiate into adipocytes (Fig. 3A). To examine the effects of TNF-α and artepillin C on 3T3-L1 differentiation in a more quantitative manner, the protein expressions of the adipose tissue-specific genes aP2 and PPARγ were determined by Western blotting analysis. In agreement with the changes in morphology, artepillin C reversed the gene and protein expressions down-regulated by TNF-α (Fig. 3B). These data suggest that artepillin C blocks the inhibitory effects of TNF-α on adipocyte differentiation.

3.4. Artepillin C increases basal and insulin-stimulated glucose consumptions in differentiated 3T3-L1 adipocytes

Since PPARγ ligands affect glucose uptake in 3T3-L1 adipocytes [28], we determined whether artepillin C had direct effects on glucose metabolism and insulin sensitivity. To determine glucose uptake in 3T3-L1 adipocytes, fully differentiated 3T3-L1 adipocytes under standard conditions were treated with artepillin C at the indicated concentrations for 48 h, and the glucose consumption was assessed. The results revealed that artepillin C enhanced glucose consumption in 3T3-L1 adipocytes ($p < 0.05$, Fig. 4A). The basal glucose consumptions in cells treated with artepillin C at 10

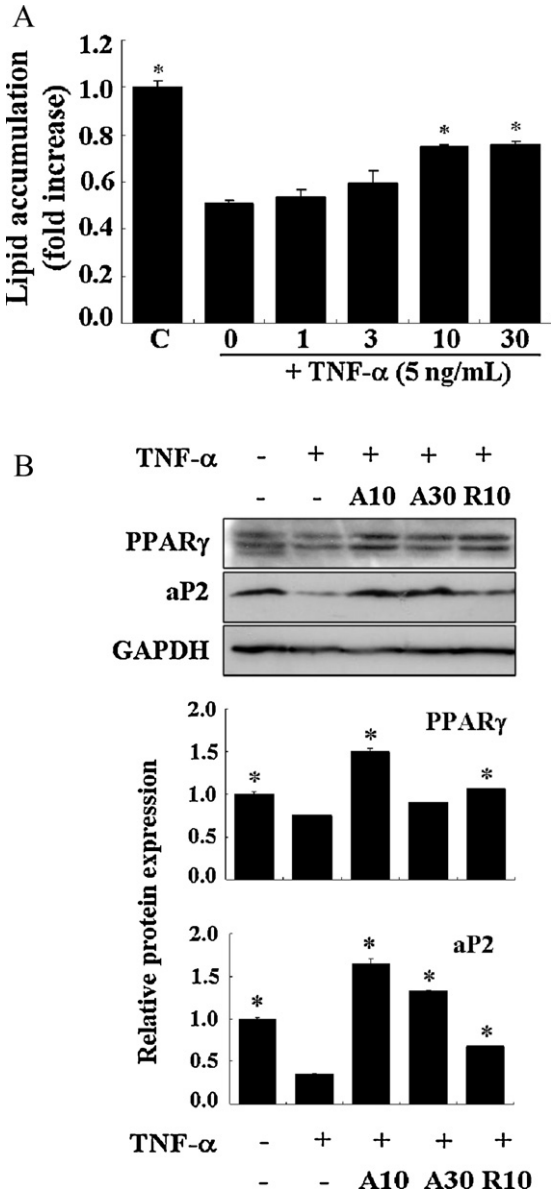

Fig. 3. Artepillin C blocks the inhibitory effects of TNF-α on adipocyte differentiation. Post-confluent 3T3-L1 preadipocytes were incubated in differentiation medium (MDI) with TNF-α (5 ng/ml) with or without the indicated concentrations of artepillin C for 2 days and the medium was then changed to DMEM containing insulin with artepillin C. The medium was replaced with DMEM after another 2 days. (A) The cells were fixed and stained with Oil Red O and eluted and quantified at 520 nm. (B) The proteins were extracted and the expressions of adipocyte-specific genes were analyzed by immunoblotting. All values are presented as means ± SE ($n = 3$). *$p < 0.05$ *vs.* the TNF-α treated group. C, control (vehicle); A10, 10 μM artepillin C; A30, 30 μM artepillin C; R10, 10 μM rosiglitazone. The data shown are representative of three independent experiments.

and 30 μM were 1.7- and 1.9-fold higher than that in vehicle-treated cells, respectively. Artepillin C also significantly stimulated insulin-stimulated glucose consumption in a dose-dependent manner. It has been reported that activation of PI3K is necessary for glucose transport [29]. Therefore, we examined the effects of wortmannin, a selective inhibitor of PI3K, on artepillin C-stimulated glucose transport. Artepillin C (30 μM)-stimulated glucose uptake in 3T3-L1 adipocytes was inhibited by treatment with 100 nM wortmannin (Fig. 4B). However, artepillin C did not produce detectable changes in the phosphorylations of IRS-1 and Akt (Fig. 4B).

Next, we investigated the effects of artepillin C on GLUT1 and GLUT4 expression and translocation, since the glucose uptake

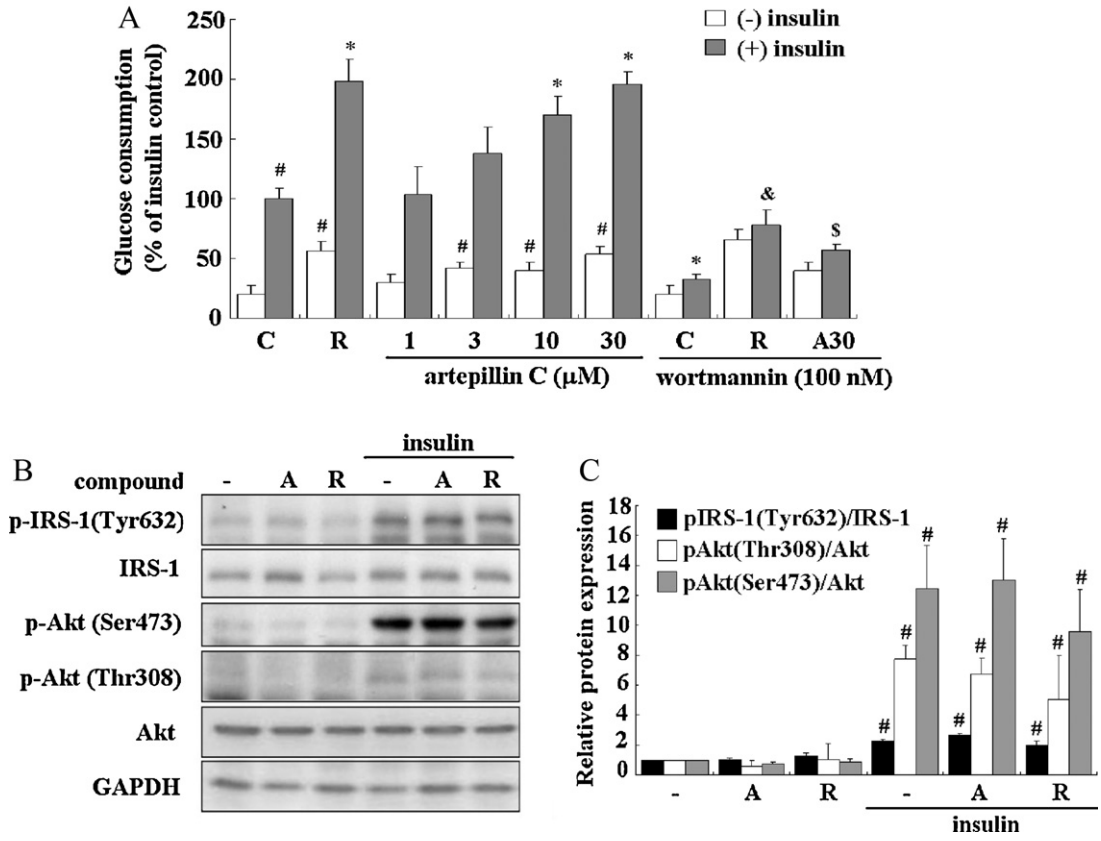

Fig. 4. Effects of artepillin C on glucose consumption and the insulin signaling pathway in 3T3-L1 adipocytes. Fully differentiated 3T3-L1 adipocytes were incubated for 48 h. (A) Glucose consumption was determined in the absence or presence of 100 nM insulin for 1 h. The glucose concentrations in the buffer were determined by the glucose oxidase method. All values are presented as means ± SE ($n = 3$). $^{\#}p < 0.05$ *vs.* the control group; $^{*}p < 0.05$ *vs.* the insulin-treated group. (B) The expressions of insulin signaling pathway proteins were analyzed by immunoblotting after treatment with artepillin C with or without 100 nM insulin for 20 min. C, control (vehicle); A30, 30 μM artepillin C; R, 1 μM rosiglitazone. The data are shown representative of three independent experiments.

activity in adipocytes is related to the expression of glucose transporters and their translocation. Artepillin C treatment increased the mRNA (Fig. 5A) and protein (Fig. 5B) expression of both GLUT1 and GLUT4 in fully differentiated adipocytes. In addition, artepillin C significantly increased the amounts of GLUT1 and GLUT4 proteins in the crude plasma membrane fraction (Fig. 5C). These results demonstrate that artepillin C promotes glucose uptake in 3T3-L1 adipocytes via the upregulation of GLUT1 and GLUT4 expression, rather than by enhancing the phosphorylation of the IRS-1/PI3K/Akt signaling pathway.

4. Discussion

Impaired adipocyte differentiation may contribute to the pathogenesis of obesity-associated conditions including insulin resistance, hyperlipidemia and type 2 diabetes. Dysregulated lipid accumulation and production of adipocytokines such as TNF-α, PAI-1 and adiponectin from enlarged adipocytes in obesity are critically involved in insulin resistance and obesity-related metabolic syndrome [30]. According to several reports, the adipocytes of type 2 diabetes patients are insulin-resistant and not capable of accumulating lipid [25,31]. Conversion of pre-adipocytes to adipocytes is controlled by various regulators such as hormones, adipogenic genes, adipocytokines and growth factors [32]. PPARγ, CCAAT/enhancer-binding protein (C/EBP) and sterol regulatory element-binding protein (SREBP) are well-established primary adipogenic transcription factors involved in adipogenesis. Among these genes, PPARγ has been the most extensively studied and is clinically validated for therapeutic use in type 2 diabetes [33]. Treatment of patients with TZDs, such as rosiglitazone and pioglitazone, consistently lowers the glucose levels and these improvements are generally accompanied by remodeling of the adipose tissue, whereby large adipocytes are replaced with small and/or insulin-sensitive cells [34,35]. Non-TZD PPARγ agonists including natural compounds have also been demonstrated to improve hyperglycemia and hypertriglyceridemia with increased adipocyte differentiation in *in vitro* and *in vivo* experiments [36]. In the present study, artepillin C weakly bound to the PPARγ ligand-binding domain and dose-dependently promoted adipocyte differentiation, albeit to a lower extent than rosiglitazone (1 μM). The gene expression of various adipogenic markers such as aP2, adiponectin, GLUT4 and PPARγ was upregulated in artepillin C-treated 3T3-L1 preadipocytes while cotreatment with GW9662 decreased artepillin C-induced adipocyte differentiation. In fully differentiated adipocytes, the expression of aP2, one of the PPARγ target genes, was increased by treatment with artepillin C. Based upon the results of the present study, we propose that artepillin C promotes adipocyte differentiation and that this effect may be related to the ability of artepillin C to directly bind to the PPARγ-binding domain and activate PPARγ as a ligand.

TNF-α is a potent cytokine with pleiotropic biological effects. Previous studies have indicated that TNF-α inhibits adipocyte differentiation by suppressing the expressions of genes that are upregulated during adipogenesis. Chronic treatment of 3T3-L1 adipocytes with TNF-α decreases glucose uptake, resulting in insulin resistance [37]. TZDs block the inhibitory effects of TNF-α on differentiation [26]. In the present study, artepillin C also blocked the inhibitory effects of TNF-α on adipogenesis in 3T3-L1 cells.

Activation of PI3K is a major event in the insulin signaling cascade that leads to glucose transporter translocation [38]. In the

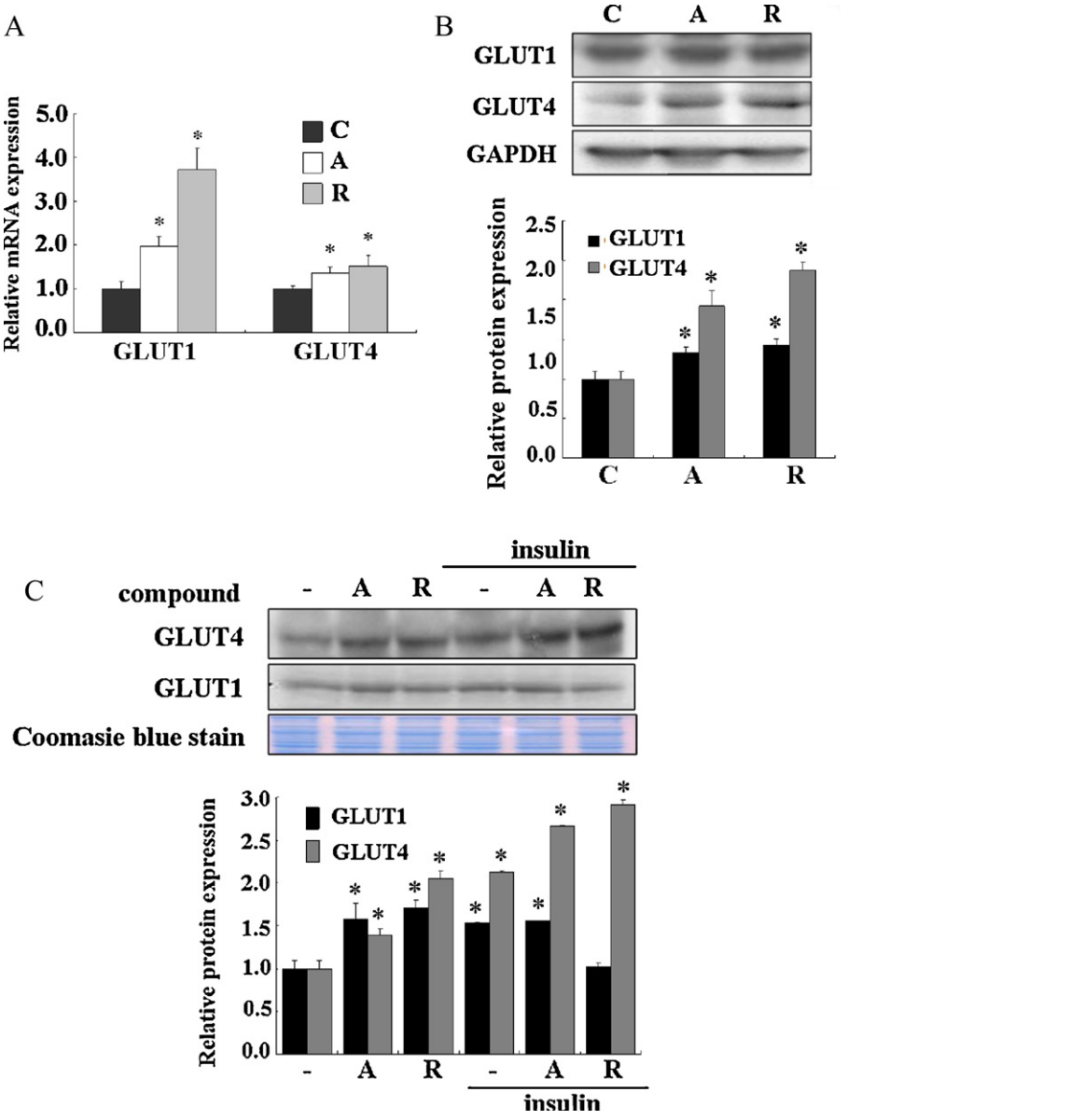

Fig. 5. Effects of artepillin C on the expression and translocation of GLUT1 and GLUT4 in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with 30 μM artepillin C or 1 μM rosiglitazone for 48 h. Total RNA and total protein were extracted and the GLUT1 and GLUT4 mRNA and protein expressions were analyzed by real-time PCR (A) and immunoblotting (B), respectively. All values are presented as means ± SE (n = 3). *$p < 0.05$ *vs.* the control group. (C) 3T3-L1 adipocytes were incubated with 30 μM artepillin C for 48 h and then incubated with 100 nM insulin for 20 min. The crude membrane fraction was extracted and analyzed by immunoblotting. C, control (vehicle); A, 30 μM artepillin C; R, 1 μM rosiglitazone. The data shown are representative of three independent experiments. All values are presented as means ± SE (n = 3). *$p < 0.05$ *vs.* the insulin-treated group.

insulin signaling pathway, autophosphorylation of the intracellular tyrosine residues of the insulin receptor β-subunit leads to the activation of downstream signaling molecules including IRS-1 and PI3K. In turn, Akt becomes phosphorylated, which results in glucose transporter translocation to the PM [38,12]. Therefore, we hypothesized that artepillin C promotes glucose uptake by enhancing the IRS-1/PI3K/Akt signaling pathway, and investigated this hypothesis using 3T3-L1 adipocytes. We found that artepillin C stimulated the basal and insulin-mediated glucose uptake in 3T3-L1 adipocytes in a concentration-dependent manner, with the maximum stimulation at 30 μM. These effects were blocked by the PI3K inhibitor wortmannin. Unfortunately, however, we found that artepillin C did not enhance the phosphorylation of IRS-1 and Akt, despite the increased concentrations of GLUT1 and GLUT4 in the crude membrane fraction. In addition, rosiglitazone did not produce detectable changes in the insulin-stimulated phosphorylation of IRS-1 and Akt, although it has been known to increase. Consequently, we thought that a distinct mechanism may be involved in the increase in glucose transport induced by artepillin C and rosiglitazone. Many studies have demonstrated that atypical protein kinase C (PKC) λ and ζ are associated with glucose transport besides Akt. PKC-λ/ζ are downstream of PI3K together with Akt and their activation is required for insulin-mediated stimulation of glucose uptake, which involves translocation of the major insulin-responsive glucose transporter GLUT4 from intracellular sites to the cell membrane [39–42]. Overexpression of a dominant-negative mutant of PKC-λ or PKC-ζ abrogates insulin-stimulated glucose transport and GLUT4 translocation in adipose [39,43] and muscle [40,44] cells. In accordance with a previous report [45], rosiglitazone led to increases in both the basal and insulin-stimulated activation of PKC-λ/ζ without accompanying increases in IRS-1/2-dependent PI3K or Akt activity in adipocytes isolated from nonobese type 2 diabetic Goto-Kakizaki rats. In addition, rosiglitazone increased the basal glucose uptake by

activating the Cbl-dependent PI3K and atypical PKC-λ pathway, rather than the IRS-1/2-dependent PI3K and Akt pathway, in 3T3-L1 adipocytes [46]. Therefore, we propose that the PI3K/PKC-λ/ζ pathway could be a possible mechanism for the artepillin C-induced glucose uptake in 3T3-L1 adipocytes, similar to the case for rosiglitazone. However, further studies are required to clarify this possibility.

The other possible mechanism underlying the finding that artepillin C promotes basal and insulin-stimulated glucose uptake is that artepillin C increased the mRNA and protein expression of GLUT1 and GLUT4 themselves. Glucose transport in insulin-sensitive tissues is closely related to the expression of glucose transporters [46,47]. In general, it is known that GLUT1 and GLUT4 are involved in basal and insulin-stimulated glucose transport in adipocytes [12,48]. GLUT1 is expressed in both preadipocytes and adipocytes and its expression level does not change during adipocyte differentiation. In contrast, GLUT4 is only expressed in adipocytes and its expression is regulated by PPARγ [32,49–51]. PPARγ ligands are known to increase glucose transport in insulin-sensitive tissues by directly regulating the expression of several genes involved in glucose metabolism such as GLUT1 and GLUT4 [25,28,48,52]. Since artepillin C was found to have PPARγ-binding activity in the present study, we hypothesized that the mechanism for the increased glucose uptake by artepillin C involved increased expression and translocation of GLUT1 and/or GLUT4. In the present study, the GLUT1 mRNA and protein expression levels were significantly increased in fully differentiated adipocytes treated with 30 μM artepillin C, but the relative protein expression compared with the control was less increased than the relative mRNA expression. At the same time, the GLUT4 mRNA and protein expression levels were also significantly increased, but the relative protein expression compared with the control was more dramatically increased than the relative mRNA expression. In addition, the concentrations of GLUT1 and GLUT4 were increased in the cytosolic fraction (data not shown). Based on these results, the increased basal and insulin-stimulated glucose uptake mediated by artepillin C may be caused by enhanced GLUT1 and GLUT4 expression, which led to increased concentrations of GLUT1 and GLUT4 on the PM without enhancing the insulin signaling pathway.

In conclusion, we found that artepillin C had a weak PPARγ ligand-binding ability and enhanced adipogenesis by increasing the PPARγ transcriptional activity. Artepillin C also reversed the inhibitory effects of TNF-α on adipocyte differentiation. In addition, we found that artepillin C augmented the basal and insulin-stimulated glucose transport in 3T3-L1 adipocytes along with increased expressions of GLUT1 and GLUT4 at the mRNA and protein levels. Taken together, our data provide evidence that artepillin C has antidiabetic and insulin-sensitizing effects on adipocytes as a PPARγ ligand. Therefore this compound could possess a concrete possibility of being used as an active pharmaceutical ingredient of new medicines as a lead compound, once its availability is enhanced by new, more efficient synthetic methodologies.

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