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Aculeatin, a coumarin derived from *Toddalia asiatica* (L.) Lam., enhances differentiation and lipolysis of 3T3-L1 adipocytes

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ABSTRACT

Toddalia asiatica (L.) Lam. (*T. asiatica*) has been utilized traditionally for medicinal purposes such as the treatment of diabetes. Currently, the extract is considered to be a good source of anti-diabetic agents, but the active compounds have yet to be identified. In this study, we investigated the effects of fractionated *T. asiatica* extracts on the differentiation of 3T3-L1 preadipocytes and identified aculeatin as a potential active agent. When 3T3-L1 preadipocytes were treated with aculeatin isolated from *T. asiatica* in the presence of insulin, aculeatin increased cellular triglyceride levels and glycerol-3-phosphate dehydrogenase activity. This indicated that aculeatin could enhance the differentiation of preadipocytes into adipocytes. Further analyses using a DNA microarray and real-time quantitative reverse-transcription PCR showed an increase in the expression of peroxisome proliferator-activated receptor-γ target genes (*Pparg*, *Ap2*, *Cd36*, *Glut4* and *Adipoq*) by aculeatin, suggesting that aculeatin enhances the differentiation of 3T3-L1 cells by modulating the expression of genes critical for adipogenesis. Interestingly, after treatment of differentiated adipocytes with aculeatin, glucose uptake and lipolysis were enhanced. Overall, our results suggested that aculeatin is an active compound in *T. asiatica* for enhancing both differentiation and lipolysis of adipocytes, which are useful for the treatment of lipid abnormalities as well as diabetes.

1. Introduction

Toddalia asiatica (L.) Lam. (*T. asiatica*), which belongs to the family Rutaceae, is a woody liana that grows in tropical and subtropical areas. *T. asiatica* is commonly referred to as "wild orange tree" and is widely recognized as a medicinal plant in Africa, India, China and Japan. All parts of the plant are believed to have medicinal properties, and its root, bark, and leaves have been used for the treatment of many diseases including diabetes [1–3]. Recent studies have supported the beneficial effects of *T. asiatica* [4], and Stephen et al. clearly showed antidiabetic properties of *T. asiatica* (ethyl acetate extract from leaves) in streptozotocin-induced diabetic rats [3]. *T. asiatica* contains many compounds, such as coumarins and alkaloids [5,6]. However, the active agents of *T. asiatica* and their mechanisms of action are still unclear.

Diabetes is caused mainly by insulin resistance. Adipocytes secrete adipocytokines, such as tumor necrosis factor-α (TNF-α) and monocyte chemoattractant protein (MCP-1), which cause insulin resistance [7]. The thiazolidinediones (TZDs) such as pioglitazone (Pio), rosiglitazone, and troglitazone are selective ligands of peroxisome proliferator-activated receptor γ (PPAR-γ) and act as insulin sensitizers [8]. Indeed, TZDs have been reported to improve insulin sensitivity by increasing adiponectin levels in diabetic patients [9], although they have several potential side effects (e.g., weight gain) [10,11]. Therefore, the screening of compounds, such as nobiletin [12], capable of enhancing not only adipocyte differentiation but also adipocyte lipolysis is desired for the development of attractive antidiabetic agents.

Abbreviations: ACN, acetonitrile; BSA, bovine serum albumin; BuOH, butanol; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; EtOAc, ethyl acetate; ext., extract; FBS, fetal bovine serum; GPDH, glycerol-3-phosphate dehydrogenase; Hex, hexane; IL, interleukin; Iso, isoproterenol; KRPH, Krebs-Ringer-phosphate-HEPES; MCP, monocyte chemoattractant protein; MeOH, methanol; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; Pio, pioglitazone; PPAR, peroxisome proliferator-activated receptor; RT-PCR, reverse transcription-PCR; TG, triglyceride; TNF, tumor necrosis factor; TOF-MS, time-of-flight mass spectrometry; Tris–HCl, tris(hydroxymethyl)aminomethane hydrochloride; TZDs, thiazolidinediones.

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Based on this background, in the present study, we investigated the effects of fractionated *T. asiatica* extracts on differentiation of 3T3-L1 preadipocytes and, for the first time, identified aculeatin as an active compound for enhancing both differentiation and lipolysis of 3T3-L1 adipocytes.

2. Materials and methods

2.1. Reagents and cells

Insulin, Dulbecco's modified Eagle's medium (DMEM) and penicillin–streptomycin were purchased from Sigma–Aldrich (Tokyo, Japan). Fetal bovine serum (FBS) was obtained from Life Technologies Japan Ltd. (Tokyo, Japan) and Pio, 2-deoxyglucose, phloretin and isoproterenol (Iso) from Wako (Osaka, Japan). All other reagents were of the highest grade commercially available.

Mouse 3T3-L1 preadipocytes were obtained from the Health Science Research Resources Bank (Osaka, Japan). The cells were cultured in DMEM containing high glucose (4500 mg/L) supplemented with 10% FBS, penicillin (100 units/mL) and streptomycin (100 μg/mL) in an atmosphere of 5% CO_2 at 37 °C.

2.2. Fractionation of T. asiatica

Dry stem chips of *T. asiatica* were purchased from the Okinawa Medical Herb Association (Okinawa, Japan). The dry stem chips (100 g) were suspended in 1 L methanol (MeOH) and heated under reflux for 2 h at 80 °C. The MeOH extract (ext.) was collected, and this extraction process was repeated twice more. The MeOH Ext. were combined and evaporated to residue (10.8 g) (Fig. 1A). The residue (10 g) was suspended in 100 mL water and extracted four times with 100 mL hexane (Hex). The Hex layers were combined and evaporated to residue (Hex ext., 1.75 g). The remaining water layer was extracted four times with 100 mL ethyl acetate (EtOAc). The EtOAc layers were combined and evaporated to residue (EtOAc ext., 0.63 g). The remaining water layer was further extracted 4 times with 100 mL butanol (BuOH). The BuOH layers were combined and evaporated to residue (BuOH ext., 1.12 g). The resultant water layer was evaporated to residue (Water ext., 1.75 g).

The Hex ext. (1.60 g) was suspended in 50 mL 20% acetonitrile (ACN) aqueous solution and subjected to Diaion HP-20 column chromatography (3.0 × 14 cm; Mitsubishi Chemical, Tokyo, Japan). The column was eluted with 300 mL 20%, 40%, 60%, 80%, and 100% ACN, yielding 20%, 40%, 60%, 80%, and 100% ACN fractions, respectively (Fig. 1A).

The 60% ACN fraction was evaporated to residue (400 mg), dissolved in 10 mL 50% ACN, and subjected to preparative high performance liquid chromatography (HPLC) using a C18 column (Wakosil-II 5C18 RS-Prep, 5 μM, 20 × 250 mm; Wako). The mobile phase was a mixture of ACN and water (50/50, v/v), and the flow rate was 12 mL/min. Eluents corresponding to components **1**, **2**, and **3** detected by UV (210 nm) were collected (Fig. 1A), and their structures were determined using ^{1}H nuclear magnetic resonance (NMR) spectroscopy and time-of-flight mass spectrometry (TOF-MS).

2.3. Preparation of the experimental medium

The test samples (Hex ext., EtOAc ext., BuOH ext., Water ext., and components **1**, **2**, and **3**) were dissolved in dimethyl sulfoxide (DMSO). The solution was diluted with 10% FBS/DMEM (high glucose) to achieve the desired final concentrations. The final concentration of DMSO in the experimental medium was less than 0.1% (v/v) and did not affect cell viability. Medium containing DMSO alone was prepared similarly and used as the control medium.

2.4. Differentiation of 3T3-L1 preadipocytes and analysis

3T3-L1 preadipocytes (2.5×10^4/well) were seeded in 24-well cell culture plates supplemented with 10% FBS/DMEM (high glucose) and incubated until confluence. Differentiation was then initiated using 10% FBS/DMEM (high glucose) supplemented with or without insulin (1 μM) and the test samples. The cells were incubated for 3 days in the medium. On day 3, the test medium was refreshed, and the cells were incubated until day 7, when the adipocytes were harvested for the analyses described below.

2.5. Oil red O staining

3T3-L1 adipocytes were stained with Oil red O using the Adipogenesis Assay Kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's protocol. Briefly, cells were treated with fixture solution for 20 min, washed twice with wash solution, and stained with filtered Oil red O solution for 20 min at room temperature. After washing twice with water and wash solution, the stained lipids were extracted, and the absorbance at 520 nm was measured.

2.6. Triglyceride (TG) levels and GPDH activity

3T3-L1 adipocytes were washed twice with ice-cold phosphate-buffered saline (PBS), harvested in ice-cold 25 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris–HCl) (pH 7.5) containing 1 mM ethylenediaminetetraacetic acid (EDTA), and lysed by brief sonication. The TG levels of the lysate were quantified using the Triglyceride E-test kit (Wako) and expressed as mg/mg protein. Glycerol-3-phosphate dehydrogenase (GPDH) activity was measured using the GPDH Activity Assay Kit (Takara Bio, Shiga, Japan) and was expressed as mU/mg protein. Protein concentration was determined using the BCA Protein Assay Kit (Thermo Fisher Scientific, Beaconsfield, UK).

2.7. DNA microarray and mRNA analyses

Total RNA from 3T3-L1 adipocytes was extracted using TRIzol reagent (Life Technologies) and the RNeasy Mini Kit (Qiagen, NV, Netherlands). Using total RNA, DNA microarray analysis (Genopal Mouse Custom Chip, Mitsubishi Rayon, Tokyo, Japan) was performed following the manufacturer's protocol. The DNA microarray used consists of 217 genes related to circadian rhythm, energy production, redox regulation, reactive oxygen species defense, the mitogen-activated protein kinase cascade, cholesterol metabolism, and protein degradation. Gene expression values were normalized against the housekeeping gene *Actb*.

For real-time quantitative reverse-transcription PCR (real-time RT-PCR), cDNA was synthesized using random primers and PrimeScript Reverse Transcriptase (Takara Bio, Shiga, Japan), and PCR amplification was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). The target cDNAs were amplified using Fast SYBR Green Master Mix (Applied Biosystems) together with gene-specific primers for *Pparg* (MA029808), *Ap2* (MA117397), *Cd36* (MA157745), *Adipoq* (MA152022), *Glut4* (MA077105), *Mcp1* (MA066003), *Ccl6* (MA153825), *Il6* (MA152279), and *Actb* (MA050368) (Takara Bio). PCR conditions were 95 °C for 20 s, 95 °C for 3 s, and 60 °C for 30 s for 40 cycles. PCR products were measured using a StepOnePlus Real-time PCR System (Applied Biosystems). Data were expressed as fold differences normalized to *Actb* levels.

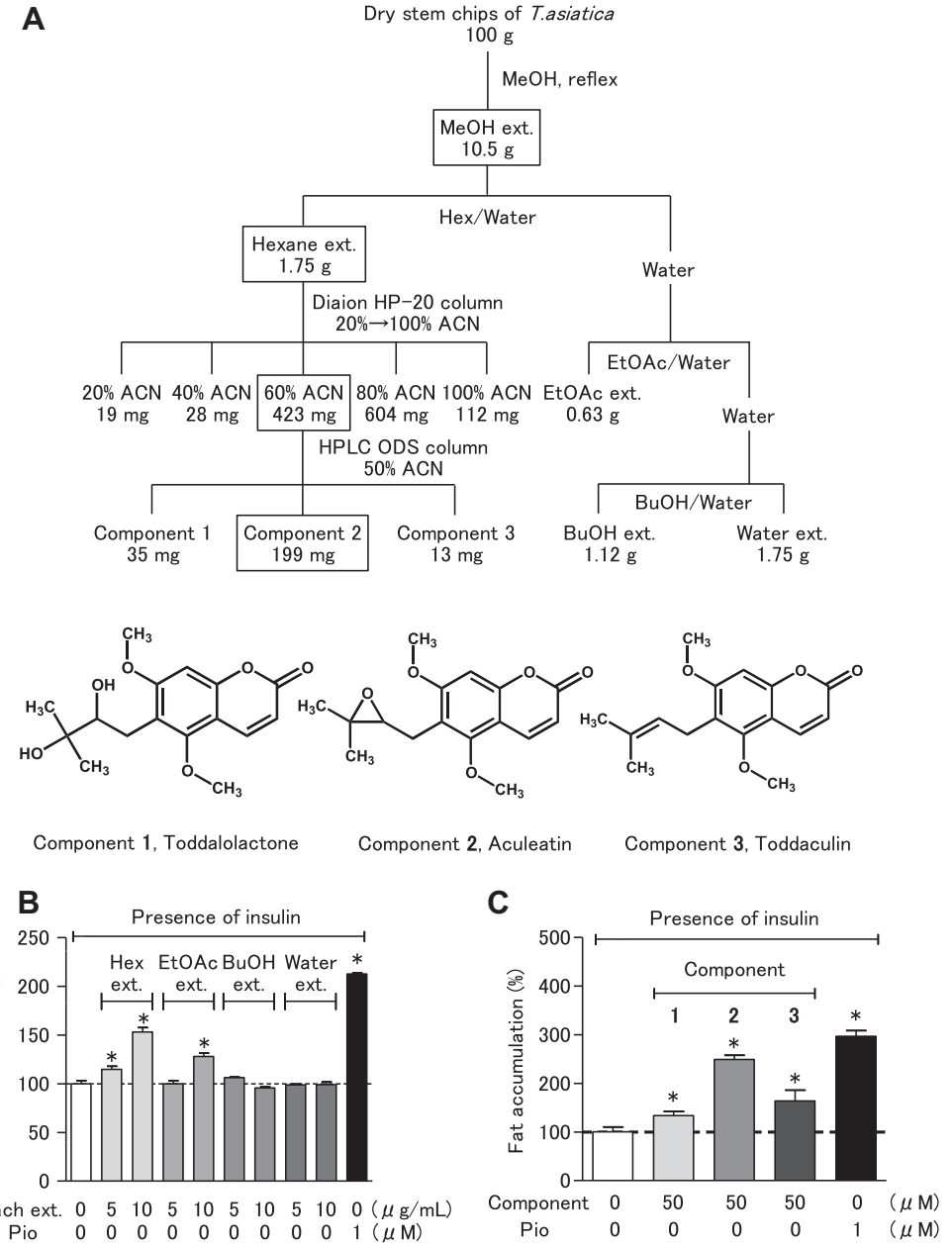

Fig. 1. Fractionation of *T. asiatica* (A) and the effects of each extract on adipocyte differentiation in 3T3-L1 cells (B). The effects of isolated component **1** (toddalolactone), component **2** (aculeatin), and component **3** (toddaculin) are shown in (C). Detailed procedures are described in Section 2. Values are expressed as means ± SEM ($n = 3$). $^{*}P < 0.05$ compared with the control in the presence of insulin.

2.8. Agonistic activity for PPAR-γ

Agonistic activity for PPAR-γ was examined using a nuclear receptor cofactor assay system (EnBio RCAS for PPAR-γ; EnBioTec Laboratories, Tokyo, Japan). This system employs a cell-free assay using nuclear receptors and cofactors to screen for chemicals. Changes in absorbance (450 nm) of the sample (aculeatin) and the positive control (Pio) were measured.

2.9. Uptake of 2-deoxyglucose

3T3-L1 preadipocytes (2.5×10^4/well) were seeded in 24-well cell culture plates supplemented with 10% FBS/DMEM (high glucose) and incubated until confluence. Differentiation was then initiated by incubating the cells in 10% FBS/DMEM (high glucose) supplemented with components (e.g., insulin, dexamethasone and isobutylmethylxanthine) from the Adipogenesis Assay Kit (Cayman Chemical) for 3 days. On days 3 and 6, the medium was refreshed, and the cells were incubated further. On day 9, 3T3-L1 adipocytes were incubated with 100 μM component **2** (aculeatin) or 1 μM Pio in 10% FBS/DMEM (high glucose) for 24 h. The medium was replaced with FBS-free DMEM, and the cells were incubated for another 6 h. After washing with Krebs Ringer phosphate HEPES (KPRH) buffer (25 mM HEPES pH 7.4, 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 1 mM KH_2PO_4) containing 2% bovine serum albumin (BSA), the cells were incubated with 1 μM insulin. After 18 min, 2-deoxyglucose dissolved in KRPH buffer was added to the wells (0.1 mM final concentration). After 20 min of incubation, cells were washed with PBS containing 200 μM phloretin and lysed in ice-cold 10 mM Tris–HCl (pH 8.0).

2-Deoxyglucose uptake was evaluated using the 2-Deoxyglucose Uptake Measurement Kit (Cosmo Bio, Tokyo, Japan) according to the manufacturer's protocol.

2.10. Lipolysis assay

3T3-L1 preadipocytes (2.5×10^4/well) were seeded in 24-well cell culture plates supplemented with 10% FBS/DMEM (high glucose) and incubated until confluence. Differentiation was then initiated by incubating the cells in 10% FBS/DMEM (high glucose) supplemented with components from the Adipogenesis Assay Kit (Cayman Chemical) for 3 days. On day 3, the medium was refreshed, and the cells were incubated further. On day 6, the stimulation of lipolysis was accomplished by incubating mature 3T3-L1 adipocytes in culture medium supplemented with 25–200 μM component **2** (aculeatin), 1 μM Pio or 10 μM Iso for 6 h. To evaluate the extent of lipolysis, glycerol concentrations in the media were measured using free glycerol reagent and glycerol standard (Sigma).

2.11. Statistical analysis

The data were expressed as means ± SEM. One-way ANOVA and Dunnett's test were performed using GraphPad Prism ver. 5.02 for Windows (GraphPad Software, San Diego, California, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Hex ext. of T. asiatica and aculeatin enhance differentiation of 3T3-L1 preadipocytes into adipocytes

First, we prepared extracts (Hex ext., EtOAc ext., BuOH ext., Water ext.) from *T. asiatica* (Fig. 1A) and investigated the effects of these extracts (5 and 10 μg/mL) on 3T3-L1 preadipocyte differentiation using Oil Red O staining. It was found that similarly to the positive control Pio, Hex ext. significantly promoted adipocyte differentiation in the presence of insulin (Fig. 1B). Therefore, activity-guided fractionation of Hex ext. was performed using Diaion HP-20 column chromatography and preparative HPLC, and three potentially active compounds (components **1**, **2**, and **3**) were separated and isolated. These compounds were identified as coumarins (component **1**, toddalolactone; component **2**, aculeatin; component **3**, toddaculin) by ^{1}H NMR and TOF-MS (data not shown) (Fig. 1A). The NMR data were consistent with those reported previously [5,6]. We performed Oil-Red O staining assay and found that these coumarins (50 μM), especially aculeatin (component **2**), significantly increased oil droplets in the presence of insulin (Fig. 1C).

Next, the dose-dependent effects of aculeatin were investigated by evaluating cellular oil droplets, TG levels, and GPDH activity. Aculeatin (12.5–100 μM) increased oil droplets dose-dependently, and importantly, the lipid accumulation was higher in the presence than in the absence of insulin (Fig. 2A). The effects of aculeatin on TG accumulation and GPDH activity were also dose-dependent (Fig. 2B and C). These results indicate that aculeatin can enhance differentiation of preadipocytes into adipocytes, especially in the presence of insulin.

3.2. Effects of aculeatin on mRNA expression in 3T3-L1 adipocytes

To investigate the mechanism by which aculeatin enhanced 3T3-L1 differentiation, we evaluated gene expression. DNA microarray analysis showed changes in the expression of certain genes after aculeatin (50 μM) treatment. For example, the expression levels of the *Pparg*, *Ap2*, *Cd36*, *Glut4* and *Adipoq* genes were up

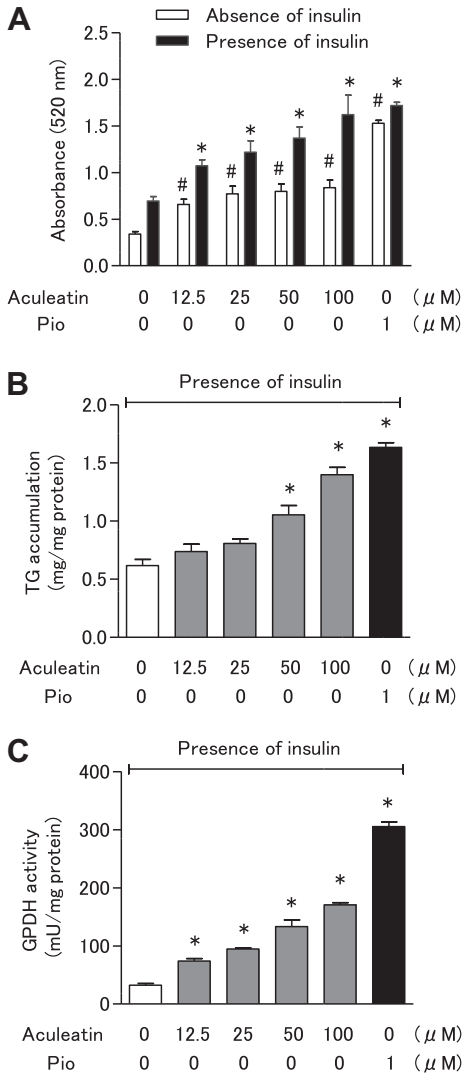

Fig. 2. (A) Effects of aculeatin on adipocyte differentiation in 3T3-L1 cells. The effects of aculeatin on (B) GPDH activity and (C) TG levels during adipocyte differentiation in 3T3-L1 cells. Detailed procedures are described in Section 2. Values are expressed as means ± SEM ($n = 3$). $^{\#}P < 0.05$ compared with the control in the absence of insulin. $^{*}P < 0.05$ compared with the control in the presence of insulin.

regulated, whereas those of *Mcp1*, *Ccl6*, and *Il6* were down regulated (data not shown). Hence, we focused on these genes and then evaluated the effects of aculeatin on mRNA expression of *Pparg*, *Ap2*, *Cd36*, *Glut4*, *Adipoq*, *Mcp1*, *Ccl6* and *Il6* using real-time RT-PCR. Even though the efficacy of aculeatin was somewhat weaker than that of Pio, PPAR-γ target gene (*Pparg*, *Ap2*, *Cd36*, *Glut4* and *Adipoq*) expression was actually increased and inflammatory gene (*Ccl6*, *Il6* and *Mcp1*) expression decreased by aculeatin (50 μM) (Fig. 3A–H). Therefore, aculeatin is expected to enhance the differentiation of 3T3-L1 cells via effects on mRNA expression related to adipogenesis and inflammatory responses. On the other hand, in the investigation of aculeatin's potential as a ligand for PPAR-γ, aculeatin showed activation, but this effect was weak (Fig. 4A).

3.3. Aculeatin stimulates glucose uptake and lipolysis in 3T3-L1 adipocytes

Finally, we performed glucose uptake and lipolysis assays using mature 3T3-L1 adipocytes. Similarly to Pio, aculeatin (100 μM) was found to enhance insulin-stimulated 2-deoxyglucose uptake in

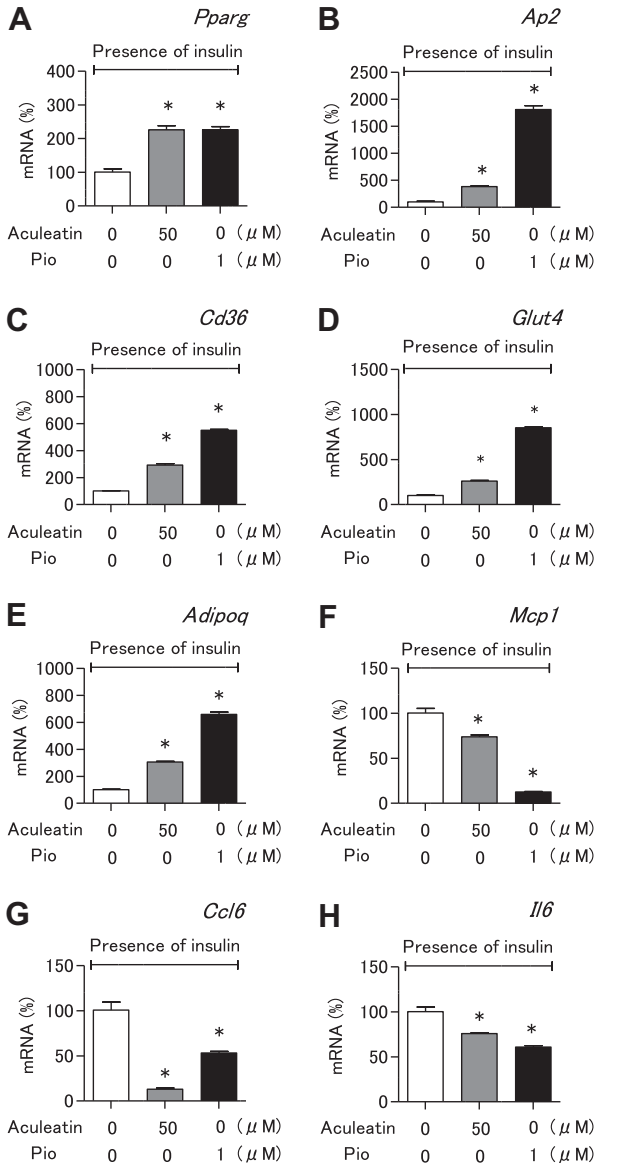

Fig. 3. Effects of aculeatin and Pio on mRNA expression ((A) *Pparg*, (B) *Ap2*, (C) *Cd36*, (D) *Glut4*, (E) *Adipoq*, (F) *Mcp1*, (G) *Ccl6*, and (H) *Il6*) related to adipocyte differentiation in 3T3-L1 cells. Detailed procedures are described in Section 2. Values are expressed as means ± SEM ($n = 3$). $^{*}P < 0.05$ compared with the control in the presence of insulin.

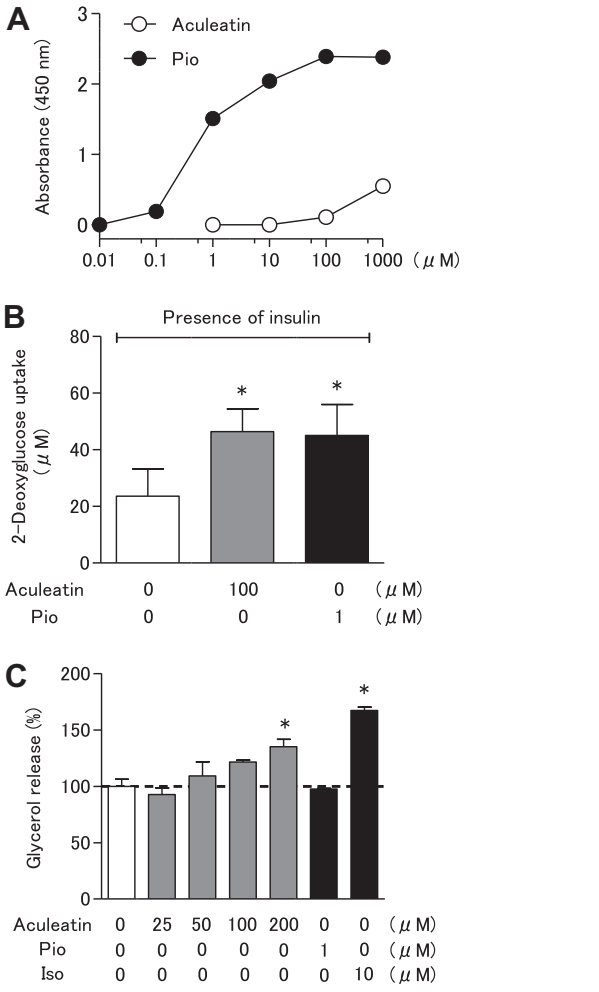

Fig. 4. (A) Effect of aculeatin on PPAR-γ ligand activity. (B) Effect of aculeatin on insulin-stimulated glucose uptake in mature 3T3-L1 adipocytes. $^{*}p < 0.05$ compared with the control in the presence of insulin. (C) Effect of aculeatin on lipolysis in mature 3T3-L1 adipocytes. Detailed procedures are described in Section 2. Analysis of PPAR-γ ligand activity was performed in duplicate. For the other data, values are expressed as means ± SEM ($n = 3$). $^{*}P < 0.05$ compared with the non-treatment control.

3T3-L1 adipocytes (Fig. 4B). Since the effect of aculeatin in the presence of insulin was greater than that of insulin alone, aculeatin may stimulate translocation of the glucose transporter GLUT4. In fact aculeatin-induced GLUT4 translocation was observed by Western blot analysis (data not shown).

In addition to glucose uptake, aculeatin (200 μM) increased glycerol release from differentiated mature 3T3-L1 cells (Fig. 4C). Similarly to aculeatin, Iso (a β-adrenergic receptor (β-AR) agonist) enhanced lipolysis, but Pio did not. These results suggest that aculeatin stimulates lipolysis through the β-AR pathway. As we anticipated, this lipolytic effect of aculeatin was inhibited by β-AR antagonists (data not shown).

4. Discussion

Insulin resistance plays a major role in the development and progression of diabetes. TZDs are potent insulin sensitizers, and currently, they are used clinically to treat diabetes. However, antidiabetic drugs such as TZDs have several potential side effects including weight gain and congestive heart failure [10,11]. Therefore, screening of new antidiabetic natural compounds with fewer side effects is desired.

In this study, we performed a screening of active compounds present in *T. asiatica* using the 3T3-L1 preadipocyte cell line and identified several coumarins as potential compounds. Since these coumarins, especially aculeatin, actually enhanced 3T3-L1 differentiation, aculeatin may be one of the major bioactive components of *T. asiatica*. The effects of aculeatin were higher in the presence than in the absence of insulin. Thus, we believe that rather than displaying insulin-like activity, aculeatin can increase insulin sensitivity. The concentration of aculeatin in the stems of *T. asiatica* was calculated to be approximately 200 mg/100 g dry weight.

Adipogenesis is accompanied by increased expression of various transcription factors and adipocyte-specific genes. We analyzed the expression of genes responsible for adipogenesis using DNA microarray analysis and RT-PCR and found that PPAR-γ expression was increased by aculeatin. PPAR-γ is essential for and serves as a master regulator of adipocyte differentiation by inducing adipocyte marker genes. As we expected, gene expression levels of PPAR-γ transcriptional targets (*Ap2*, *Cd36*, *Glut4* and *Adipoq*) were increased by aculeatin.

Both obesity and insulin resistance are associated with inflammation. Adipose tissue associated with obesity is characterized by enhanced infiltration of macrophages, which produce various inflammatory proteins including MCP-1 and interleukin 6 (IL-6). These proteins cause insulin resistance and stimulate cytokine expression in adipocytes, thereby aggravating inflammation. In this study, the expression of inflammatory genes (*Mcp1*, *Ccl6*, and *Il6*) was decreased by aculeatin. Therefore, it is thought that aculeatin enhances differentiation of 3T3-L1 cells by modulating the expression of genes critical for both adipogenesis and inflammatory responses.

Several natural compounds have been reported to act as ligands for PPAR-γ [13], but the PPAR-γ ligand activity of aculeatin was found to be weak in this study. Thus, aculeatin does not appear to act as a PPAR-γ ligand. It may enhance the expression of PPAR-γ transcriptional targets via a certain mechanism (e.g., up-regulation of PPAR-γ signaling cascades), and this possibility needs to be investigated in future studies.

Aculeatin led to increased glucose uptake in the presence of insulin. In insulin-sensitive tissues, glucose uptake is associated directly with a number of glucose transporters. It is therefore plausible that aculeatin sensitizes adipocytes to insulin and promotes GLUT4 translocation in 3T3-L1 adipocytes. Aculeatin also increased the release of glycerol into the medium. In adipose tissue, β-AR agonists such as Iso reportedly increased cAMP-induced lipolysis [14]. Since the lipolytic effects of aculeatin were inhibited by the β-AR antagonists propranolol and pindolol (data not shown), aculeatin may enhance lipolysis in 3T3-L1 adipocytes through the β-AR pathway. On the other hand, Pio did not affect lipolysis. Saito et al. reported that nobiletin enhanced lipolysis of 3T3-L1 adipocytes [12]. In consideration of these observations, the effects of aculeatin seem to resemble those of nobiletin, but not Pio. Because Lee et al. showed that nobiletin could improve hyperglycemia, obesity and insulin resistance in animal studies [15,16], we can anticipate that aculeatin also may exert these effects in vivo. The latter assumption is currently undergoing investigation in our laboratory.

In conclusion, our results demonstrated that aculeatin can promote adipocyte differentiation, improve glucose uptake and enhance adipocyte lipolysis. Hence, aculeatin and/or *T. asiatica* extracts would be useful for the treatment of lipid abnormalities as well as diabetes. To the best of our knowledge, this is the first study to elucidate the bioactivity of aculeatin using cultured cells. Further studies, especially those performed in vivo, are needed to better understand the function of aculeatin inside the body.

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