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Aucubin, a naturally occurring iridoid glycoside inhibits TNF-α-induced inflammatory responses through suppression of NF-κB activation in 3T3-L1 adipocytes

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ABSTRACT

Obesity is closely associated with a state of chronic, low-grade inflammation characterized by abnormal cytokine production and activation of inflammatory signaling pathways in adipose tissue. Tumor necrosis factor (TNF)-α is chronically elevated in adipose tissues of obese rodents and humans. Increased levels of TNF-α are implicated in the induction of atherogenic adipokines, such as plasminogen activator inhibitor (PAI)-1, adipose-tissue-derived monocyte chemoattractant protein (MCP)-1, and interleukin (IL)-6. Aucubin, an iridoid glycoside existing in medicinal plants, has been reported to show an anti-inflammatory activity by suppression of TNF-α production in murine macrophages. The present study is aimed to investigate the effects of aucubin on TNF-α-induced atherogenic changes of the adipokines in differentiated 3T3-L1 cells. Aucubin significantly inhibited TNF-α-induced secretion and mRNA synthesis of the atherogenic adipokines including PAI-1, MCP-1, and IL-6. Further investigation of the molecular mechanism revealed that pretreatment with aucubin suppressed extracellular signal-regulated kinase (ERK) activation, inhibitory kappa Bα (IκBα) degradation, and subsequent nuclear factor kappa B (NF-κB) activation. These findings suggest that aucubin may improve obesity-induced atherosclerosis by attenuating TNF-α-induced inflammatory responses.

1. Introduction

Obesity, which is characterized by excessive accumulation of abdominal fat, is casually associated with the premature development of atherosclerosis, increased risk of stroke, and development of congestive heart failure [1]. Recent studies have indicated that the adipocyte secretes a variety of adipokines involved in energy metabolism, inflammation, and cardiovascular functions [2]. The cellular mechanisms linking obesity and atherosclerosis are complex and have not been fully elucidated. However, increasing evidences suggest that the changes of adipokines including PAI-1, MCP-1, and IL-6 due to excess adipose tissue may be a cause of atherosclerosis [3].

Plasminogen activator inhibitor (PAI)-1 is the primary inhibitor of plasminogen activation. Plasma levels of PAI-1 are markedly elevated in obese individuals as well as in patients with insulin resistance, type 2 diabetes, and cardiovascular disease (CVD) [4,5]. PAI-1 is thought to be the link between obesity and increased risk for CVDs [6]. Although several tissues are known to produce PAI-1, adipose tissue appears to be the major contributor to elevated PAI-1 levels observed in cases of obesity [7,8]. Monocyte chemoattractant protein (MCP)-1 chemotactically recruits monocytes to sites of inflammation. Although this protein is traditionally thought to be expressed mainly endothelial cells and macrophages, it has been shown to be primarily expressed by adipose tissues [9,10]. Adipocyte-derived MCP-1 induces macrophage infiltration into adipose tissues and thus secretes inflammatory cytokines including tumor necrosis factor (TNF)-α, which in turn leads to the dysfunction of adipocytes [11]. In addition, MCP-1 inhibits insulin-dependent glucose uptake and the expression of adipogenic genes [10].

Aucubin is a natural constituent with a monoterpene cyclic ring system found in a wide range of some insects and higher plants such as *Aucuba japonica* and *Plantago asiatica* [12,13]. In recent years, a variety of bio-activities of aucubin have been reported; liver-protective activities against hepato-toxicants, stimulation of bile acid excretion, anti-microbial activities, anti-tumor activities, antidotal activities for noxious amanita mushroom poisoning, anti-viral activities against hepatitis B virus, and anti-inflammatory activities [14–22]. Interestingly, a number of plants containing aucubin have been used long as medicinal herbs for anti-inflammatics and anti-rheumatics in Oriental and Occidental hemispheres. As for the molecular mechanism for anti-inflammatory

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activities of aucubin, it was reported that aucubin inhibits the production of TNF-α resulting from the both IκBα degradation and the nuclear translocation of NF-κB in RAW 264.7 cells [21]. However, the inhibitory effects of aucubin on TNF-α-induced inflammatory responses in adipocytes have not been reported previously.

The present study was designed to determine whether it attenuates TNF-α-induced secretion and mRNA production of the atherogenic adipokines including PAI-1, MCP-1, and IL-6 in differentiated 3T3-L1 adipocytes. In addition, the possible mechanisms for inhibitory effects of aucubin on obesity-related inflammatory responses were examined.

2. Materials and methods

2.1. Reagents

Aucubin with 99.5% purity was purchased from Wako Pure Chemical Industries Ltd., (Osaka, Japan). Recombinant murine tumor necrosis factor (TNF)-α was from R&D Systems (Minneapolis, MN, USA). Insulin, dexamethasone, and 3-isobutyl-1-methylxanthine were from Sigma (St. Louis, MO, USA). All tissue culture materials were from Gibco-BRL (Rockville, MD, USA). Primary anti-mouse antibodies for extracellular signal-regulated kinases (ERK), phospho-ERK, IκBα, phospho-IκBα, and β-actin were obtained from Cell Signaling Technology (Beverly, MA, USA). Poly (ADP-ribose) polymerase (PARP) and p65 monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. 3T3-L1 cell culture and treatment

3T3-L1 preadipocytes (American Type Culture Collection, Rockville, MD) were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin–streptomycin (10,000 U/mL penicillin and 10,000 cg/mL streptomycin in 0.85% saline), and 1% (v/v) 100 μM pyruvate at 37 °C in 95% air 10% CO_2. Differentiation of 2-day postconfluent preadipocytes (designated as day 0) was initiated with 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1 μg/mL insulin in DMEM supplemented with 10% fetal bovine serum. After 48 h (day 2), the culture medium was replaced with DMEM supplemented with 10% fetal bovine serum and 1 μg/mL insulin, and the cells were then fed every other day with DMEM containing 10% fetal bovine serum. Aucubin was reconstituted, filter sterilized, and stored at −20 °C. For each experiment, cells received aucubin premixed with culture medium. TNF-α treatment was carried out after a 6-h pretreatment with aucubin. Aucubin was dissolved in dimethyl sulfoxide (DMSO) before it was added to culture medium. The final concentration of DMSO in culture medium was 0.5% (v/v).

2.3. Measurement of adipokines by ELISA

The conditioned culture medium from 3T3-L1 adipocytes was collected from each sample. The concentrations of MCP-1, PAI-1, and IL-6 were assayed using a mouse MCP-1 ELISA kit (R&D Systems, Minneapolis, MN, USA), a mouse PAI-1 ELISA kit (Molecular Innovations Inc., Southfield, MI, USA), and a mouse IL-6 ELISA kit (Biosource Inc., Camarillo, CA, USA), respectively. All assays were performed according to the manufacturer's instructions.

2.4. Evaluation of gene expression levels by quantitative real-time RT-PCR

For real-time RT-PCR, cDNA was prepared from 1 μg total RNA in a final reaction volume of 15 μL. Real-time RT-PCR was performed using 2 μL cDNA diluted with each of the gene-specific primer sets (Bioneer Inc., Korea). Each primer set was used at a concentration of 150 nM in a final reaction volume of 20 μL and reactions were performed on the Lightcycler 480 SYBR Green I Master (Roche Applied Science, Germany). The sequences for primers are: MCP-1 (5′-GCCCCACTCACCTGCTGCTACT-3′ and 5′-CCTGCTGCTGGTGATCCTCTTGT-3′), PAI-1 (5′-AGGATCGAGGTAAACGAGAGC-3′ and 5′-GCGGGCTGAGATGACAAA-3′), and IL-6 (5′-GCTACCAAACTGGATATAATCAGGA-3′ and 5′-CCAGGTAGCTATGGTACTCCAGAA-3′). Levels of mRNA, expressed as relative mRNA levels compared with control, were calculated after normalization to β-actin.

2.5. Nuclear extraction

Nuclear fractions of cells were prepared as previously described [23]. Briefly, cells were scraped and suspended in 400 μL of cold buffer A (HEPES 10 mM; pH 7.9; KCl 10 mM; EDTA 0.1 mM; EGTA 0.1 mM; dithiothreitol 1 mM; phenylmethylsulfonyl fluoride 0.5 mM; pepstatin A 1 μg/mL; leupeptin 10 μg/mL; aprotinin

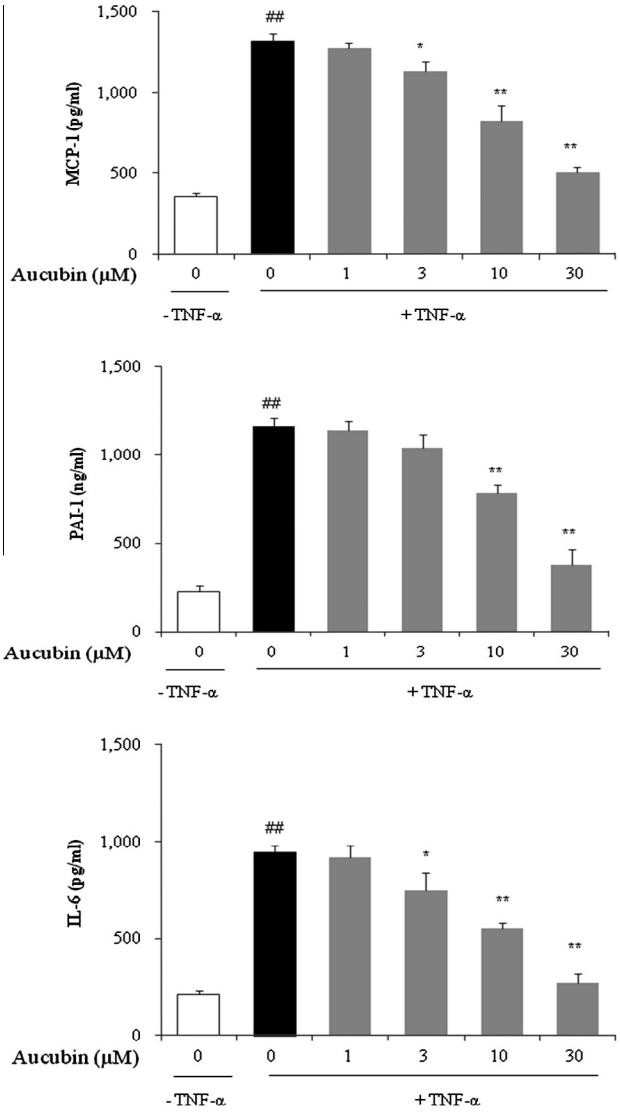

Fig. 1. Effects of aucubin on TNF-α-stimulated adipokine production in 3T3-L1 adipocytes. 3T3-L1 adipocytes were exposed or not to increasing concentrations of aucubin (1, 3, 10, and 30 μM) for 6 h and then were stimulated for 24 h by the addition of 10 ng/mL TNF-α. The secreted antigens of MCP-1, PAI-1, and IL-6 in conditioned medium were measured as described in Section 2. Results represent the mean ± SD from three independent experiments. $^{\#\#}p < 0.01$ versus untreated control; $^{*}p < 0.05$, $^{**}p < 0.01$ versus TNF-α-stimulated control.

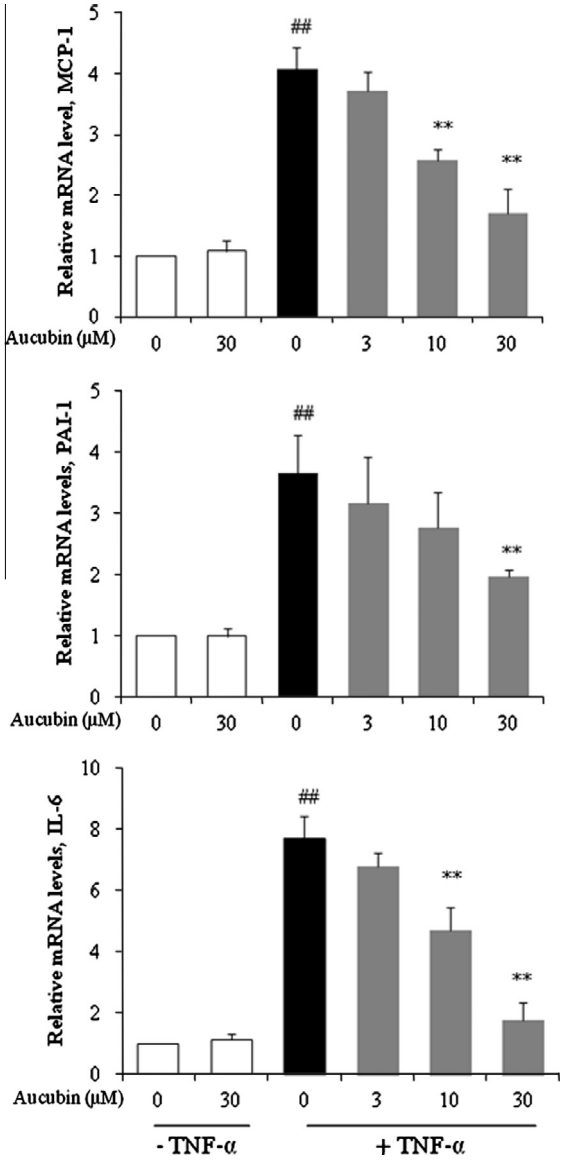

Fig. 2. Effects of aucubin on TNF-α-induced adipokine mRNA synthesis in 3T3-L1 adipocytes. After exposure to various concentrations of aucubin (1, 3, 10, and 30 μM) for 6 h, 3T3-L1 adipocytes were stimulated with 10 ng/mL TNF-α for 24 h. Then cells were harvested, and total RNA was extracted for measuring the mRNA levels of MCP-1, PAI-1, and IL-6 by quantitative RT-PCR. Values are normalized to β-actin RNA expression levels and expressed relative to untreated control cells. Results represent the mean ± SD from three independent experiments. $^{\#\#}p < 0.01$ versus untreated control; $^{**}p < 0.01$ versus TNF-α-stimulated control.

10 μg/mL) in ice bath under the presence of 25 μL 1% IGEPALCA-630 (Sigma). Then, the above samples were vortexed and centrifuged for 1 min at 10,000*g*. The precipitated cell pellet were used as the nuclear fraction for immunoblot assay. And the precipitated cell pellet was re-suspended with shaking in 100 μL of buffer B (HEPES 20 mM; pH 7.9; NaCl 400 mM; EDTA 1 mM; EGTA 1 mM; dithiothreitol 1 mM; phenylmethylsulfonyl fluoride 0.5 mM; pepstatin A 1 μg/mL; leupeptin 10 μg/mL; aprotinin 10 μg/mL), and centrifuged for 15 min at 10,000*g*. The supernatant aliquots (70 μL) were used for assay.

2.6. Western blot analysis

Cell lysates were collected, and then protein concentrations were determined with bicinchoninic acid (BCA) protein assay reagents (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of cell extracts were separated by electrophoresis using a 10% SDS–polyacrylamide gel and transferred to nitrocellulose membranes (Whatman Protran, Whatman GmbH, Dassel, Germany). After being blocked at room temperature in 5% non-fat dry milk with Tris-buffered saline Tween-20 (TBST) buffer for 2 h, the blots were probed with the primary antibody against each target protein at a concentration of 1:500–1:1000 in 5% non-fat dry milk in TBST overnight at 4 °C. Bound antibodies were detected by horseradish peroxidase-conjugated secondary antibodies and signals were detected and quantified using a chemiluminescent detection system, LAS-3000 (Fujifilm, Japan).

2.7. Statistics

Data expressed as mean ± SD were obtained from three separate experiments. Statistical analysis of the data was performed by one-way analysis of variance followed by Dunnett's test. $P < 0.05$ was considered to have significant difference.

3. Results

3.1. Effects of aucubin on the TNF-α-induced secretion of adipokines in 3T3-L1 adipocytes

To examine the effects of aucubin on TNF-α-induced secretion of adipokines, 3T3-L1 adipocytes were pretreated with various concentrations of aucubin for 6 h and then incubated with 10 ng/mL TNF-α for 24 h. After incubation, conditioned medium was collected for ELISA assay. Treatment with aucubin inhibited TNF-α-induced increase in the secretion of MCP-1, PAI-1, and IL-6 in dose-dependent manner with IC50 of 8.20, 9.94, and 6.85 μM, respectively (Fig. 1).

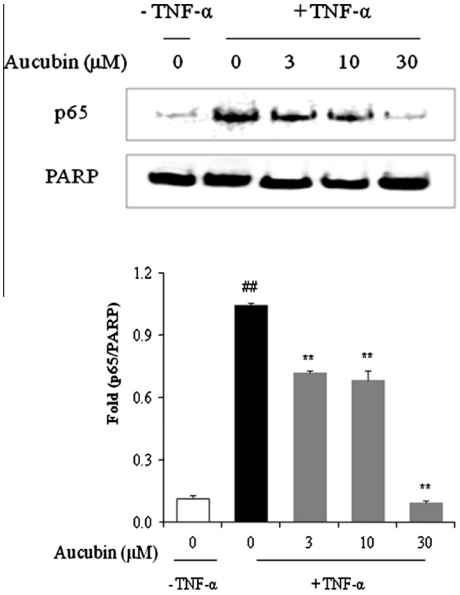

Fig. 3. Effects of aucubin on TNF-α-induced nuclear translocation of NF-κB. Nuclear extracts, prepared from control or pretreated with different concentrations (1, 3, 10, and 30 μM) of aucubin for 6 h, then TNF-α (10 ng/mL) for 24 h, were prepared for the Western blotting of p65 of NF-κB using specific anti-p65 monoclonal antibody. PARP was used as internal controls. Density ratios versus PARP were determined by densitometry. The data shown are representative of three independent experiments. The values shown are means ± SD. $^{\#\#}p < 0.01$ versus untreated control; $^{**}p < 0.01$ versus TNF-α-only treated control.

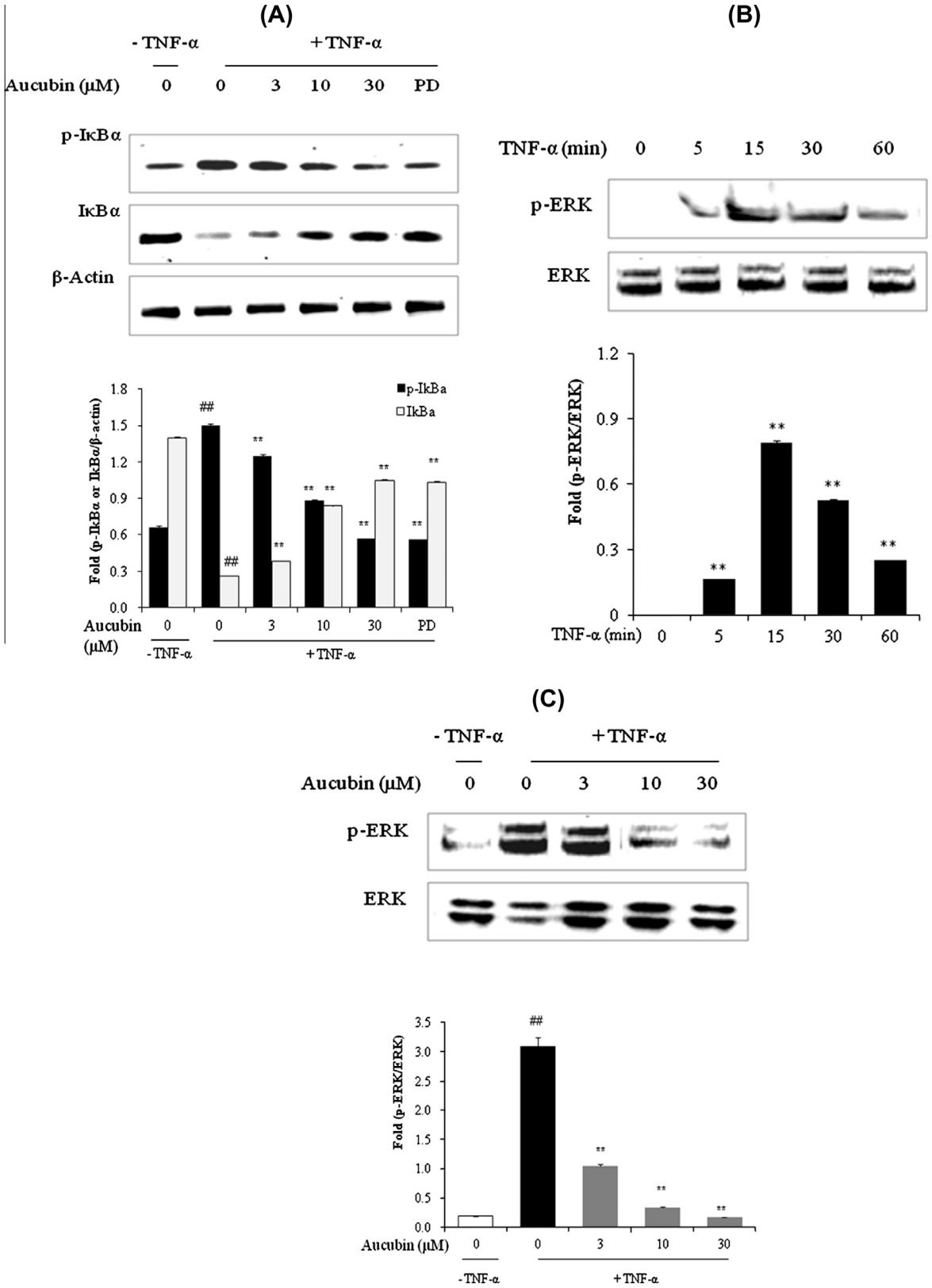

Fig. 4. Effects of aucubin on TNF-α-induced IκBα degradation and ERK phosphorylation. (A) Effects of aucubin on IκBα protein phosphorylation and degradation. Following pretreatment with aucubin (1, 3, 10, and 30 μM) for 6 h, cells were treated with TNF-α (10 ng/mL) for 15 min. Total proteins were prepared and Western blotting was performed using specific p-IκBα and IκBα antibodies. PD98059 (PD, 20 μM) was used as an ERK1/2-specific inhibitor. (B) Time-dependent changes of TNF-α-induced ERK phosphorylation. After stimulation with TNF-α (10 ng/mL), whole cell lysates prepared at different times (5, 15, 30, 60 min) were analyzed by Western blotting using specific p-ERK and ERK antibodies. (C) Effects of aucubin on TNF-α-induced ERK phosphorylation. Following pretreatment with aucubin (1, 3, 10, and 30 μM) for 6 h, cells were treated with TNF-α (10 ng/mL) for 15 min. Whole cell lysates were prepared and Western blotting was performed using specific p-ERK and ERK antibodies. Density ratios versus β-actin or ERK were determined by densitometry. The data shown are representative of three independent experiments. The values are represented as means ± SD. $^{\#\#}p < 0.01$ versus untreated control; $^{**}p < 0.01$ versus TNF-α-only treated control.

3.2. Effects of aucubin on the TNF-α-induced expression of adipokine genes in 3T3-L1 adipocytes

To investigate whether aucubin inhibited TNF-α-induced secretion of adipokines by altering the gene expression, real-time quantitative RT-PCR was carried out with total RNA extracted from 3T3-L1 adipocytes of each group. The gene expression of adipokines was measured after 3T3-L1 adipocytes were exposed to aucubin for 6 h prior to TNF-α (10 ng/mL) treatment for 24 h. The enhanced production of MCP-1, PAI-1, and IL-6 mRNA by TNF-α was suppressed by aucubin pretreatment (Fig. 2). At a aucubin concentration of 10 μM, the mRNA production of MCP-1, PAI-1, and IL-6 was suppressed by 36.5%, 24.3%, and 39.0%, respectively.

3.3. Effects of aucubin on the TNF-α-induced nuclear translocation of NF-κB

Since NF-κB is a major transcription factor that modulates the expression of pro-inflammatory cytokines, it was investigated whether aucubin prevents the translocation of the p65 subunit of NF-κB from cytosol into the nucleus using Western blotting. It was found that aucubin prior to TNF-α significantly attenuated p65 level in the nuclear fraction (Fig. 3).

3.4. Effects of aucubin on the TNF-α-induced phosphorylation of IκBα and ERK

In unstimulated adipocytes, NF-κB is sequested in the cytosol by its inhibitor IκBα, and when cells are stimulated with TNF-α, IκB is phosphorylated, ubiquitinated, and rapidly degraded via 26S proteasome, which results in the release of NF-κB. Therefore, the effects of aucubin on the TNF-α-induced phosphorylation and degradation of IκBα was examined using Western blotting. Aucubin significantly reduced the TNF-α-induced IκBα phosphorylation in a dose-dependent manner. Furthermore, aucubin pretreatment blocked TNF-α-induced IκBα degradation (Fig. 4A). Next, the effects of ERK1/2-specific inhibitor PD98059 (PD) on TNF-α-induced IκBα phosphorylation and degradation were investigated. In parallel with the effects of aucubin, pretreatment with 20 μM PD98059 reduced the TNF-α-induced IκBα phosphorylation and degradation in adipocytes (Fig. 4A). These observations suggest that the phosphorylation and degradation of IκBα is affected by ERK1/2 signal pathway. In fact, it has been known that activation of IκBα degradation is a downstream event following the activation of the ERK1/2 pathway [24]. Thus, it was investigated whether aucubin regulates the phosphorylation of ERK using Western blotting. Challenge with TNF-α brought about the ERK phosphorylation within 15 min; then p-ERK band was gradually restored to basal level (Fig. 4B). Therefore, Western blotting for ERK was carried out after treating with TNF-α (10 ng/mL) for 15 min in the presence or absence of aucubin. 3T3-L1 adipocytes were pretreated with aucubin in the indicated concentrations for 6 h and then stimulated with TNF-α (10 ng/mL) for 15 min. Total cell lysates were then probed with phosphospecific antibodies for ERK1/2. The phosphorylation of ERK1/2 was increased in cells treated with TNF-α alone. However, aucubin significantly inhibited phosphorylated ERK1/2 levels in TNF-α-induced 3T3-L1 adipocytes in a concentration-dependent manner (Fig. 4C).

4. Discussions

Aucubin [1,4a,5,7a-tetra-5-hydroxy-7-(hydroxymethyl)cyclopenta(c)pyran-1-yl-β-D-glucopyranoside] is a common iridoid glycoside, found in a wide range of plants, which are used in folk medicine and traditional Chinese medicine. Those medicinal plants containing aucubin show a variety of biological activities as aforementioned. Of pharmaceutical products as well as dietary supplements containing such medicinal plant materials, agents for treating inflammatory ailments including rheumatic inflammation have been marketed and used for long time. As for the molecular mechanism for anti-inflammatory activities of aucubin, it was reported that aucubin inhibits the production of TNF-α resulting from the both IκBα degradation and the nuclear translocation of NF-κB in RAW 264.7 cells [21].

It was previously reported that the increased levels of TNF-α associated with obesity may significantly contribute to elevated plasma and adipose tissue expression levels of adipokines [25]. This study demonstrated that aucubin effectively inhibited TNF-α-stimulated increases in the secretion of MCP-1, PAI-1, and IL-6 in 3T3-L1 adipocytes (Fig. 1). The mRNA production of each adipokines was suppressed in parallel with its secretion regulated by aucubin (Fig. 2).

Transcription factor NF-κB has been evidenced to play a major role in the TNF-α-induced expression of adipokines in adipocytes [26]. NF-κB is associated and controlled by IκBα, which presents an inactive form in the cytoplasm. Upon stimulation, IκBα becomes phosphorylated, undergoes degradation, and allows NF-κB translocation into the nucleus, where it activates proinflammatory genes and participates in the inflammatory response [27]. In the present study, aucubin suppressed the nuclear translocation of NF-κB (Fig. 3), which suggests that aucubin may decrease NF-κB activity by inhibition of IκBα degradation. Furthermore, it revealed that ERK1/2-specific inhibitor PD98059 blocked degradation of IκBα upon TNF-α stimulation, implying that IκBα degradation is a downstream event following the activation of the ERK1/2 pathway (Fig. 4A). Therefore, to confirm the inhibitory mechanisms of IκBα degradation by aucubin, the effects of aucubin on TNF-α-induced ERK phosphorylation were investigated. It was found that aucubin exhibited significant inhibition on TNF-α-stimulated ERK1/2 phosphorylation in a concentration-dependent fashion (Fig. 4C).

In conclusion, aucubin can attenuate TNF-α-stimulated increases in gene expression and secretion of adipokines in 3T3-L1 adipocytes. The effects of aucubin were mediated by suppression of ERK phosphorylation, IκBα degradation, and subsequent NF-κB activation. The present study may contribute to the understanding of anti-inflammatory effects of aucubin and provide a novel mechanism of aucubin in preventing obesity-related pathologies.

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