Original Research

Blockade of visfatin induction by oleanolic acid via disturbing IL-6-TRAF6-NF-κB signaling of adipocytes

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Abstract

Oleanolic acid is a pentacyclic triterpenoid naturally present in foods and medicinal plants with anticancer, antioxidant, and antiaging properties. The current study elucidated that oleanolic acid inhibited the production of insulin-mimetic and inflammatory adipokine of visfatin during adipogenic differentiation of 3T3-L1 adipocytes. Adipocytes were cultured in an adipogenic media with and without 1–25 μM oleanolic acid up to 8 days for differentiation. The cellular expression and secretion of visfatin was markedly enhanced in differentiating adipocytes, which was dose-dependently attenuated by 1–25 μM oleanolic acid. Secretion of interleukin (IL)-6 and macrophage inflammatory protein (MIP)-2 was highly elevated during differentiation, which was much earlier than visfatin production of adipocytes. The visfatin production was secondary to inflammatory IL-6 and MIP-2. This study further elucidated that nuclear factor-κB (NF-κB) signaling was responsible for cellular production of visfatin. NF-κB was activated by translocating into the nucleus with increased phosphorylation of inhibitory κB (IκB), which was disturbed by oleanolic acid. Cellular expression of tumor necrosis factor receptor associated factor 6 (TRAF6), a NF-κB upstream, was upregulated in parallel with transactivation with NF-κB. The TRAF6 induction required the auto-stimulation of inflammatory IL-6 and MIP-2. These results demonstrate that oleanolic acid inhibited visfatin and its inflammatory response during adipocyte differentiation through blocking IL-6-TRAF6-NF-κB signaling. Therefore, oleanolic acid may be a potent therapeutic agent targeting against adipogenesis and visfatin-linked inflammation.

Keywords: Adipocyte differentiation, obesity, oleanolic acid, visfatin, nuclear factor-κB, TRAF6

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Introduction

It has been demonstrated that there is a strong association between obesity and inflammatory diseases.[1] Adipose tissue is a major endocrine organ releasing signaling and mediator proteins of adipokines that facilitate adipose tissue to communicate with other organs.[2] Expansion of adipose tissue in obesity alters adipokine secretion which may contribute to the regulation of pathological processes.[3] Visfatin/Pre-B-cell colony-enhancing factor/nicotinamide phosphoribosyl transferase was an adipokine recently found in high levels in visceral fat, which exerts insulin-mimetic and sensitizing effects by binding to and activating the insulin receptor.[4] Although visfatin has been suggested to be a beneficial adipokine due to its insulin-sensitizing effect, the regulation of visfatin production and its physiological importance in the conditions of obesity and type 2 diabetes mellitus are still not completely understood. The various functions of visfatin have been shown towards involvements in the pathophysiology of several diseases.[4]

New insight into the role of adipokines makes them attractive targets for novel therapeutic strategies in chronic inflammatory diseases or subclinical inflammation relating to obesity and various metabolic abnormalities. Among various adipokines, resistin and visfatin are proposed as important pro-inflammatory mediators interfering with the central regulation of insulin sensitivity.[5] Visfatin is reported to be involved in the regulation of inflammatory processes.[6–8] The cytokine functions of visfatin are mainly pro-inflammatory as it induces potently various other pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α or interleukin-6 (IL-6).[6] However, the definite role of visfatin in various metabolic, inflammatory, and malignant diseases remains largely unknown.

Oleanolic acid (Figure 1(a)), a pentacyclic triterpene naturally found in fruits, vegetables, and medicinal plants, is shown to have anticancer, antioxidant, and antiaging properties.[9] Oleanolic acid ameliorates visceral adiposity and improves glucose tolerance in high-fat diet-induced obesity in mice, indicating its antiobese potential through modulation of carbohydrate and fat metabolism.[10] Our previous study showed that oleanolic acid suppressed adipocyte differentiation and adipokine resistin production.[11,12] It has been reported that natural compounds are effective in targeting inflammation in the treatment of obesity-induced insulin resistance and metabolic dysfunctions.[13] Quercetin-rich onion peel extract supplementation influences expression of adipokines including visfatin particularly in mesenteric fat.[14] In addition, quercetin and resveratrol but not epigallocatechin gallate decrease visfatin secretion from human Simpson-Golabi-Behmel syndrome (SGBS) adipocytes.[15] However, it is deemed that the mechanism(s) by which natural compounds inhibit adipokine production are diverse. Ellagic acid in pomegranate inhibits resistin secretion by a novel mechanism involving the degradation of intracellular resistin protein in murine adipocytes.[16] Curcumin down-regulates visfatin gene expression in human breast cancer cells by a nuclear factor-κB (NF-κB)-dependent mechanism, contributing to obesity-linked breast cancer development and progression.[17]

Based on previous evidence that oleanolic acid inhibits the differentiation and resistin secretion of adipocytes,[11,12] this study examined whether oleanolic acid may disturb obesity-related inflammation by inhibiting cellular production of visfatin in 3T3-L1 adipocytes. Furthermore, the mechanisms by which oleanolic acid attenuated visfatin production during adipocyte differentiation were explored. This study revealed that oleanolic acid suppressed visfatin production in adipocytes and adipogenesis-related inflammation through blocking IL-6-TRAF6-NF-κB signaling.

Materials and methods

Materials

Mus musculus (mouse) fibroblast preadipocyte cell line 3T3-L1 was provided by American Type Culture Collection (Manassas, VA). Dulbecco modified Eagle medium

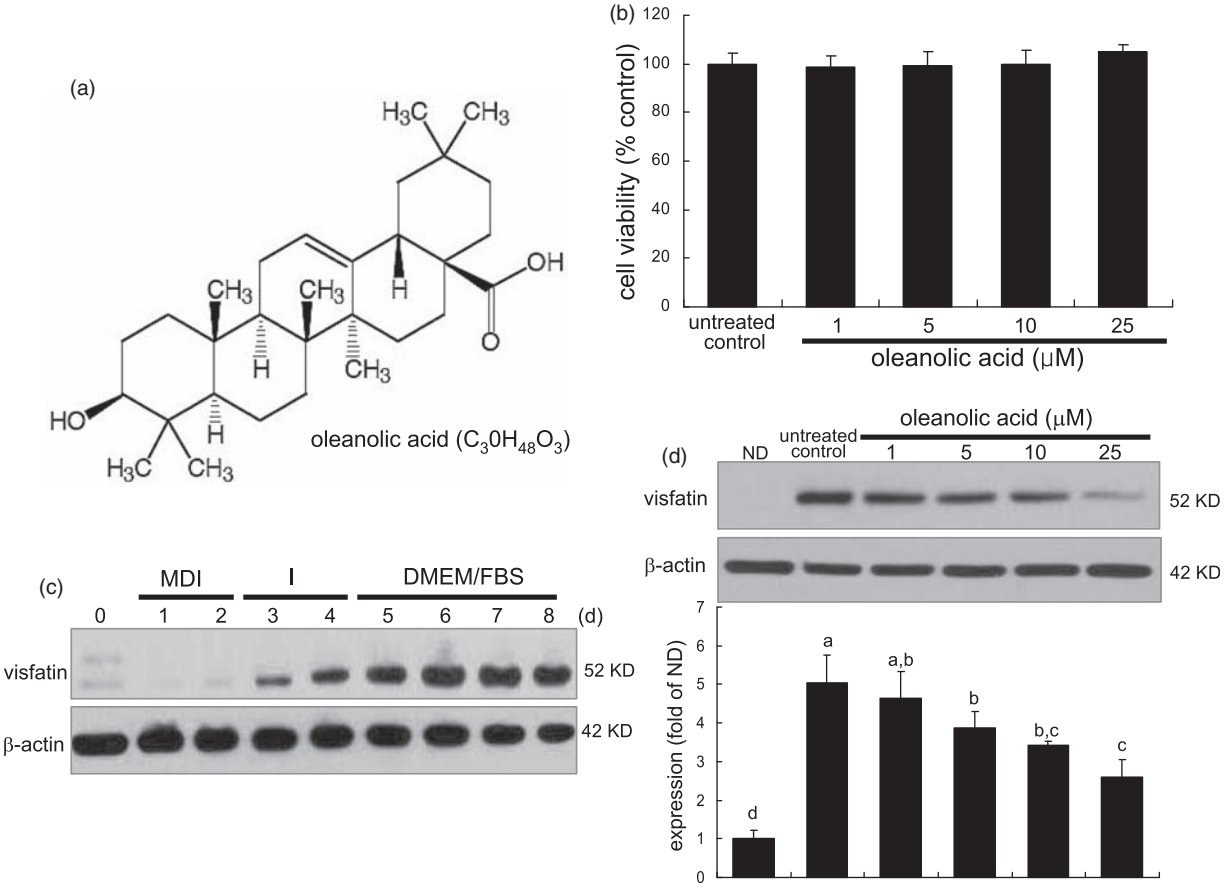

Figure 1 Chemical structure of oleanolic acid (a) and cytotoxicity of oleanolic acid (b). 3T3-L1 cells were incubated with 1–25 μM oleanolic acid during differentiation process. Cell viability was measured using MTT assay. Values (mean ± SEM, $n = 5$) are expressed as percent of oleanolic acid-untreated control. Time-course response of visfatin expression (c) and visfatin inhibition by oleanolic acid (d). 3T3-L1 adipocytes were maintained in DMEM supplemented with 10% inactivated NCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Adipocyte differentiation was initiated on post-confluence using DMEM with 10% FBS, 0.5 mM 3-isobytyl-1-methylxanthine (M), 0.5 μM dexamethasone (D), and 5 μg/mL insulin (I). Cells were maintained in differentiating DMEM in the absence or presence of 1–25 μM oleanolic acid for 5 days. Western blot analysis was performed with a primary antibody against visfatin. β-Actin was used as an internal control. Bar graphs (bottom panel, C and D) represent densitometric results of bands. ND, undifferentiated control. Values in the bar graphs (mean ± SEM, $n = 4$) not sharing a common letter are significantly different at $P < 0.05$

(DMEM; high glucose with L-glutamine and sodium pyruvate), culture chemicals, insulin, 3-isobytyl-1-methylxanthine, dexamethasone, 3-(4, 5-dimetylthiazol-yl)-diphenyl tetrazolium bromide (MTT), and oleanolic acid were provided by Sigma-Aldrich Chemical (St. Louis, MO). Newborn calf serum (NCS) was obtained from Invitrogen (Auckland, New Zealand). Fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA were purchased from Cambrex (East Rutherford, NJ). Mouse visfatin antibody was obtained from Phoenix Pharmaceuticals (Belmont, CA). Antibodies against inhibitory κB (IκB)-α, phospho-IκB-α were provided by Cell Signaling Technology (Beverly, MA) and nuclear factor-kappa B (NF-κB), lamin B, and tumor necrosis factor receptor associated factor 6 (TRAF6) were provided by Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant mouse IL-6 protein and mouse macrophage inflammatory protein (MIP)-2 antibody was obtained from (R&D Systems, Minneapolis, MN). β-Actin antibody was purchased from Sigma-Aldrich Chemical. Horseradish peroxidase-conjugated goat antirabbit IgG and donkey antigoat IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Reverse transcriptase and *Taq* DNA polymerase were purchased from Promega (Madison, WI). NF-κB inhibitor SN50 was purchased from Enzo Life Sciences (Farmingdale, NY).

The cytotoxicity of oleanolic acid was determined using a modified MTT assay. After 2-day cell culture protocols with 1−25 μM oleanolic acid, 3T3-L1 adipocytes were labeled with 1 mg/mL MTT solution at 37°C for 3 h and the resulting formazan was solubilized with 0.04 N HCl/isopropanol. The absorption was measured in an ELISA reader at $\lambda = 570$ nm. Oleanolic acid at ≤25 μM was not toxic to 3T3-L1 adipocytes (Figure 1(b)).

Preadipocyte differentiation

3T3-L1 preadipocytes were maintained in DMEM supplemented with 10% inactivated NCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The adipocyte differentiation was initiated on post-confluence in DMEM containing 10% FBS, 0.5 mM 3-isobytyl-1-methylxanthine, 0.5 μM dexamethasone, and 5 μg/mL insulin.[11,18] After 2-day incubation, culture media were changed to DMEM containing 10% FBS and 5 μg/mL insulin. After cells were incubated for additional 2 days, cells were continuously cultured in DMEM supplemented with 10% FBS, and media were changed every other day for the following 4 days.

Western blot analysis

Undifferentiated and differentiated 3T3-L1 adipocytes were washed with ice-cold phosphate buffered saline (PBS) and lysed with 1 M Tris-HCl (pH 6.8) buffer containing 10% sodium dodecyl sulfate (SDS), 1% β-glycerophosphate, 0.1 M Na3VO4, 0.5 M NaF, and protease inhibitor cocktail. Collected culture media was centrifuged at $800 \times g$ for 10 min and resulting supernatants were taken. Equal volumes of supernatants and equal amounts of lysate protein samples were run on 8–15% SDS-PAGE and transferred onto a nitrocellulose membrane (0.2 μm pore size, Whatman, Germany). Non-specific binding was blocked by soaking the membrane in Tris buffered saline-Tween 20 (TBS-T) buffer (0.5 M Tris-HCl [pH 7.5], 1.5 M NaCl, and 0.1% Tween 20) containing 5% non-fat dry milk for 3 h. The membrane was incubated overnight at 4°C with polyclonal rabbit anti-mouse visfatin, polyclonal rabbit anti-mouse phospho-IκB-α, polyclonal rabbit anti-mouse NF-κB, polyclonal goat anti-mouse macrophage inflammatory protein (MIP)-2, polyclonal goat anti-mouse lamin B, and polyclonal rabbit-anti mouse TRAF6. After 3 washes with TBS-T buffer, the membrane was incubated for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG or donkey anti-goat IgG. The protein levels were determined by using Supersignal West Pico chemiluminescence detection reagents (Pierce Biotechnology, Rockford, IL) and AGFA medical X-ray film (Gevaert, Belgium). Incubation with antibodies of mouse β-actin and lamin B was performed for the comparative control.

Reverse transcriptase-polymerase chain reaction analysis

Total RNA was isolated from undifferentiated and differentiated adipocytes using a commercially available Trizol reagent kit (Invitrogen, Carlsbad, CA), as previously described elsewhere.[19] The RNA (5 μg) was reversibly transcribed with 200 units of reverse transcriptase and 0.5 mg/mL oligo-$(dT)_{15}$ primer (Bioneer, Daejeon, Korea). Expression of mRNA transcripts of visfatin (forward primer: 5′-CAGTGCCTGTGTCTGTGGTCA-3′, reverse primer: 5′-CTAATGAGGTGCCACGTCCTG-3′) and β-actin (forward primer: 5′-GACTACCTCATGAAGATC-3′, reverse primer: 5′-GATCCACATCTGCTGGAA-3′) were evaluated by reverse transcriptase-polymerase chain reaction (RT-PCR) with a slight modification. PCR was performed in 25 μL Tris-HCl buffer (10 mM, pH 9.0) containing 25 mM $MgCl_2$, 10 mM dNTP, 5 units of *Taq* DNA polymerase, and 10 μM of each primer, and then terminated by heating at 94°C for 10 min. After thermocycling and electrophoresis of 25 μL PCR product on 1% agarose gel, bands were visualized using a TFX-20 M model-UV transilluminator (Vilber-Lourmat, Marne-la-Vallée, France), and gel microphotographs were obtained.

Nuclear extract preparation

Nuclear protein extract were prepared by a detergent lysis procedure to assay the translocation of NF-κB.[19] Adipocytes were lysed in a buffer of 20 mM HEPES (pH 7.9), 1 mM EDTA, 10 mM NaCl, 1 mM dithiothreitol, 1 mg/mL Nonidet P40, 0.4 mM phenylmethylsulfonylfluoride, 0.01 ng/mL leupeptin, and 200 units aprotinin and incubated on ice for 10 min. Proteins were extracted from nuclear pellets by incubation with a high-salt buffer containing 420 mM NaCl, 1 mM EDTA, 20 mM HEPES (pH 7.9), 25% glycerol, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 0.01 ng/mL leupeptin, and 200 units of aprotinin with vigorous shaking. The nuclear debris was pelleted by a brief centrifugation, and the supernatant was stored at −70°C for Western blot analysis.

Enzyme-linked immunoadsorbent assay

To measure the secretion of IL-6 and MIP-2 from differentiating 3T3-L1 adipocytes, collected culture media were assayed by using enzyme-linked immunoadsorbent assay (ELISA) kits of IL-6 and MIP-2 (R&D Systems) according to the manufacturer's instructions.

Statistical analysis

Results were expressed as mean ± SEM. The SPSS release 14.0 software package (SPSS, Chicago, IL) was used for the statistical analyses. Differences between the groups using one-way ANOVA were tested by Duncan's multiple range tests at $P < 0.05$.

Results

Suppression of visfatin induction by oleanolic acid

This study elucidated that oleanolic acid suppressed visfatin induction in differentiating adipocytes. Visfatin was induced in a temporal fashion during 8-day-differentiation of 3T3-L1 adipocytes, with initial expression on the third day of differentiation and thereafter the induction being sustained throughout (Figure 1(c)). The cellular expression of visfatin from 3T3-L1 cells differentiated for 5 days was dose-dependently attenuated by 1–25 μM oleanolic acid (Figure 1(d)). Additionally, visfatin secretion was diminished by oleanolic acid in a similar manner to its cellular expression (Figure 2(a)). This indicates that submicromolar oleanolic acid was effective in inhibiting the visfatin induction responsible for adipogenesis and inflammation.

RT-PCR analysis was performed to determine inhibitory effects of 1–25 μM oleanolic acid on visfatin transcription. Differentiation of 3T3-L1 adipocytes increased the visfatin mRNA level, compared to that of undifferentiated control (Figure 2(b)). Oleanolic acid suppressed the elevated transcriptional expression of visfatin in a dose-dependent manner, indicating that oleanolic acid may inhibit the visfatin induction at its transcriptional level.

Inhibition of production of IL-6 and MIP-2 by oleanolic acid

IL-6 secretion was highly enhanced at the very early stage of differentiation for 2 days and thereafter such secretion was rapidly vanished (Figure 3(a)). Oleanolic acid dose-dependently attenuated the IL-6 secretion markedly elevated on the first day of differentiation (Figure 3(b)). In addition, MIP-2 secretion increased at the early stage of differentiation (Figure 3(c)). The highly enhanced MIP secretion was observed on the second day of differentiation. Western blot analysis revealed that such secretion was suppressed by oleanolic acid in a dose-dependent manner (Figure 3(d)). Accordingly, oleanolic acid may blunt obesity-associated inflammation by demoting production of inflammatory mediators from adipocytes.

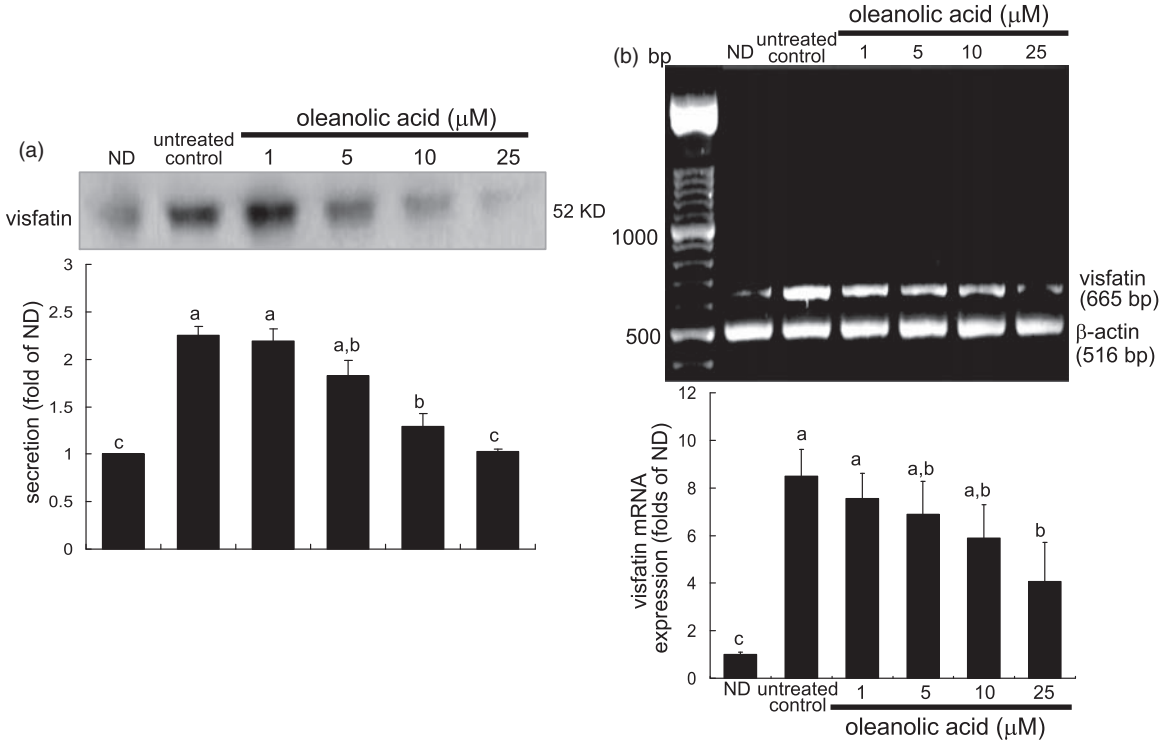

Figure 2 Suppressive effect of oleanolic acid on visfatin production (a) and transcription (b). For the measurement of visfatin secretion (a), culture media were collected. 3T3-L1 adipocytes were maintained in DMEM supplemented with 10% inactivated NCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Adipocyte differentiation was initiated on post-confluence using DMEM with 10% FBS, 0.5 mM 3-isobytyl-1-methylxanthine (M), 0.5 μM dexamethasone (D), and 5 μg/mL insulin (I). Cells were maintained in differentiating DMEM in the absence or presence of 1-25 μM oleanolic acid for 5 days. Western blot analysis was performed with a primary antibody against visfatin (a). β-Actin was used as an internal control. In RT-PCR analysis beta-Actin gene was used as an internal control for the co-amplification (b). Bar graphs (bottom panels, a and b) represent densitometric results of blot bands. ND, undifferentiated control. Values in the bar graphs (mean ± SEM, $n = 4$) not sharing a common letter are significantly different at $P < 0.05$

Involvement of IL-6 and MIP-2 in visfatin induction

This study further examined that the visfatin induction entailed the stimulation of IL-6 and MIP-2 secreted during adipocyte differentiation. The exposure of 3T3-L1 adipocytes to 10 μg/mL MIP-2 mAb during differentiation near-completely abolished the enhanced visfatin induction as 20 μM oleanolic acid (Figure 4). When 3T3-L1 preadipocytes were treated with 10 ng/mL IL-6 for 5 days without differentiation, the visfatin induction was highly increased as if differentiation occurred (Figure 4). Accordingly, inflammatory IL-6 and MIP-2 appeared to be responsible for visfatin induction in an autocrine manner.

Disturbance of NF-κB signaling by oleanolic acid

This study attempted to reveal that oleanolic acid blocked NF-κB activation during adipocyte differentiation. The NF-κB transactivation was observed from the third day of differentiation of 3T3-L1 adipocytes, with its activation being sustained throughout for 8-day-differentiation (Figure 5(a)). Similarly, the initial IκB phosphorylation occurred on the third day of differentiation. When 3T3-L1 adipocytes were treated with 1−25 μM oleanolic acid during 5-day-differentiation, nuclear NF-κB level was significantly diminished by ≥5 μM oleanolic acid, compared with that of untreated control (Figure 5(b)). The IκB phosphorylation was also markedly suppressed by adding oleanolic acid to differentiating adipocytes.

The current study tested whether the visfatin induction of adipocytes was mediated through activating NF-κB signaling. The cellular expression of visfatin was abolished by adding 10 μM SN50, a NF-κB inhibitor, to differentiating adipocytes (Figure 6(a)). This reveals that NF-κB signaling may be involved in the visfatin induction during adipocyte differentiation, which was blocked by supplementing submicromolar oleanolic acid to differentiating adipocytes.

Oleanolic acid inhibition of TRAP6 induction

This study attempted to reveal that oleanolic acid blocked TRAF6 induction for NF-κB signaling during adipocyte differentiation. When 3T3-L1 adipocytes were differentiated

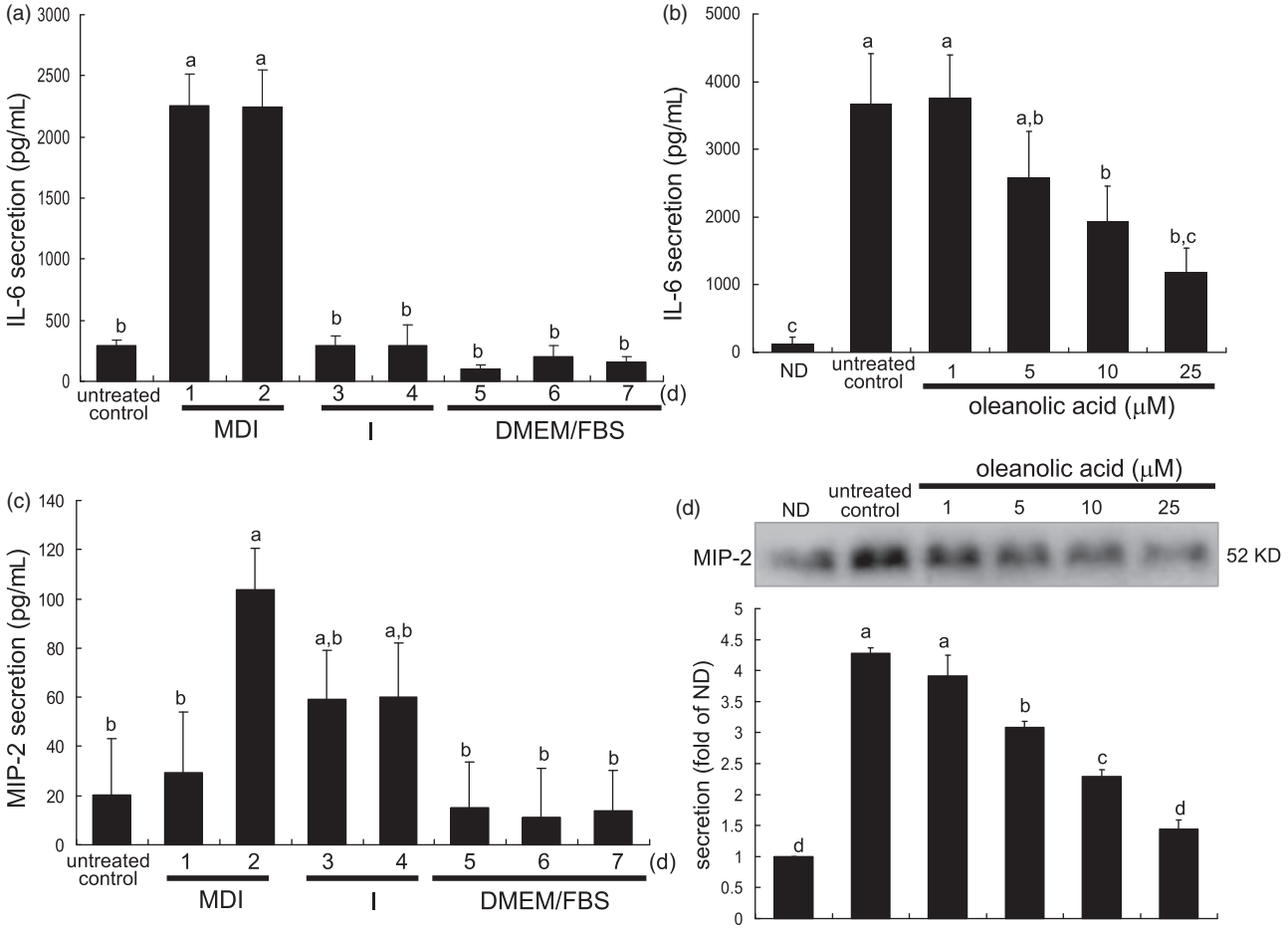

Figure 3 Time course response of secretion of IL-6 and MIP-2 (a and c) and inhibition of secretion of IL-6 and MIP-2 by oleanolic acid (b and d). 3T3-L1 adipocytes were maintained in DMEM supplemented with 10% inactivated NCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Adipocyte differentiation was initiated on post-confluence using DMEM with 10% FBS, 0.5 mM 3-isobytyl-1-methylxanthine (M), 0.5 μM dexamethasone (D), and 5 μg/mL insulin (I). Cells were maintained in differentiating DMEM in the absence or presence of 1–25 μM oleanolic acid. To examine the inhibitory effects on secretion of IL-6 and MIP-2, cells were cultured for 1 day or 2 days. The secretion of IL-6 and MIP-2 was measured by using ELISA kits for IL-6 and MIP-2 (a–c). Western blot analysis was performed with a primary antibody against MIP-2 (d). Bar graphs (bottom panel, d) represent densitometric results of bands. ND, undifferentiated control. Values in the bar graphs (mean ± SEM, $n = 4$) not sharing a common letter are significantly different at $P < 0.05$. IL-6: interleukin-6; MIP-2: macrophage inflammatory protein-2; DMEM; Dulbecco modified Eagle medium; FBS: fetal bovine serum

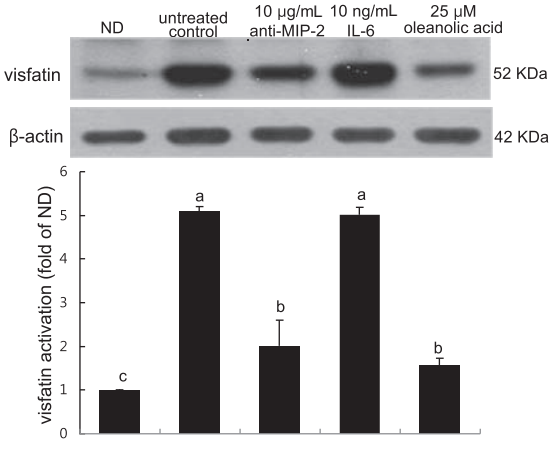

Figure 4 Visfatin induction by IL-6 and its inhibition by MIP-2 neutralization. 3T3-L1 adipocytes were maintained in DMEM supplemented with 10% inactivated NCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. IL-6 was treated to 3T3-L1 adipocytes in DMEM supplemented with 10% inactivated NCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Cells were maintained in differentiating DMEM in the absence or presence of 10 μg/mL anti-MIP-2 or 25 μM oleanolic acid for 5 days. Another set of cells were maintained in DMEM containing 10 ng/mL IL-6 for 5 days. Western blot analysis was performed with a primary antibody against visfatin and TRAF6. Bar graphs represent densitometric results of blot bands. β-Actin was used as an internal control as nuclear control. Values are mean ± SEM ($n = 3$) and means without a common letter differ, $P < 0.05$. IL-6: interleukin-6; MIP-2: macrophage inflammatory protein-2

during 5 days, TRAF6 was significantly induced (Figure 6(b)). However, TRAF6 induction was dose-dependently attenuated by 1−25 μM oleanolic acid, compared with that of untreated control (Figure 6(b)).

This study elucidated that the adipocyte differentiation-associated TRAF6 induction required the auto-stimulation of IL-6 and MIP-2. When 3T3-L1 adipocytes were neutralized with 10 μg/mL MIP-2 mAb during 5-day differentiation, the TRAF6 induction was dampened (Figure 6(c)). In addition, the treatment of 3T3-L1 preadipocytes with 10 ng/mL IL-6 for 5 days without differentiation markedly promoted TRAF6 induction (Figure 6(c)). Thus, TRAF-NF-κB signaling required for visfatin induction may be triggered by inflammatory IL-6 and MIP-2 secreted in early stages of differentiation (Figure 3).

Discussion

Five major findings were observed in this study. (1) Non-cytotoxic oleanolic acid suppressed cellular production of visfatin in differentiating 3T3-L1 adipocytes at its transcriptional level. (2) Inflammatory IL-6 and MIP-2 were highly secreted at the early phase of differentiation, which was dose-dependently attenuated by oleanolic acid. (3) Cellular production of IL-6 and MIP-2 occurred prior to initiating cellular production of visfatin. The production of these cytokines was responsible for visfatin induction during adipocyte differentiation. (4) NF-κB was transactivated concomitantly with phosphorylation of IκB during differentiation and such activation was blocked by incubating adipocytes with oleanolic acid. (5) TRAF6 expression was markedly upregulated at same point of time to NF-κB activation. When oleanolic acid was supplied to adipocytes, the TRAF6 induction was retarded. These results demonstrate that visfatin was primarily secreted from mature adipocytes and modulates adipogenesis-linked inflammation and insulin-mimetic glucose uptake in 3T3-L1 adipocytes. Additionally, submicromolar oleanolic acid encumbered adipocyte differentiation-associated cellular production of visfatin and inflammation through disturbing TRAF6-NF-κB signaling. Unfortunately, this study did not examine mechanisms involving the degradation of cellular visfatin during adipocyte differentiation.

Oleanolic acid is one of naturally occurring triterpene widely distributed in *campanulaceae* plants and is reported to have chemopreventive, hepatoprotective, and anti-inflammatory activities.[9] In our previous studies, oleanolic acid blunted adipocyte differentiation and resistin production.[11,12] Oleanolic acid reduced cellular expression of the adipogenic biomarkers of peroxisome proliferators-activated receptor γ (PPARγ) and cytidine-cytidine-adenosine-adenosine-thymidine (CCAAT) enhancer binding protein α (C/EBPα).[11] The current study investigated that submicromolar oleanolic acid inhibited adipocyte production of visfatin. It should be noted that the visfatin induction initiated much earlier than the completion of adipocyte differentiation. Oleanolic acid blunted the cellular expression of visfatin highly enhanced on fifth day after initiating differentiation at its transcriptional level. Accordingly, it is assumed that oleanolic acid inhibiting visfatin production may ameliorate obesity-induced metabolic dysfunctions. Plant polyphenols such as quercetin decrease visfatin secretion from human adipocytes without influencing leptin and adiponectin secretion.[15] Supplementation of quercetin rich in onion peel extract lowered transcription of adiponectin and IL-6 in the visceral adipose tissue of rats.[14] Similarly, ellagic acid rich in pomegranate inhibits secretion of another adipokine resistin at the protein levels of murine adipocytes.[16]

Adipose tissue is a major endocrine organ instigating cellular signaling and releasing adipokines making adipose tissue communicate with other organs.[2] Expansion of adipose tissue in obesity influences adipokine secretion which may contribute to the regulation of pathophysiological processes.[3] Visfatin was a novel adipokine found in high levels in visceral fat, exercising insulin-mimetic activity through binding to insulin receptors.[4] It has been suggested that visfatin is a beneficial adipokine with insulin-mimicking/-sensitizing effects enhancing insulin sensitivity. However, visfatin has pro-inflammatory properties in the conditions of obesity and type 2 diabetes mellitus. Whether visfatin binds to insulin receptor and exerts insulin mimetic activity is still a controversy. However, elevated level of visfatin is found in the systemic circulation of patients with a variety of inflammatory diseases.[6,20,21] Visfatin is considered a pro-inflammatory adipocytokine activating human leukocytes and inducing production of cytokines such as TNF-α, IL-1β, and IL-6.[20] In the current study, inflammatory IL-6 and MIP-2 were highly secreted at the early phase of differentiation, which occurred earlier than the cellular induction of visfatin. The initial production of IL-6 and MIP-2 appeared to trigger the visfatin induction during

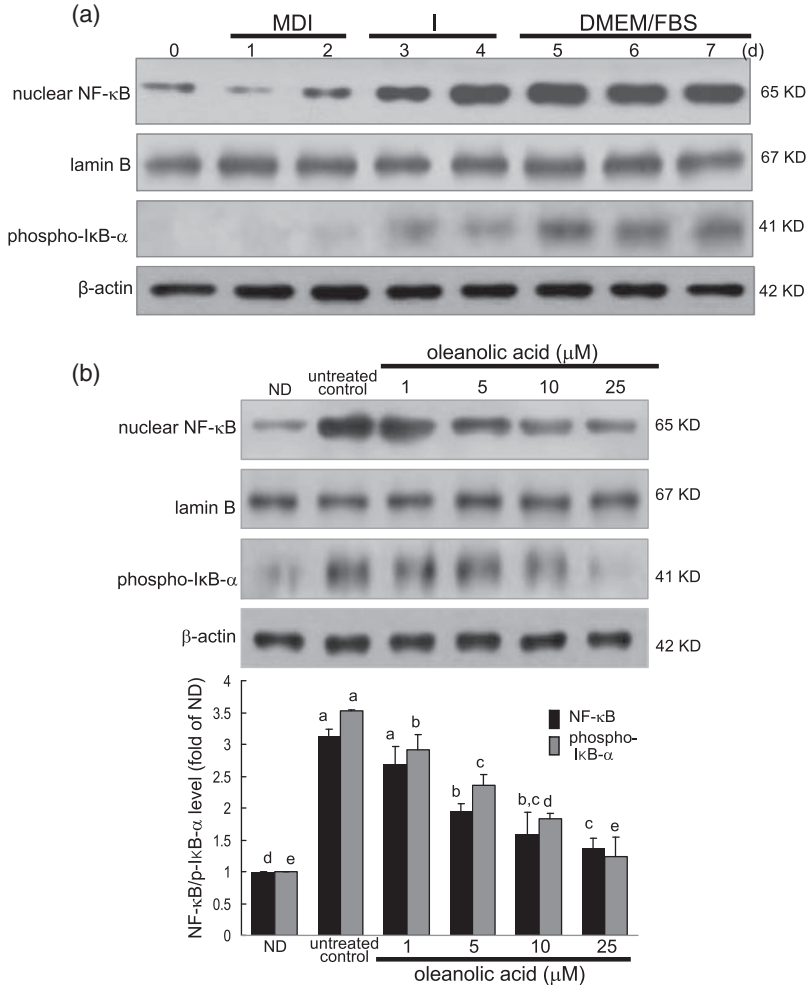

Figure 5 Time course responses of NF-κB transactivation and IκB phosphorylation (a) and inhibition of NF-κB transactivation and IκB phosphorylation by oleanolic acid (b). 3T3-L1 adipocytes were maintained in DMEM supplemented with 10% inactivated NCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Adipocyte differentiation was initiated on post-confluence using DMEM with 10% FBS, 0.5 mM 3-isobytyl-1-methylxanthine (M), 0.5 μM dexamethasone (D), and 5 μg/mL insulin (I). Cells were maintained in differentiating DMEM in the absence or presence of 1–25 μM oleanolic acid for 5 days. Western blot analysis was performed with a primary antibody against nuclear NF-κB and phosphorylated IκB (p-IκB)-α. Bar graphs (bottom panel, b) represent densitometric results of blot bands. β-Actin was used as an internal control and lamin B as nuclear control. ND, undifferentiated control. Values are mean ± SEM ($n = 3$) and means without a common letter differ, $P < 0.05$. DMEM; Dulbecco modified Eagle medium; FBS: fetal bovine serum; NF-κB: nuclear factor-κB; IκB: inhibitory κB

adipocyte differentiation. Visfatin-linked release of other inflammatory cytokines would address the modulatory effect of this substance on obesity-induced inflammation.[20] Accordingly, visfatin is clinically relevant as a biomarker for adipose tissue function, insulin sensitivity and chronic inflammation, and as potential therapeutic tools or targets in metabolic and cardiovascular diseases. Inhibition of inflammatory cytokine secretion might therefore have therapeutic efficacy in immune-mediated inflammatory disorders.

Circulating visfatin level is enhanced in insulin-resistant states such as type 2 diabetes and obesity, thus linking visfatin with the pathogenesis of insulin resistance.[22,23] It has been shown that the visfatin/insulin ratio declining with increasing visceral obesity may predispose to the development of insulin resistance.[22] Clinical and experimental studies have shown that the expression and secretion of visfatin are up-regulated during inflammation and in response to pro-inflammatory cytokines.[5,6,24] In the current study, inflammatory IL-6 and MIP-2 were highly secreted at the early phase of differentiation, which occurred earlier than the cellular induction of visfatin. The initial production of IL-6 and MIP-2 appeared to trigger the visfatin induction during adipocyte differentiation. It can be assumed that adipocyte differentiation would be an inflammatory process.

New insight into the potential role of visfatin makes it an attractive target for novel therapeutic strategies in chronic or subclinical inflammation relating to obesity. In our previous study, oleanolic acid disabled Tyk2-STAT signaling to be activated and suppressed inflammatory resistin expression at its transcriptional level.[12] A recent study has reported that curcumin down-regulated adipokines of resistin and leptin, being accompanied by blocking NF-κB, STAT3, and Wnt/β-catenin signaling.[25] This study revealed that oleanolic acid encumbered TRAF6-NF-κB signaling responsible for visfatin induction. It is deemed that inflammatory IL-6 and MIP-2 triggered TRAF6 signaling

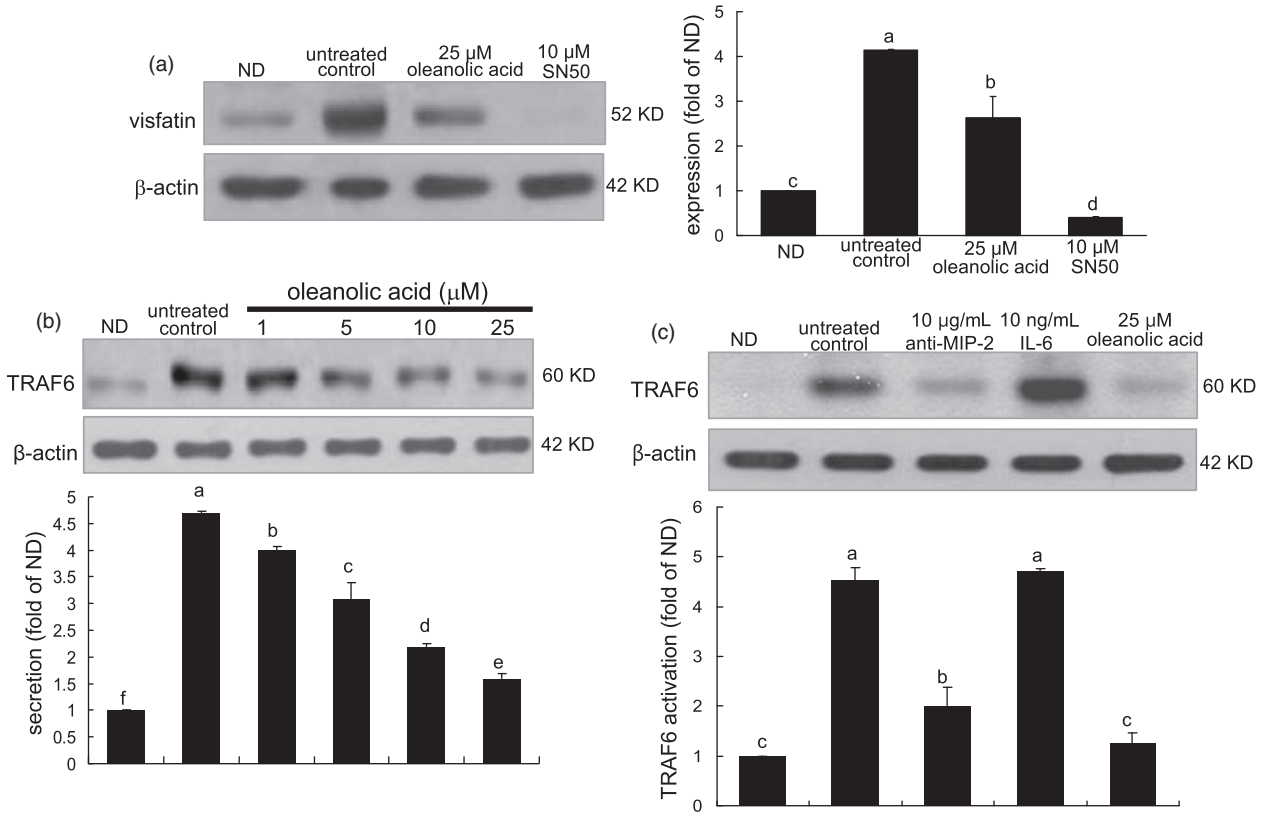

Figure 6 Inhibition of visfatin induction and TRAF6 induction by NF-κB blockade (a), oleanolic acid (b), and TRAF6 induction by IL-6 (c). 3T3-L1 adipocytes were maintained in DMEM supplemented with 10% inactivated NCS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Adipocyte differentiation was initiated on post-confluence using DMEM with 10% FBS, 0.5 mM 3-isobytyl-1-methylxanthine (M), 0.5 μM dexamethasone (D), and 5 μg/mL insulin (I). Cells were maintained in differentiating DMEM in the absence or presence of 1–25 μM oleanolic acid, 10 μM SN50, or 10 μg/mL anti-MIP-2 for 5 days. Another set of cells were maintained in DMEM containing 10 ng/mL IL-6 for 5 days. Western blot analysis was performed with a primary antibody against visfatin and TRAF6. Bar graphs (bottom panels, b and c) represent densitometric results of blot bands. β-Actin was used as an internal control. ND, undifferentiated control. Values are mean ± SEM ($n = 3$) and means without a common letter differ, $P < 0.05$.IL-6: interleukin-6; MIP-2: macrophage inflammatory protein-2; TRAF6: tumor necrosis factor receptor associated factor

and downstream activation of NF-κB which in turn enhanced cellular induction of visfatin during adipocyte differentiation.

In summary, the current study revealed that oleanolic acid antagonized visfatin production and its inflammation during adipogenesis. The cellular expression of visfatin appeared to be secondary to IL-6 and MIP-2 secreted from adipocytes at early phase of differentiation. Oleanolic acid blunted the visfatin production through blocking IL-6-TRAF6-NF-κB pathways in differentiating adipocytes. The NF-κB signaling was sustained to be highly activated throughout the completion of differentiation. Therefore, oleanolic acid may be a potential therapeutic compound targeting adipogenesis-linked inflammation and insulin resistance.

Author contributions: H-sK, S-yH and Y-hK (YH Kang) conceived and designed the experiments. H-sK, S-yH and S-hP performed the experiments. M-kK and S-jH analyzed the data. H-yS and Y-hK wrote the paper.

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