Ursolic Acid Suppresses Oncostatin M Expression through Blockade of PI3K/Akt/NF-κB Signaling Processes in Neutrophil-Like Differentiated HL-60 Cells

Na-Ra Han 1,2, Seong-Gyu Ko 2,3, Hi-Joon Park 4 and Phil-Dong Moon 5,*

1 College of Korean Medicine, Kyung Hee University, Seoul 02447, Korea; nrhan@khu.ac.kr
2 Korean Medicine-Based Drug Repositioning Cancer Research Center, College of Korean Medicine, Kyung Hee University, Seoul 02447, Korea; epiko@khu.ac.kr
3 Department of Preventive Medicine, College of Korean Medicine, Kyung Hee University, Seoul 02447, Korea
4 Department of Anatomy & Information Sciences, College of Korean Medicine, Kyung Hee University, Seoul 02447, Korea; acusinde@khu.ac.kr
5 Center for Converging Humanities, Kyung Hee University, Seoul 02447, Korea
* Correspondence: pdmoon@khu.ac.kr; Tel.: +82-2-961-0897

Abstract: Cytokine oncostatin M (OSM) plays an important role in a variety of inflammatory reactions and is mainly produced in neutrophils in inflammatory diseases. While natural pentacyclic triterpenoid ursolic acid (UA) possesses a wide range of beneficial effects, such as anti-oxidant, anti-tumor, and anti-inflammatory, the regulatory processes of OSM suppression by UA in neutrophils are still poorly understood. This study was aimed at examining how UA regulates OSM expression in neutrophil-like differentiated (d)HL-60 cells. Enzyme-linked immunosorbent assay, quantitative polymerase chain reaction, and immunoblotting were employed to analyze the effects of UA. Whereas stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF) led to elevations of OSM production and mRNA expression, these elevations were lowered by treatment with UA in neutrophil-like dHL-60 cells. When the cells were exposed to GM-CSF, phosphorylated levels of phosphatidylinositol 3-kinase, Akt, and nuclear factor-κB were upregulated. However, the upregulations were diminished by treatment with UA in neutrophil-like dHL-60 cells. The results of this study proposed that UA might relieve inflammatory diseases via inhibition of OSM.

Keywords: oncostatin M; ursolic acid; neutrophil-like differentiated HL-60 cells; phosphatidylinositol 3-kinase; Akt; nuclear factor-κB

1. Introduction

Oncostatin M (OSM) is known as a tumor-related cytokine that exhibits high expression levels in cancer patients [1,2]. On the other hand, OSM is known as an inflammatory cytokine and is secreted from diverse cells, inclusive of T cells, monocytes, dendritic cells, macrophages, and neutrophils [3–7]. OSM is involved in diverse pathophysiologic conditions, inclusive of tumor progression, extracellular matrix remodeling, hemocytopenia, liver regeneration, heart disorder, and inflammation [2,8,9]. Cytokine OSM is regarded as an important factor in a variety of inflammatory reactions [2]. High expression of OSM is implicated in inflammatory responses in arthritis and liver abnormality [2,10]. Additionally, OSM is correlated with respiratory inflammatory diseases, such as rhinitis and asthma [11,12]. Stimulation with recombinant human OSM resulted in elevated expressions of inflammatory cytokine and chemokine in normal human colonic stromal cells [3]. Liu and colleagues adduced that recombinant human OSM treatment increases inflammatory cytokine interleukin (IL)-1β release from...
HaCaT cells [7]. Our previous study also showed that stimulation with OSM protein elevates IL-1β production in human keratinocyte cell line HaCaT cells, suggesting a contribution of OSM to inflammatory reactions [13]. It was reported that neutrophils produce most OSM in pulmonary inflammation [12]. Thus, we studied whether ursolic acid (UA) could inhibit OSM production in neutrophil-like differentiated (d)HL-60 cells, because there is no report showing OSM inhibition by UA.

In general, phosphatidylinositol 3-kinase (PI3K) plays a crucial role in controlling a wide range of intracellular signaling courses [14]. Akt also plays an essential role in inflammatory reactions as a downstream molecule of PI3K [15]. Chang and colleagues suggested that the PI3K/Akt signal pathway is pivotal in the cytokine signaling network [16]. However, other pathways, for example, JAK/STAT and MEK/ERK, also play common and important roles in the intracellular signaling of cytokines. Almost all reports used OSM as a stimulator or activator or inducer, and thus revealed that OSM induces activation of JAK/STAT or MEK/ERK [17,18]. Just two reports suggested that OSM is produced by ERK and p38 MAPK, as well as the MEK/ERK pathway [19,20]. On the other hand, many reports suggested that OSM is produced by the PI3K/Akt/NF-κB pathway [15,21–23]. Wu et al. [21] reported that OSM production is mediated by PI3K in osteoblasts. OSM expression was regulated by PI3K/Akt/NF-κB signaling processes in osteoblasts [23]. Treatment with PI3K siRNA or p65 siRNA absolutely reduced OSM production [22]. In addition, treatment with Akt inhibitor or Akt siRNA decreased OSM production [15]. It was suggested that PI3K/AKT signaling is essential in inflammatory diseases [24]. NF-κB activation resulted from the activation of Akt (an upstream molecule of NF-κB) [16]. Su et al. [23] asserted that NF-κB is a pivotal mediator in inflammatory reactions. Thus, we adopted the PI3K/Akt/NF-κB pathway because many researchers have studied it and a dependency of the pathway in OSM production is clearer than other pathways.

Ursolic acid (UA; Figure 1), a natural pentacyclic triterpenoid found in apples, bilberries, cranberries, and holy basil [25–27], exerts a variety of beneficial effects, including anti-oxidant, cholesterol-lowering, anti-depressant, anti-fibrotic, anti-microbial, anti-osteoporosis, anti-inflammatory, anti-obesogenic, and anti-tumor [28–33]. In a recent report, Yan and colleagues [34] reported that UA exerts a hepatoprotective effect in a murine model. However, the regulatory mechanism of UA on the OSM expression has not been fully examined. Hence, we investigated how UA suppresses OSM expression in neutrophil-like dHL-60 cells.

![Chemical structure of UA](image)

**Figure 1.** Chemical structure of UA.

## 2. Materials and Methods

### 2.1. Materials

UA (C30H48O3) (FUJIFILM Wako Chemical Corp., Miyazaki, Japan) was dissolved in dimethyl sulfoxide (DMSO) and further diluted with phosphate-buffered saline (PBS) for
cell treatment. OSM capture antibody, OSM detection antibody, recombinant OSM, and recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from R&D Systems (Minneapolis, MN, USA). In Western blot analysis, phosphorylated (p)-PI3K p85 was purchased from Cell Signaling Technology (Danvers, MA, USA) and the others were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell Culture and Differentiation

HL-60 cells were purchased from Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI 1640 medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Welgene, Daegu, Korea) and 1% penicillin/streptomycin at 37 °C in 5% CO₂. To differentiate into the neutrophilic phenotype dHL-60 cells, HL-60 cells were incubated with DMSO (1.3%) for 7 days with a medium change (fresh RPMI 1640 medium containing 1.3% DMSO on 4th day) at 37 °C in 5% CO₂. The cells were stimulated with recombinant human GM-CSF (5 ng/mL) considering reports of Elbjeirami et al. [35] and Han et al. [13]. In brief, 5 ng/mL of GM-CSF was more efficient than other doses in OSM upregulation. The maximum levels were shown 15 min (PI3K), 30 min (Akt), 60 min (p65), 60 min (OSM mRNA expression), and 4 h (OSM production) after GM-CSF stimulation.

2.3. Cell Viability Assessment

The cells (1 × 10⁵ cells/mL) were seeded in 24-well plate and pretreated with UA or PBS for 60 min, and then stimulated with GM-CSF for 4 h. The cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Co., Burlington, MA, USA) solution at 37 °C for 4 h. Next, we added 1 mL of dimethyl sulfoxide to dissolve the MTT formazan and transferred 100 μL of supernatant into a new 96-well microplate. A microplate reader (540 nm, Versa Max, Molecular Devices, Sunnyvale, CA, USA) was used to measure the absorbance of formazan dissolved in DMSO [36–40].

2.4. OSM Measurement

The cells (5 × 10⁵ cells/mL) were treated with UA or PBS for 60 min and exposed to 5 ng/mL of GM-CSF for 4 h. An enzyme-linked immunoassorbent assay method was utilized to measure OSM values as described previously [36,41]. In brief, the capture antibody (R&D Systems Inc., Minneapolis, MN, USA) was pre-coated in a 96-well plate. PBS containing 10% FBS was added to block the plate for 2 h. After washing the plate by means of PBS containing Tween 20 (PBST), cell supernatants were added into the plate for 2 h. After washing the plate with PBST, the plate was treated with biotinylated detection antibody (R&D Systems Inc.) for 2 h and then incubated with avidin conjugated to horseradish peroxidase (Sigma-Aldrich Co.) for 30 min. Absorbance by TMB substrate (BD Pharmingen, San Jose, CA, USA) was measured by a microplate reader (405 nm, Versa Max).

2.5. Quantitative Polymerase Chain Reaction (PCR)

The cells (1 × 10⁵ cells/mL) were seeded in a 6-well plate and pretreated with UA or PBS for 60 min, and then stimulated with GM-CSF for 60 min. The harvested cells were used to isolate total RNA by means of an RNA extraction reagent (iNtRON, Seongnam, Korea), as previously described [42–47]. The first-strand cDNA from total RNA was synthesized with cDNA synthesis reagents (Bioneer, Daejeon, Korea). The following designed primers were used for the real time PCR (Applied Biosystems, Foster City, CA, USA) by using Power SYBR® Green Master Mix (Applied Biosystems): OSM: 5′-GCTCAACACAGGACCGCTG-3′, 5′-GGAGCACCGTGACTTCTT-3′; GAPDH: 5′-TCGACAGTCGCCGATCTTCTT-3′, 5′-ACCAATTCGGACTCCGACCT-3′.
The PCR program steps were 95 °C for 10 min, 40 cycles of 95 °C for 15 s, 60 °C for 1 min. The relative expression of mRNA for OSM was normalized by GAPDH and measured by using the 2^ΔΔCt method.

2.6. Immunoblot Analysis

The cells (5 × 10^5 cells/mL) were seeded in a 60 mm dish and pretreated with UA or PBS for 60 min, and then stimulated with GM-CSF for 15 min (PI3K) or 30 min (Akt) or 60 min (NF-κB). Immunoblotting was performed as described previously [48–50]. In brief, an ice-cold cell lysis buffer (Sigma-Aldrich Co.) was used to lyse the harvested cells. Cell extracts were prepared with sampling buffer (Laemmli’s 2×, ELPIS BIOTECH. INC., Daejeon, Korea) and heated at 95 °C for 5 min. Proteins were subjected to electrophoresis using 10–15% gel containing sodium dodecyl sulfate and transferred to nitrocellulose membranes (Amersham™, Chicago IL, USA). PBST containing 5% bovine serum albumin (Sigma-Aldrich Co.) was used to block the membranes afterwards and relevant primary antibodies (p-PI3K, Cat. No. 17366, 1:1000 dilution, Cell Signaling Technology, Danvers, MA, USA; PI3K, Cat. No. sc-423, 1:500 dilution, p-Akt, Cat. No. sc-514032, 1:500 dilution, Akt, Cat. No. sc-81434, 1:500 dilution, p-65, Cat. No. sc-136548, 1:500 dilution, p65, Cat. No. sc-8008, 1:500 dilution, and GAPDH, Cat. No. sc-32233, 1:500 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used. Secondary antibodies (m-IgGκ BP-HR, Cat. No. sc-516102, 1:5000 dilution, mouse anti-rabbit IgG-HRP, Cat. No. sc-2357, 1:5000 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added for incubation of the membranes for 1 h at room temperature after washing with PBST. Specific bands were detected by an enhanced chemiluminescence solution (DoGenBio Co., Seoul, Korea). Band intensities were calculated with the ImageJ program (National Institutes of Health, Bethesda, MD, USA).

2.7. Data Analysis

The results are represented as average ± SEM. SPSS version 25 (Armonk, NY, USA) and one-way ANOVA, followed by Tukey post hoc test, were applied to analyze the statistical significance (p < 0.05).

3. Results

3.1. UA Suppresses OSM Release in Neutrophil-like dHL-60 Cells

To assess whether UA is able to suppress OSM production in neutrophil-like dHL-60 cells, we treated various concentrations of UA (2 to 200 ng/mL) for 60 min prior to stimulation with GM-CSF (5 ng/mL) at 37 °C in 5% CO2. The concentrations of UA (2 to 200 ng/mL) were determined considering a report of Moon et al. [25]. GM-CSF stimulation for 4 h induced elevations of OSM levels in neutrophil-like dHL-60 cells (Figure 2a) in ways consistent with a previous report [13]. These elevated OSM levels were lowered by treatment with UA (Figure 2a). The OSM production values at doses of 2 to 200 ng/mL were 32.267 ± 1.130, 30.283 ± 0.630, and 28.574 ± 1.051, respectively (Figure 2a). The values of OSM production in the control and blank groups were 35.550 ± 1.240 and 25.633 ± 0.966, respectively. There was no cytotoxicity in UA-treated groups (Figure 2b).
Figure 2. Effects of UA on the OSM production in neutrophil-like dHL-60 cells. (a) The cells (5 × 10⁶) were treated with UA (2 to 200 ng/mL) for 60 min, and then exposed to 5 ng/mL of GM-CSF for 4 h. (b) Cytotoxicity was measured by means of an MTT assessment. Results are expressed as the mean ± SEM (n = 3). B, PBS-pretreated, and no added cells; C, PBS-pretreated, and GM-CSF-added cells (control). * p < 0.05 vs. control.

3.2. UA Inhibits OSM mRNA Expression in Neutrophil-like dHL-60 Cells

To investigate whether UA is able to decrease the OSM mRNA expression in neutrophil-like dHL-60 cells, we treated UA for 60 min prior to stimulation with GM-CSF (5 ng/mL) at 37 °C in 5% CO₂. OSM mRNA expressions were upregulated by GM-CSF stimulation (Figure 3) in ways consistent with a previous report [13]. These upregulated OSM mRNA levels were lowered by UA (Figure 3). The OSM mRNA expression values at doses of 2 to 200 ng/mL were 0.547 ± 0.027, 0.461 ± 0.015, and 0.424 ± 0.013, respectively. The values in the control and blank groups were 0.568 ± 0.021 and 0.311 ± 0.013, respectively. We investigated the effect of 200 ng/mL of UA in the subsequent experiment (immunoblotting), because the regulatory effect of 200 ng/mL of UA was better than those of 2 and 20 ng/mL.

Figure 3. Effects of UA on the OSM mRNA expression in neutrophil-like dHL-60 cells. The cells (1 × 10⁶) were treated with UA (2 to 200 ng/mL) for 60 min, and then exposed to GM-CSF (5 ng/mL) for 60 min. Results are expressed as the mean ± SEM (n = 3). B, PBS-pretreated, and no added cells; C, PBS-pretreated, and GM-CSF-added cells (control). * p < 0.05 vs. control.

3.3. UA Attenuates Phosphorylation of PI3K in Neutrophil-like dHL-60 Cells

To examine the regulatory process of OSM suppression by UA, we treated neutrophil-like dHL-60 cells with UA for 60 min prior to stimulation with GM-CSF (5 ng/mL) at 37 °C in 5% CO₂. Stimulation with GM-CSF for 15 min resulted in elevated PI3K phosphorylation (Figure 4) in ways consistent with a previous report [13]. However, this elevated phosphorylation of PI3K was lowered by treatment with UA (Figure 4).
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Figure 4. Effects of UA on the PI3K phosphorylation in neutrophil-like dHL-60 cells. (a) The cells (5 × 10^6) were treated with UA for 60 min, and then exposed to GM-CSF (5 ng/mL) for 15 min. (b) Histograms show relative expression levels of p-PI3K protein. Band intensities were calculated with the ImageJ program (National Institutes of Health, Bethesda, MD, USA). Results are expressed as the mean ± SEM (n = 3). B, PBS-pretreated, and no added cells; C, PBS-pretreated, and GM-CSF-added cells (control); UA, UA-pretreated, and GM-CSF-added cells; p-PI3K, phosphorylated PI3K; GAPDH, endogenous control. * p < 0.05 vs. control.

3.4. UA Reduces Phosphorylation of Akt in Neutrophil-like dHL-60 Cells

To assess the regulatory process of OSM suppression by UA, we treated neutrophil-like dHL-60 cells with UA for 60 min prior to stimulation with GM-CSF (5 ng/mL) at 37 °C in 5% CO₂. Similar to a previous report [13], stimulation with GM-CSF for 30 min induced upregulated Akt phosphorylation, whereas this upregulated phosphorylation of Akt was lowered by treatment with UA (Figure 5).

Figure 5. Effects of UA on the Akt phosphorylation in neutrophil-like dHL-60 cells. (a) The cells (5 × 10^6) were treated with UA for 60 min, and then exposed to GM-CSF (5 ng/mL) for 30 min. (b) Histograms show relative expression levels of p-Akt protein. Band intensities were calculated with the ImageJ program (National Institutes of Health, Bethesda, MD, USA). Results are expressed as the mean ± SEM (n = 3). B, PBS-pretreated, and no added cells; C, PBS-pretreated, and GM-CSF-added cells (control); UA, UA-pretreated, and GM-CSF-added cells; p-Akt, phosphorylated Akt; GAPDH, endogenous control. * p < 0.05 vs. control.

3.5. UA Decreases Phosphorylation of NF-κB in Neutrophil-like dHL-60 Cells

To find out the regulatory process of OSM suppression by UA, we treated neutrophil-like dHL-60 cells with UA for 60 min prior to stimulation with GM-CSF (5 ng/mL) at 37 °C in 5% CO₂. Increased NF-κB phosphorylation resulted from stimulation with GM-CSF for 60 min (Figure 6) in ways consistent with a previous report [13]. However, this increased phosphorylation of NF-κB was reduced by treatment with UA (Figure 6).
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Figure 6. Effects of UA on the NF-κB phosphorylation in neutrophil-like dHL-60 cells. (a) dHL-60 cells (5 × 10^6) were treated with UA for 60 min, and then exposed to GM-CSF (5 ng/mL) for 60 min. (b) Histograms show relative expression levels of p-p65 protein. Band intensities were calculated with the Image program (National Institutes of Health, Bethesda, MD, USA). Results are expressed as the mean ± SEM (n = 3). B, PBS-pretreated, and no added cells; C, PBS-pretreated, and GM-CSF-added cells (control); UA, UA-pretreated, and GM-CSF-added cells; p-p65, phosphorylated p65; GAPDH, endogenous control. *p < 0.05 vs. control.

4. Discussion

It has been known that high expression of OSM was exhibited in a wide range of inflammatory diseases, inclusive of asthma and chronic rhinosinusitis [12,51,52]. Upregulated mRNA expression of OSM resulted from stimulation with GM-CSF [53]. In addition, a lot of reports proposed that stimulation with recombinant human GM-CSF leads to an increase in OSM expression in isolated human neutrophils [12,35,54,55]. Our results showed that stimulation with recombinant human GM-CSF increases the production and mRNA expression of OSM (Figures 2a and 3) in ways consistent with a previous report [13]. The elevated levels of OSM production and mRNA expression were downregulated by treatment with UA (Figure 2a and 3). Mozaffarian and colleagues [56] suggested that recombinant OSM treatment upregulated infiltration of inflammatory cells and elevated levels of inflammatory cytokine and chemokine in an experimental model. Skin inflammation was aggravated when recombinant OSM was administered subcutaneously in mice [57]. Botelho et al. [58] proposed that overexpression of OSM leads to pulmonary inflammation in mice. Patients with asthma showed elevated values of OSM mRNA and protein, while control subjects showed no detection of OSM [52]. Moreover, colonic inflammation was diminished by treatment with OSM-neutralizing antibody as well as in OSM-deleted mice [3]. Thus, we assume that UA may possess the therapeutic potential for inflammatory diseases via suppression of OSM.

Signaling processes PI3K/AKT are regarded as crucial processes in inflammatory responses [14–16,24]. Transcription factor NF-κB plays an essential role in inflammatory reactions [23]. Su et al. [23] proposed that OSM production is regulated by PI3K/Akt/NF-κB signaling processes in osteoblasts. In addition, our previous study showed that OSM is produced by PI3K/Akt/NF-κB signaling processes in neutrophil-like dHL-60 cells [13]. The values of mRNA and protein of a wide range of inflammatory cytokines, inclusive of IL-1β, IL-6, and tumor necrosis factor (TNF)-α, were lowered by addition of PI3K inhibitor in an in vitro experiment [59]. Additionally, an alleviation of osteoarthritis resulted from suppressing of PI3K/Akt signaling processes in a murine model [60]. Decreased airway hyperresponsiveness and inflammation resulted from treatment with famous PI3K inhibitors, inclusive of wortmannin, LY-294002, and IC87114 in an ovalbumin-induced asthma model [61,62]. Moreover, administration of Akt inhibitor diminished airway hyperresponsiveness and inflammation in a murine model of asthma [63]. Suppressed airway inflammation and hyperresponsiveness resulted from NF-κB inhibition in asthmatic mice [63,64]. The results of the present study showed that treatment with UA suppressed phosphorylations of PI3K, Akt, and NF-κB (Figures 4–6). Hence, we presuppose that downregulation of OSM by UA, at least
partly, might be regulated by PI3K/Akt/NF-κB signaling processes in neutrophil-like dHL-60 cells.

A transcription factor, NF-κB, plays a crucial role in the expression of inflammatory cytokines. The NF-κB family consists of five different molecules, NFKB1 (p105/50), NFKB2 (p100/p52), Rel A (p65), Rel B, and c-Rel. Most commonly, NF-κB dimers are composed of subunits of Rel A (p65) and NFKB1 (p50) or NFKB2 (p52) [65,66]. In addition, previous reports suggested that OSM production is mediated by NF-κB p65 [13,22,23]. Thus, we selected NF-κB p65 in the present study. Since the signal transduction of NF-κB can be undertaken through not only Rel A/p65 but also Rel B, further studies will be needed to clarify the phosphorylation of NF-κB.

Finally, daily administration of 1000 mg/kg of UA for 90 days showed no toxicity in rats [67]. In the present study, we treated 200 ng/mL of UA (approximately 0.2 mg/kg). Therefore, we could presume that UA may not be toxic to humans at the dose of 200 ng/mL.

5. Conclusions

In conclusion, we demonstrated that UA decreased OSM expression through suppressing of PI3K/Akt/NF-κB signaling processes in neutrophil-like dHL-60 cells (Figure 7). These results propose that UA may possess the therapeutic potential for inflammatory diseases.

Figure 7. A schematic diagram of the proposed processes of OSM regulation by UA. GM-CSF, granulocyte-macrophage colony-stimulating factor; PI3K, phosphatidylinositol 3-Kinase; NF-κB, nuclear factor-κB, OSM, oncostatin M, UA, ursolic acid.

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