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Protective role of betulinic acid on TNF-α-induced cell adhesion molecules in vascular endothelial cells

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ABSTRACT

Vascular inflammation is an important event in the development of vascular diseases such as tumor progression and atherosclerosis. In the present study, betulinic acid (BA) treatment was found to show potent inhibitory effect on vascular inflammation process by TNF-α in human umbilical vein endothelial cells (HUVEC). Pretreatment of HUVEC with BA was blocked TNF-α induced expression level of cell adhesion molecules such as intracellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), endothelial cell selectin (E-selectin) as well as gelatinase in TNF-α-activated HUVEC in a dose-dependent manner. When preincubated with BA, the adhesion of HL-60 cells to TNF-α-induced HUVEC was significantly decreased in a concentration-dependent manner. TNF-α-induced intracellular ROS was markedly decreased by pretreatment with BA. Furthermore, BA significantly inhibited the translocation and transcriptional activity of NF-κB increased by TNF-α. In conclusion, these results suggested a vascular protective role of BA via inhibition of ROS and NF-κB activation in HUVEC.

Introduction

Natural products derived from plant resources have been used extensively in traditional medicine for treatment of vascular disorders such atherosclerosis. We previously reported that water extract from the leaves of *Lycopus lucidus* has an anti-inflammatory property [1]. The Labiatae plant, *L. lucidus* TURCZ is a perennial herb and widely distributed in Korea and China. Several chemical constituents such as flavonoids, triterpenes, and tannins were isolated from the leaves of *L. lucidus* [2]. Among them, betulinic acid (BA), a pentacyclic triterpene, has been reported to have a variety of biological effects, such as anti-neoplastic activity in several human cancer cell line, anti-HIV, anti-inflammatory and immunomodulatory properties [3–5]. However, the underlying mechanisms for inhibitory effect of BA in vascular inflammation process are not well understood.

Vascular inflammation process has been suggested to play an important risk factor in the initiation and development of atherosclerosis [6]. Endothelial cells play an important role in maintaining the vessel homeostasis in response to altered flow. Activation of these cells after changes induced by pro-inflammatory cytokines promotes the expression of adhesion molecules and the recruitment of inflammatory cells, followed by proliferation and migration of vascular smooth muscles, resulting in vascular remodeling [7]. TNF-α, endothelial cell-derived cytokine, is commonly found in atherosclerotic lesions and can induce expression of cell adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), and endothelial-selectin (E-selectin), which subsequently contribute to the inflammatory process [8]. Although it is reported that several natural compounds isolated from herbal extracts play significant roles in the prevention of vascular inflammation, it is not determined for the vascular protective role of BA in endothelial cells.

Transcriptional activation of NF-κB is an important process involved in the expression of cell adhesion molecules in inflammation responses [9,10]. Crucial enzymes involved in this process are the matrix metalloproteinase (MMP)-2, -9, called "gelatinase", the transcription of which is regulated by NF-κB [11,12]. A number of anti-oxidants and free radical quenchers have also been shown to block the NF-κB activation. Many natural compounds could scavenge intracellular ROS and inhibit endothelial adhesiveness to monocytes by reducing the expression of various adhesion molecules [13]. Thus, the present study investigates the inhibitory effects of BA on TNF-α-induced vascular inflammation in human umbilical vein endothelial cells (HUVEC).

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Materials and methods

Materials. Betulinic acid was purchased from Calbiochem (San Diego, CA). TNF-α, ICAM-1, VCAM-1, and E-selectin antibodies were purchased from R&D Systems (Minneapolis, MN). 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxy-methylester (BCECF-AM), and CM-H_2DCFDA were purchased from Invitrogen, Inc (Eugene, OR). Anti-NF-κB p65, phospho-IκB-α, IκB-α, MMP-2, MMP-9, β-actin and HRP-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). All other reagents used in this study were of the highest purity commercially available.

Cell cultures. Primary cultured HUVEC and endothelial cell growth medium (EGM-2) were purchased from Cambrex (Cambrex Co., East Rutherford, NJ), which contains 2.5% fetal bovine serum and growth supplements such as recombinant epidermal growth factor (rEGF), human fibroblast growth factor-basic, VEGF, ascorbic acid, hydrocortisone, human recombinant insulin-like growth factor, heparin, gentamicin and amphotericin [14]. HUVEC which were used between passages 3 and 8, were maintained in EGM-2 medium in a humidified chamber containing 5% CO_2 at 37 °C.

Determination of cell based-ELISA. Cell based-ELISA was performed as previously reported with minor modifications [15]. Briefly, HUVEC on 96-well plates were pretreated with/without BA for 18 h, which was followed by TNF-α treatment for 6 h at 37 °C. After the treatments, the cells fixed by 1% paraformaldehyde and exposed to the mouse anti-human ICAM-1, VCAM-1 or E-selectin antibodies at 1:1000 dilution in PBS containing 1% bovine serum albumin (BSA) for 2 h at room temperature. The cells were washed and incubated with the horseradish peroxidase-conjugated secondary antibody. Finally, ICAM-1, VCAM-1 or E-selectin expression levels were quantified by adding a peroxidase substrate solution and measuring the absorbance of each well at 490 nm using a microplate reader (Bio-Rad 3550, Hercules, CA).

Monocyte-HUVEC adhesion assay. HUVEC were grown to confluence in 24-well culture plates, pretreated with BA for 18 h, and stimulated with TNF-α for 6 h. Then the HL-60 cells were labeled with 10 μM BCECF-AM for 1 h at 37 °C and washed twice with growth medium. This was followed by adding 2.5×10^5 of the labeled HL-60 cells to the HUVEC and incubated them in CO_2 incubator for 1 h. The non-adherent HL-60 cells were removed from the plate by washing with PBS, and the HL-60 cells bound to the HUVEC were measured by fluorescence microscopy, and then lysed with 50 mM Tris–HCl, pH 8.0, containing 0.1% SDS. The fluorescent intensity was measured using a spectrofluorometer (F-2500, Hitachi, Tokyo, Japan) at an excitation and emission wavelength of 485 and 535 nm, respectively. The adhesion data were represented in terms of percent change compared with the TNF-α value.

Western blot analysis. Cell homogenates were separated on 10% SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Blots were then blocked with 5% skimmed milk powder in TBST [10 mM Tris–HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20] for 1 h and incubated with the appropriate primary antibody at dilutions recommended by the supplier. Then the membrane was washed, and primary antibodies were detected with goat anti-rabbit-IgG or anti-mouse-IgG conjugated to horse-

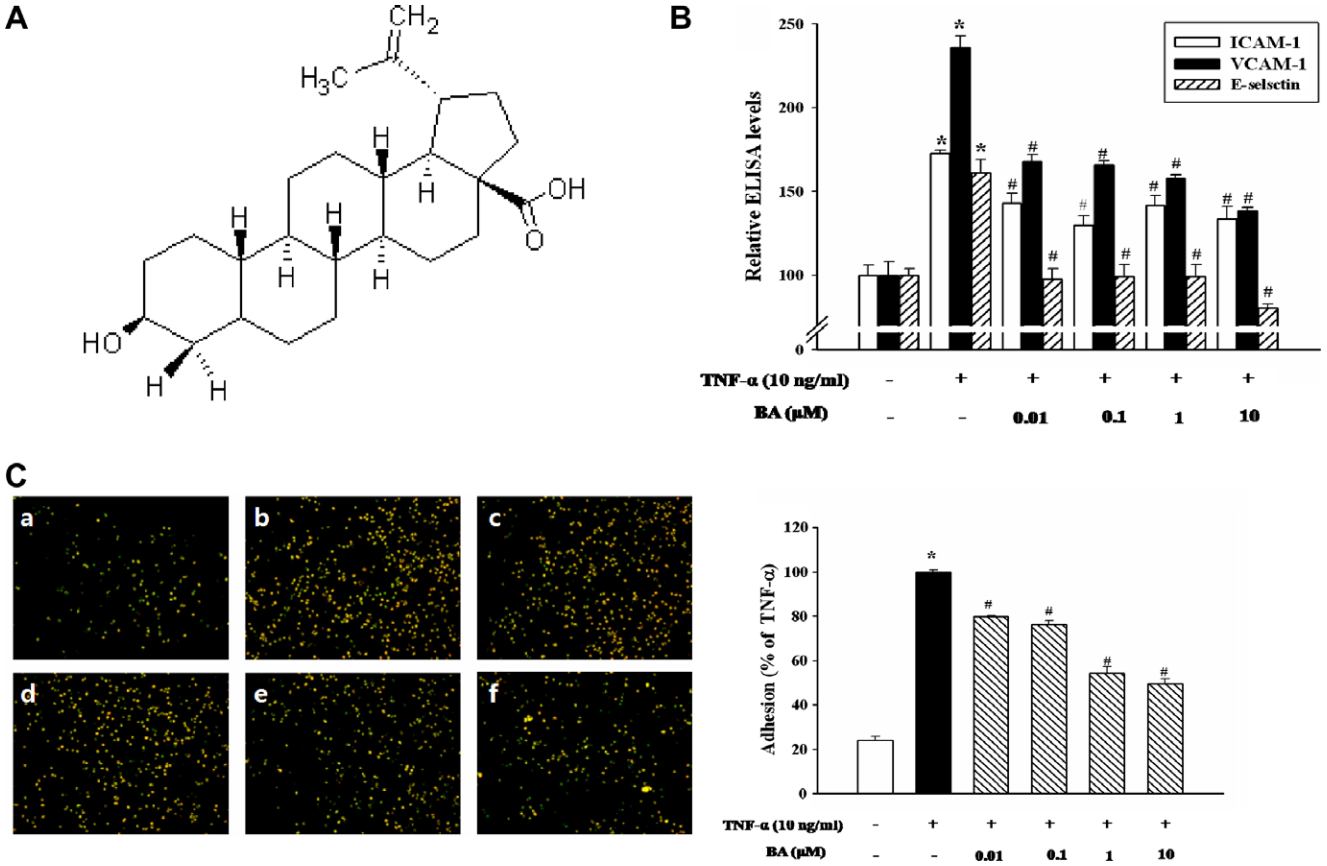

Fig. 1. Chemical structure of betulinic acid (A) and the effects of betulinic acid (BA) on TNF-α-induced cell adhesion molecules expression and monocyte adhesion. HUVEC were pretreated with BA (0.01–10 μM) for 18 h and then stimulated with TNF-α (10 ng/ml) for 6 h. Cell surface expressions (B) of ICAM-1, VCAM-1, and E-selectin were analyzed as described in *Materials and methods*. Adhesion of fluorescence-labeled HL-60 cells were added to the HUVEC and allowed to adhere for 1 h (C). a, Control; b, TNF-α (10 ng/ml); c, co-treated with 0.01 μM BA and TNF-α; d, co-treated with 0.1 μM BA and TNF-α; e, co-treated with 1 μM BA and TNF-α; f, co-treated with 10 μM BA and TNF-α. The amounts of adherent HL-60 cells were monitored by fluorescence microscopy. Values are expressed as mean ± SE. $^*p < 0.01$ vs. control; $^\#p < 0.01$ vs. TNF-α alone.

radish peroxidase, and the bands were visualized with enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using the Chemi-doc image analyzer (Bio-Rad, Hercules, CA).

Preparation of cytoplasmic and nucleus extracts. The cells were rapidly harvested by sedimentation and nuclear and cytoplasmic extracts were prepared on ice as previously described by the method of Dschietzig et al. [16]. Cells were harvested and washed with 1 ml buffer A (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 19 mM KCl) for 5 min at 600*g*. The cells were then resuspended in buffer A and 0.1% NP 40, left for 10 min on ice to lyse the cells and then centrifuged at 600*g* for 3 min. The supernatant was saved as cytosolic extract. The nuclear pellet was then washed in 1 ml buffer A at 4200*g* for 3 min, resuspended in 30 μl buffer C (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA), rotated for 30 min at 4 °C, then centrifuged at 14,300*g* for 20 min. The supernatant was used as nucleus extract.

Gelatin zymography. MMP-2 and MMP-9 enzymatic activities were assayed by gelatin zymography [17]. Samples were electrophoresed on 1 mg/ml gelatin containing 10% SDS–polyacrylamide gel. After electrophoresis, the gel was washed twice with washing buffer (50 mM Tris–HCl, pH 7.5, 100 mM NaCl, 2.5% Triton X-100), followed by a brief rinsing in washing buffer without Triton X-100. The gel was incubated with incubation buffer (50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 0.02% NaN_3, 1 μM $ZnCl_2$) at 37 °C. After incubation, the gel was stained with Commassie brilliant blue R-250 and destained. A clear zone of gelatin digestion was represented with the MMP activity.

Intracellular ROS production assay. The fluorescent probe, CM-H_2DCFDA, was used to determine the intracellular generation of ROS. Briefly, the confluent HUVEC in the 24-well culture plates were pretreated with BA for 1 h. After removing from the wells, the HUVEC were incubated with 20 μM CM-H_2DCFDA for 1 h. and then stimulated with TNF-α. The fluorescence intensity was measured by flow cytometry on FACScalibur (BD, San Diego, CA) and spectrofluorometer.

H_2O_2 release. The levels of H_2O_2 were determined by a modification of the method of Zhou et al. [18]. The cells washed twice with ice-cold PBS and harvested cells by microcentrifugation were resuspended in a Krebs–Ringer phosphate solution. One hundred microliter of the reaction mixture (50 μM Amplex Red reagent containing 0.1 U/ml HRP in KRPG) was added into each microplate well and then prewarm at 37 °C for 10 min. After then, reaction was started as adding resuspended cells in 20 μl of KRPG. Fluorescence readings became stable within 30 min of starting of reaction. The fluorescence intensities of reaction mixtures were measured at 30 min using fluorescence microplate reader (Multiskan, Thermo labsystems Inc, Franklin, MA) equipped for absorbance at ~560 nm.

Statistical analysis. All the experiments were repeated at least three times. The results were expressed as a mean ± SE, and the data were analyzed using one-way ANOVA followed by a Dunnett's test or Student's *t*-test to determine any significant differences. $p < 0.05$ was considered as statistically significance.

Results

Effect of BA on TNF-α-induced cell adhesion molecules

As the expression of cell adhesion molecules on endothelial cells is a prerequisite for adhesion of monocytes, the effect of BA (Fig. 1A) on TNF-α-induced cell adhesion molecules were investigated at non-cytotoxic concentrations in HUVEC (Fig. S1). The results using cell based-ELISA demonstrated that TNF-α (10 ng/ml) increased ICAM-1, VCAM-1, and E-selectin expression levels. However, pretreatment with BA significantly decreased TNF-α-induced ICAM-1, VCAM-1, and E-selectin expression levels (Fig. 1B). Wes-

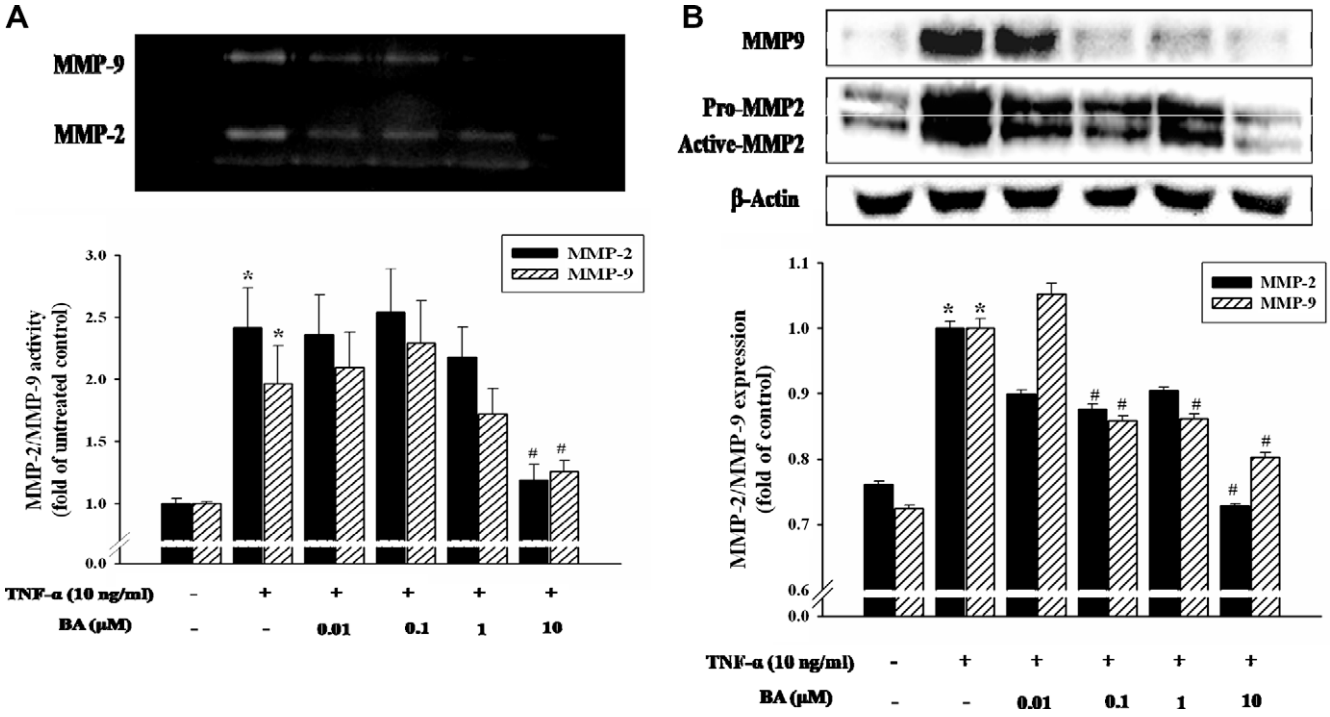

Fig. 2. Effects of BA on TNF-α-induced gelatinase activity in HUVEC. HUVEC were pretreated with BA (0.01–10 μM) for 18 h and then stimulated with TNF-α (10 ng/ml) for 6 h. The levels of MMP-2/-9 in conditioned medium were analyzed by gelatin zymography (A). Data are expressed as fold of basal value and are the means ± SE. of three independent experiments, performed in triplicate. Western blots of MMP-2/MMP-9 are detected as described in *Materials and methods* (B). The blots are representative of three independents and densitometric quantification of MMP-2 and MMP-9 expression. Values are expressed as mean ± SE. $^{*}p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. TNF-α alone.

tern blot analysis also showed that BA decreased TNF-α-induced ICAM-1, VCAM-1, and E-selectin expression levels (Fig. S2).

Next, we evaluated the inhibitory effect of BA on HL-60 cells adhesion to HUVEC under static conditions. As shown in Fig. 1C, few HL-60 cells were adhered to unstimulated HUVEC in free TNF-α. There was heavy staining on the TNF-α-alone-induced HUVEC, indicative of a marked increase in the HL-60 adherence to the activated HUVEC. However, the treatment of TNF-α-induced cells with BA (0.01–10 μM) significantly inhibited monocyte adherence in a dose-dependent manner.

Effect of BA on TNF-α-induced gelatinase activity

Since gelatinase has been correlated with clinical manifestations of vascular lesions. Thus, we measured that TNF-α-induced MMP-2 and MMP-9 proteolytic activity and protein expression using zymography and western blotting, respectively. HUVEC was treated with TNF-α (10 ng/ml), in presence of various concentrations of BA. As shown in Fig. 2A, MMP-2 and MMP-9 proteolytic activity were increased in TNF-α-induced HUVEC. However, pretreatment with BA (0.01–10 μM) decreased their activity in a dose-dependent manner. Coincident with a gelatin zymography, western blot analysis showed that TNF-α increased MMP-2 and MMP-9 expression levels. BA significantly decreased TNF-α-induced MMP-2/-9 expression levels (Fig. 2B).

Anti-oxidant effect of BA on TNF-α-induced ROS production

To confirm that inhibitory effect of BA on TNF-α-induced oxidative stress, HUVEC were labeled with a cell-permeable fluorescent dye, CM-H_2DCFDA and analyzed by flow cytometry or spectrofluorometer. As shown in Fig. 3A and B, intracellular ROS levels were higher after TNF-α treatment as compared untreated cells (control). However, pretreatment with BA significantly decreased

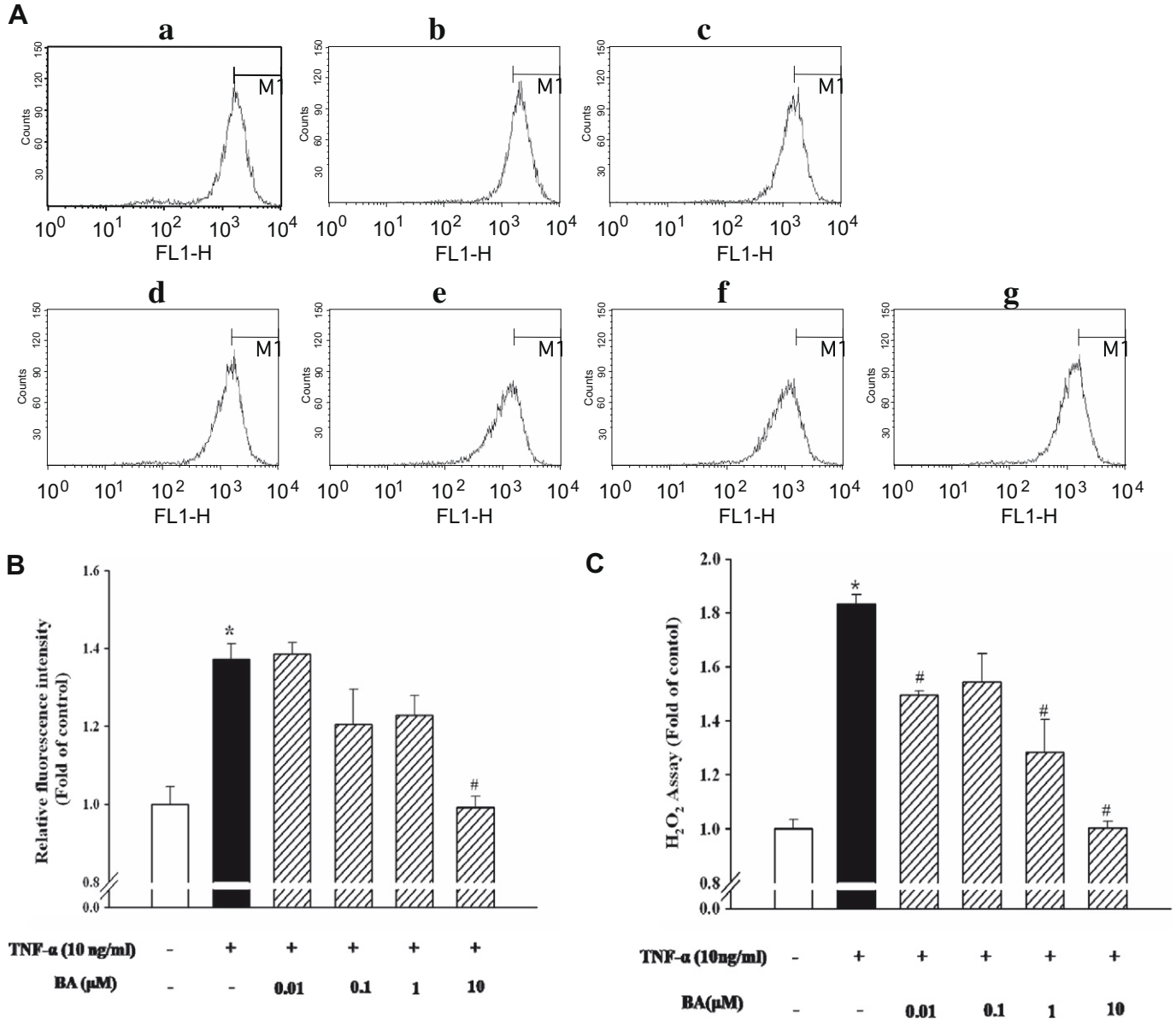

Fig. 3. Effects of BA on TNF-α-induced ROS production in HUVEC. Cells were pretreated with BA (0.01–10 μM) for 1 h and then stimulated with TNF-α (10 ng/ml) for 4 h. CM-H_2DCFDA (20 μM) was added for 30 min and ROS production was measured by FACS-analysis (A). Alternatively, DCF-sensitive ROS assay (B) and H_2O_2 assay (C) were measured in a 96-well plate reader and fold induction was calculated in relation to unstimulated HUVEC (B). a, control; b, TNF-α (10 ng/ml); c, 0.01 μM BA + TNF-α; d, 0.1 μM BA + TNF-α; e, 1 μM BA + TNF-α; f, 10 μM BA + TNF-α; g, 50 μM NAC + TNF-α. Values are expressed as mean ± SE. $^{*}p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. TNF-α alone.

TNF-α-induced ROS levels. Coincident with the DCF-sensitive ROS analysis, TNF-α-induced cellular hydrogen peroxide was markedly decreased by pretreatment with BA (Fig. 3C).

Effect of BA on TNF-α-induced NF-κB activation in HUVEC

When cells stimulated by inflammatory cytokines, NF-κB is activated by stimulating phosphorylation and degradation of IκB-α. Then the activated NF-κB is translocated into the nucleus, and leads to transcriptional expression of genes associated with cellular growth properties. Thus, we measured whether BA could suppress or not the NF-κB activation and translocation into the nuclear fraction in HUVEC. As shown in Fig. 4A, NF-κB activation and translocation of NF-κB in the nuclear fractions of HUVEC was significantly increased by treatment with TNF-α. However, pretreatment of BA inhibited NF-κB activation and translocation of NF-κB into the nucleus in a dose-dependent manner. In addition, western blot analysis revealed that TNF-α-activated HUVEC exhibited markered decrease in IκB-α level and increase in phospho-IκB-α level. Furthermore, BA blocked TNF-α-induced IκB-α phosphorylation to activate NF-κB (Fig. 4B).

Discussion

To our knowledge, this study is the first to produce an evidence for a vascular protective role of BA in primary cultured HUVEC. BA is one of pentacyclic triterpenes including ursolic acid, oleanolic acid and was isolated from the methanol extracts of the leaves of *L. lucidus*. Especially, BA exhibited cholesterol acyltransferase inhibitory activity for treatment hypercholesterolemia or atherosclerosis [3]. Recently, Yun et al. [5] reported that immunomodulatory activity of BA in LPS-stimulated RAW 264.7 cells and peritoneal macrophages. Thus, we postulated anti-inflammatory effects of BA in vascular endothelial cells. One of the earliest events in atherosclerosis associated with vascular inflammation is the adhesion of monocytes to the endothelium, which is followed by their infiltration and differentiation into macrophages. This key step is mediated by an interaction between monocytes and the adhesion molecules expressed on the endothelial cell surface [19]. In our ELISA and western blotting, results showed that pretreatment with BA decreased the TNF-α-induced increase of ICAM-1, VCAM-1, and E-selectin expression levels. Adhesion assay suggested that TNF-α-induced HUVEC and HL-60 interaction was decreased by BA. In present study, BA had no cytotoxicity at 0.01–10 μM concentration although BA was known as a cytotoxic agent in cancer cell line [2]. Thus, we firstly revealed that BA exhibited low toxicity in vascular endothelial cells and could prevent the early pathogenesis of atherosclerosis by modulating vascular inflammation.

Gelatinase inhibitors could prevent vascular dysfunction and vascular remodeling in development of atherosclerosis [20,21]. We examined whether BA affects the synthesis and release of MMPs in HUVEC. In gelatin zymography and western blotting, we found that pretreatment with BA decreased TNF-α-induced MMP-2 and MMP-9 proteolytic activity in a dose-dependent manner, suggesting an anti-atherosclerotic property. Once MMPs activated, those are specifically inhibited by a group of endogenous protease inhibitors known as the tissue inhibitors of metalloproteinases (TIMPs), which bind to the active site inhibiting catalytic activity [22]. Here, we demonstrated that BA has an anti-atherosclerotic activity, further study is needed to investigate a possible mechanism of MMPs–TIMPs by BA in HUVEC.

In our results, BA also inhibited TNF-α-induced production of intracellular ROS in HUVEC. Oxidative stress induces the expression of the cell adhesion molecules via the activation of redox-sensitive transcriptional factors such as NF-κB [23], and ROS-mediated

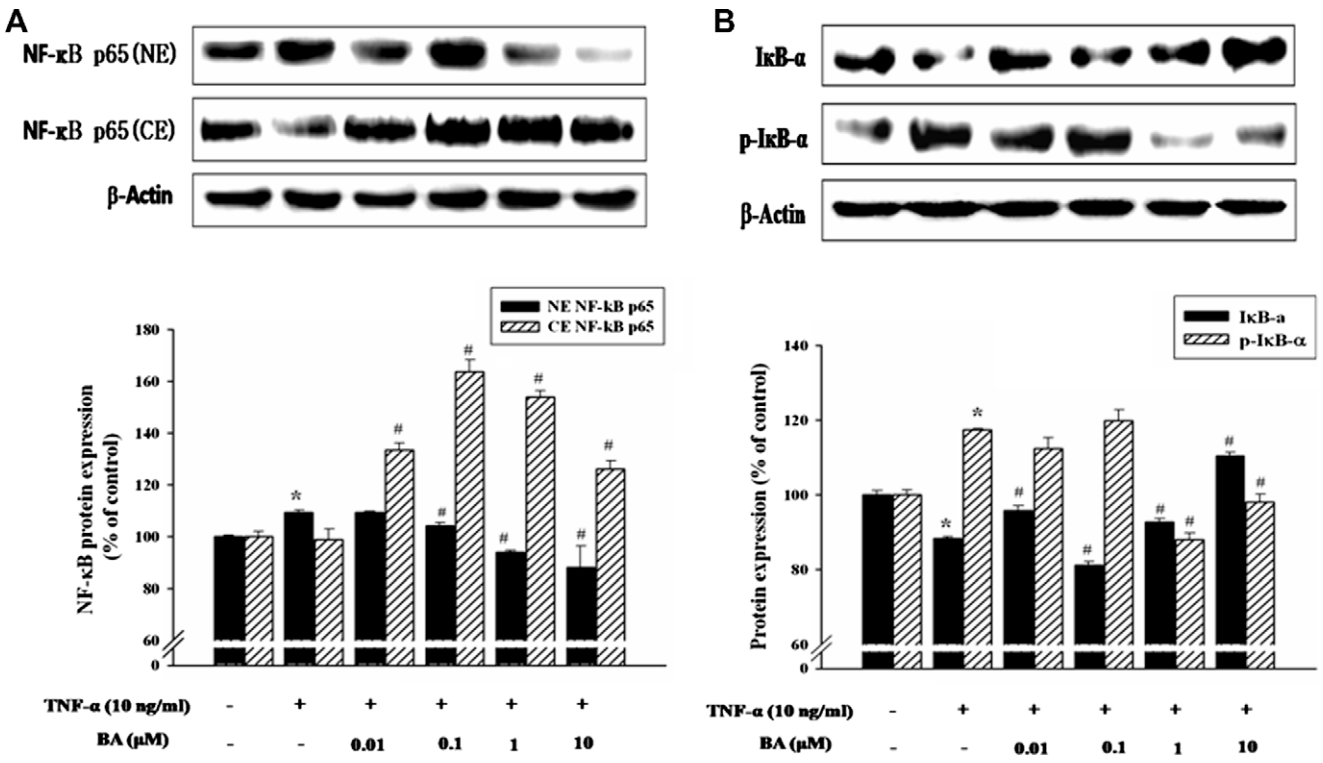

Fig. 4. Effects of BA on TNF-α-induced NF-κB translocation into nucleus in HUVEC. Cells were pretreated with BA (0.01–10 μM) for 18 h and then stimulated with TNF-α (10 ng/ml) for 6 h. Cytoplasmic (CE) and nuclear (NE) fractions were extracted and protein levels determined by western blot analysis. Bands indicate p65 NF-κB of 65 kDa (A) and IκB-α, p-IκB-α of 41 kDa (B). The blots are representative of three independents and densitometric quantification of NF-κB and IκB-α expression. $^{*}p < 0.01$ vs. control; $^{\#}p < 0.01$ vs. TNF-α alone.

NF-κB activation plays an important role in the pathogenesis of atherosclerosis [24]. It was shown that cytokine-activated endothelial expressions of the cell adhesion molecules and monocytes adhesion to HUVEC are inhibited by natural anti-oxidants via the suppression of NF-κB p65 activation [25]. TNF-α increased intracellular ROS level and pretreatment with BA significantly decreased TNF-α-induced those level, suggesting a role of anti-oxidant activity. Similar result was reported by Liby et al. [2] that over 10 μM concentration of BA decreased ROS level in human cancer cell lines. In our results, flow cytometry analysis and hydrogen peroxidase assay showed that BA has a potent anti-oxidant property in a dose-dependent manner and we suspected that anti-oxidant effect of BA is more specific to vascular endothelial cells.

NF-κB is involved in the development of atherosclerosis [26]. It has been also shown that activation of transcription factor NF-κB by TNF-α is required for the transcriptional activation of endothelial cell adhesion molecules and MMP-9 [27]. The present study showed that pretreatment with EGE inhibited TNF-α-stimulated translocation of NF-κB p65 to nucleus and TNF-α-induced activation of IκB-α phosphorylation and degradation. We demonstrated that the vascular protective role of BA correlated with the suppression of TNF-α-induced IκB-α phosphorylation and degradation, p65 NF-κB nuclear translocation. Overall, our results indicated that BA inhibits activation of NF-κB and NF-κB regulated gene expression such cell adhesion molecules or MMP-9 induced by inflammatory stimuli TNF-α in vascular endothelial cells. It is likewise possible that the anti-cancer effects of BA are mediated through the suppression of MMP-9 gene expression in HCT 116 cell line [4]. Interestingly, we found that BA decreased both MMP-2 and MMP-9 activity in HUVEC. These results provided a possibility of an unknown regulatory protein induced by BA in the inhibition of gelatinase activity in vascular endothelial cells.

In summary, our data indicated that BA, at physiologically relevant concentrations, could contribute to improvement of vascular inflammation associated with regulation of adhesion molecules and gelatinases expression through inhibition of ROS-NF-κB pathway in HUVEC These findings may provide a molecular basis for the ability of BA to suppress vascular inflammation, and further prevent atherosclerosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.009.

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