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Inhibition of lipopolysaccharide-induced expression of inducible nitric oxide synthase by butein in RAW 264.7 cells

Sung Hee Lee[a], Geom Seog Seo[b], Dong Hwan Sohn[a,*]

[a] Medicinal Resources Research Center, College of Pharmacy, Wonkwang University, Iksan, Jeonbuk 570-749, Republic of Korea
[b] Department of Internal Medicine, Wonkwang University Medical School, Iksan, Jeonbuk 570-749, Republic of Korea

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Abstract

Butein has been reported to exert anti-inflammatory effect but the possible mechanism involved is still unclear. Here, we report the inhibitory effect of butein on nitric oxide (NO) production and inducible nitric oxide synthase (iNOS) gene expression. Butein also inhibited the induction of tumor necrosis factor-α and cyclooxygenase 2 by LPS. To further investigate the mechanism responsible for the inhibition of iNOS gene expression by butein, we examined the effect of butein on LPS-induced nuclear factor-κB (NF-κB) activation. The LPS-induced DNA binding activity of NF-κB was significantly inhibited by butein, and this effect was mediated through inhibition of the degradation of inhibitory factor-κB and phosphorylation of Erk1/2 MAP kinase. Furthermore, increased binding of the osteopontin αvβ3 integrin receptor by butein may explain its inhibitory effect on LPS-mediated NO production. Taken together, these results suggest that butein inhibits iNOS gene expression, providing possible mechanisms for its anti-inflammatory action.

Keywords: Butein; Inducible nitric oxide synthase; Nuclear factor-κB; Erk1/2 MAP kinase; Osteopontin; αvβ3 integrin

Butein (3,4,2′,4′-tetrahydroxychalcone), a plant polyphenol, has traditionally been used for the treatment of pain, parasitic, and thrombotic disease [1]. Previous studies have shown that butein has beneficial effects including antioxidant and anti-inflammatory activities [2,3], inhibition of protein kinase activity [4,5], inhibitory activity against HIV-1 protease [6], and antifibrogenic effects of hepatic fibrosis [7,8].

Nitric oxide (NO) is a short-lived free radical and intercellular messenger that mediates a variety of biological functions, including vascular homeostasis, neurotransmission, antimicrobial defence, and antitumor activities [9]. NO is known to be synthesized from L-arginine by nitric oxide synthase (NOS) [10]. To date, three isoforms of NOS have been identified [10–13]: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). Of the three NO synthases, iNOS, the high-output isoform, is the most widely expressed in various cell types after its transcriptional activation. Most importantly, iNOS is highly expressed in lipopolysaccharide (LPS)-activated macrophages, and this contributes to the pathogenesis of septic shock [14,15]. In some cases, the induction of iNOS by other stimuli leads to organ destruction in some inflammatory [16] and autoimmune diseases [17]. Thus, the inhibition of NO production by blocking iNOS expression may present a useful strategy for the treatment of various inflammatory diseases.

Because butein has been described as an antioxidant with anti-inflammatory activity, we investigated the effect of butein on the LPS-mediated induction of NO production and on iNOS gene expression, and attempted to clarify its mechanism of action in cultures

* Corresponding author. Fax: +82 63 854 6038.
E-mail address: dhsohn@wonkwang.ac.kr (D.H. Sohn).

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of the murine macrophage cell line RAW 264.7. Our results demonstrated that butein inhibits the LPS-induced increase of NO production and activation of iNOS gene expression by inactivating of nuclear factor-κB (NF-κB) and increasing in the expression of αvβ3 integrin.

Materials and methods

Cell culture. The murine macrophage cell line, RAW 264.7, was obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, antibiotics (100 U/ml of penicillin A and 100 U/ml of streptomycin), and 10% heat-inactivated fetal bovine serum (Gibco-BRL, Gaithersburg, MD, USA) and maintained in a 37 °C humidified incubator containing 5% CO_2.

Nitrite quantification. NO_2^- accumulation was used as an indicator of NO production in the medium as described previously [18]. RAW 264.7 cells were plated at 5×10^5 cells/ml, and stimulated with LPS (200 ng/ml, *Escherichia coli* 026:B6; Difco, Detroit, MI, USA) in the presence or absence of butein for 24 h. The isolated supernatants were mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% *N*-1-naphthylethylenediamine dihydrochloride, and 2% phosphoric acid) and incubated at room temperature for 10 min. $NaNO_2$ was used to generate a standard curve and nitrite production was determined by measuring optical density at 550 nm.

Measurement of tumor necrosis factor. RAW 264.7 cells were preincubated at 37 °C for 1 h in medium containing butein and then further incubated with LPS (200 ng/ml) for 6 h. tumor necrosis factor (TNF-α) levels in the conditioned medium were determined using a TNF-α enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, USA) according to the manufacturer's instructions.

Northern blot analysis. Total RNA was separated electrophoretically on a 1% agarose gel containing 5.4% formaldehyde, transferred to nylon membranes (Hybond-N; Amersham–Pharmacia Biotech, Uppsala, Sweden) by electroblotting, and fixed with UV irradiation. The RNA was hybridized with randomly primed [^{32}P]cDNA-specific for iNOS, TNF-α, cyclooxygenase 2 (COX-2) or GAPDH. Specific cDNA probes were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using the selective primers and cloned in a TA vector (Promega, Madison, WI, USA). The primers used were as follows: iNOS sense, 5′-TCT GCG CCT TTG CTC ATG AC-3′ and antisense, 5′-TAA AGGC TCC GGG CTC TG-3′ (254 bp); TNF-α sense, 5′-TAC TGA ACT TCG GGG TGA TCG GTC C-3′ and antisense, 5′-CAG CCT TGT CCC TTG AAG AGA ACC-3′ (295 bp); and COX-2 sense, 5′-TCT CCA ACC TCT CCT ACT AC-3′ and antisense, 5′-GCA CGT AGT CTT CGA TCA CT-3′ (624 bp). Prehybridization and hybridization were performed in a solution of 50 mM PIPES, 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA, and 5% SDS at 65 °C overnight. After hybridization, the nylon membrane was rinsed twice in 1× SSC containing 5% SDS at room temperature for 10 min, and subsequently washed in 0.1× SSC containing 5% SDS at 65 °C for 30 min. Reactive bands were detected by autoradiography using X-ray film (Agfa-Gevaert, Belgium).

Nuclear and cytosolic extracts. Nuclear extracts were prepared according to a modified procedure of Lo et al. [19]. Briefly, cells were scraped, washed with PBS (pH 7.4), resuspended in hypotonic buffer containing 10 mM Hepes (pH 7.9), 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM PMSF, 1.5 mM $MgCl_2$, and 1.2% Nonidet P-40 (Sigma, St. Louis, MO, USA), and allowed to swell on ice for 10 min. Lysates were separated by spinning at 3300*g* for 5 min at 4 °C and the supernatant was used as cytosolic extract. The nuclear pellets were extracted in nuclear extraction buffer containing 20 mM Hepes (pH 7.9), 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, and 0.5 mM DTT for 30 min on ice, and centrifuged at 12,000*g* for 30 min. The supernatant was used as nuclear extract and stored at −70 °C prior to use.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assay (EMSA) was carried out according to the protocol accompanying Gel Shift Assay System (Promega). Briefly, double-stranded oligonucleotide probes containing NF-κB-binding sequences were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The nuclear extract (5 μg) was incubated at room temperature for 20 min with ^{32}P-labeled probe in a binding buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, 0.25 mg/ml poly(dI–dC), and 20% glycerol. DNA/nuclear protein complexes were separated from the DNA probe by electrophoresis on a native 4% acrylamide gel, and the gel was vacuum-dried and subjected to autoradiography.

Western blot analysis. Whole-cell lysate (20 μg, for Erk1/2, SAPK/c-Jun N-terminal kinase (JNK), and p38 MAP kinase), 20 μg nuclear extract (for NF-κB), or 20 μg cytosolic extract (for I-κBα) were separated by 10% SDS–polyacrylamide gel electrophoresis, and electrotransferred to nitrocellulose membranes (Amersham–Pharmacia Biotech). The membranes were preincubated for 1 h at room temperature in Tris-buffered saline, pH 7.6, containing 0.05% Tween 20 and 5% skim milk. Nitrocellulose membranes were then incubated with specific antibodies against NF-κB, I-κBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or the phosphorylated forms of Erk1/2, SAPK/JNK, or p38 MAP kinase (Cell Signaling Technology, Beverly, MA, USA).

To determine the amount of secreted osteopontin (OPN), equal volumes of conditioned medium were concentrated 10-fold in 10 kDa microcentrifuge concentrators (Millipore, Bedford, MA, USA). Equal amounts of protein were resolved on SDS–polyacrylamide gel electrophoresis, blotted onto a nitrocellulose membrane, and incubated with a primary anti-mouse OPN antibody (R&D Systems). Immunoreactive bands were then detected by incubating with conjugates of anti-rabbit or anti-mouse IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham–Pharmacia Biotech).

Flow cytometry. To analyze cell surface expression, flow cytometry was performed as previously described [20]. Cells were harvested by weakly trypsinizing with 0.025% trypsin for 10 s and washing with PBS containing 0.1% bovine serum albumin (BSA), then 1×10^6 cells were suspended in PBS/BSA and incubated with biotin conjugated anti-integrin αv or anti-integrin β3 (BD Biosciences, KY, USA) for 1 h on ice. Cells were then washed twice in PBS/BSA and incubated with streptavidin–FITC (BD Biosciences) for 45 min on ice. Finally, cells were washed three times and immediately analyzed using a flow cytometer (FACS Caliber, Becton–Dickinson).

Statistical analysis. Data were analyzed with one-way ANOVA and Tukey's multiple comparison tests when comparing more than three means. Calculations were performed with the GraphPad Prism program (GraphPad Software, San Diego, CA, USA).

Results and discussion

Inhibition of iNOS protein and mRNA expression

To investigate the effect of butein on NO production, we measured the accumulation of nitrite, the stable metabolite of NO, in culture media. NO production was monitored in RAW 264.7 cells stimulated by LPS in the presence or absence of butein for 24 h. LPS (200 ng/ml) evoked a 4.7-fold induction of nitrite production versus the naive control, and this induction was inhibited by butein treatment in a dose-dependent manner (Fig. 1A). Examination of the cytotoxicity of butein in RAW 264.7 macrophages by MTT assay

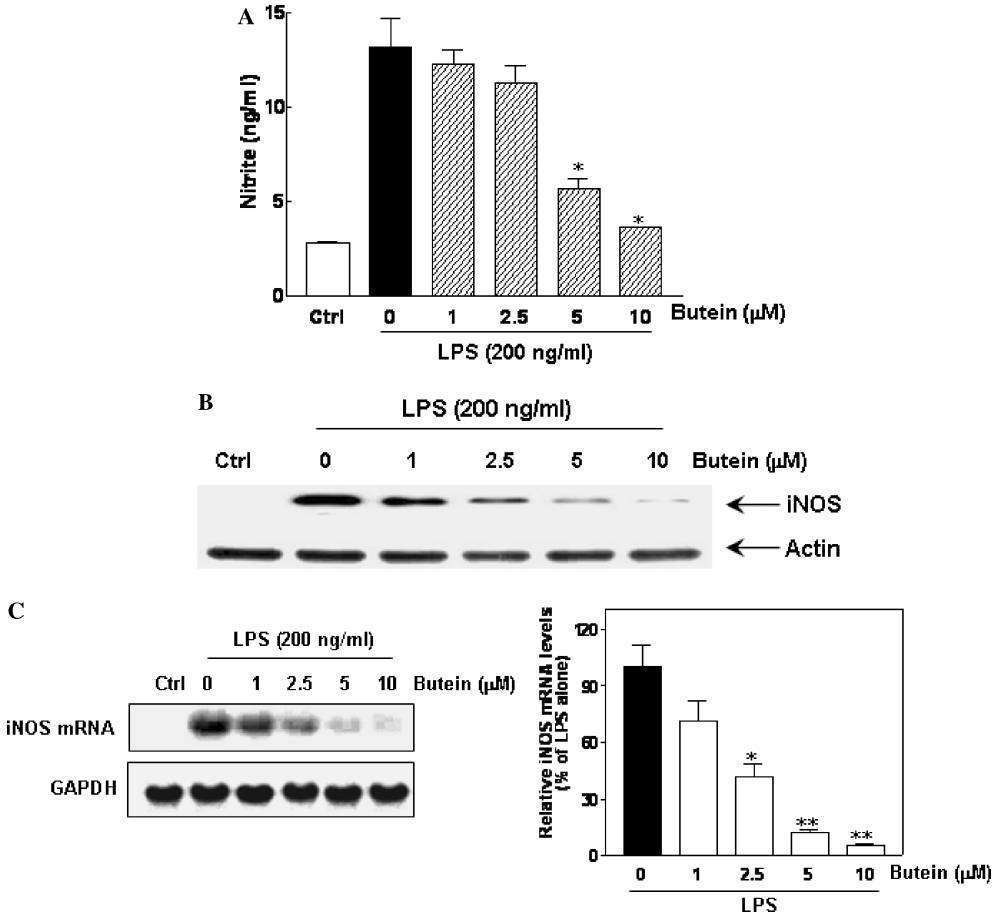

Fig. 1. (A) Inhibition of nitrite production by butein in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentrations of butein for 1 h before being incubated with LPS (200 ng/ml) for 24 h. Control cells were incubated with vehicle alone. The culture supernatants were subsequently isolated and analyzed for nitrite production. Each column shows the mean ± SD of triplicate determinations. Statistical significance: $*p < 0.001$ vs. LPS alone, as analyzed by one-way ANOVA and Tukey's multiple comparison tests. (B) Inhibition of iNOS protein expression by butein. The level of iNOS protein was monitored 24 h after treatment of cells with LPS (200 ng/ml), with or without butein. Cell lysates were then prepared and subjected to Western immunoblotting using an antibody specific for murine iNOS. Equal loading of proteins was verified by actin immunoblotting. One of two representative experiments is shown. (C) The effects of butein on the iNOS mRNA level in RAW 264.7 cells stimulated with LPS (200 ng/ml). Northern blot analysis was performed to determine iNOS mRNA in total RNA fractions (50 μg each) isolated from cells treated with LPS in the presence or absence of butein. Cells were pretreated with butein for 1 h followed by the addition of LPS, and the iNOS mRNA level was assessed 12 h after addition of LPS. The amount of RNA loaded in each lane was confirmed by rehybridization of the stripped membrane with a ^{32}P-labeled probe complementary to GAPDH. The results presented are representative of three independent experiments. Results are means ± SD. Statistical significance: $*p < 0.01$ and $**p < 0.001$ vs. LPS alone, as analyzed by one-way ANOVA and Tukey's multiple comparison tests.

indicated that, even at 10 μM, butein did not affect the viability of RAW 264.7 cells (data not shown). Therefore, inhibition of LPS-induced nitrite production by butein was not the result of a cytotoxic effect on these cells.

Several studies have demonstrated that induction of iNOS produces a large amount of NO during endotoxemia and under inflammatory conditions. Therefore, drugs that inhibit iNOS expression and/or enzyme activity, resulting in decreased NO generation, may be beneficial in treating diseases caused by an overproduction of NO [21,22]. In view of the involvement of iNOS in the inflammatory process, we monitored iNOS gene expression in macrophages exposed to butein. The effect of butein on iNOS protein expression was examined by Western blot. As shown in Fig. 1B, expression of the iNOS protein was barely detectable in unstimulated cells, but markedly increased 24 h after LPS (200 ng/ml) treatment. Consistent with previous results, treatment with butein showed a concentration-dependent inhibition of iNOS protein expression in LPS-stimulated RAW 264.7 cells.

To assess the effect of butein on iNOS mRNA expression, we measured mRNA levels by Northern blot analysis. The expression of iNOS mRNA was hardly detectable in unstimulated cells. However, RAW 264.7 cells expressed high levels of iNOS mRNA when stimulated with LPS (200 ng/ml) for 12 h. Furthermore, butein inhibited this LPS-stimulated iNOS mRNA production in a dose-dependent manner (Fig. 1C).

Northern and Western blot analyses revealed that iNOS expression by butein was in parallel with the

comparable inhibition of NO production. As stated earlier, NO plays an important role in the pathogenesis of various inflammatory diseases. Therefore, the inhibitory effect of butein on iNOS gene expression suggests that this is one of the mechanisms responsible for the anti-inflammatory action of butein.

Inhibition of LPS-inducible TNF-α expression

TNF-α is the principal mediator of responses to LPS, such as tissue injury and shock, and may play a role in the innate immune response [23]. We studied the effect of butein on LPS-inducible TNF-α expression, to determine the effects of butein on acute inflammation. Production of TNF-α was measured in the medium of RAW 264.7 cells cultured with LPS (200 ng/ml) in the presence or absence of butein for 6 h. Butein treatment showed a concentration-dependent inhibition of TNF-α production in LPS-stimulated RAW 264.7 cells (Fig. 2A). RAW 264.7 cells were incubated for 3 h in medium containing LPS (200 ng/ml) and various concentrations of butein, and the levels of TNF-α mRNA were determined by Northern blot. Butein also inhibited the increase in TNF-α mRNA by LPS (Fig. 2B). These

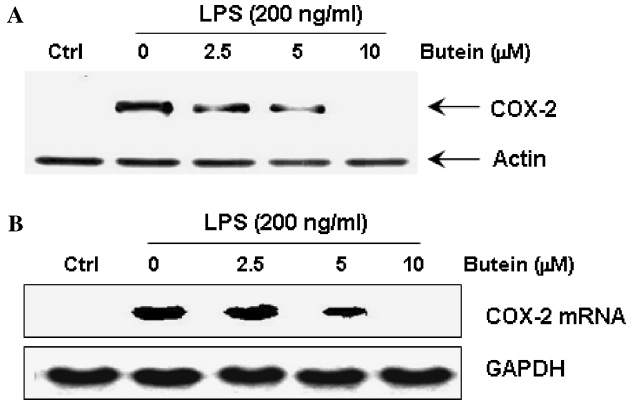

Fig. 3. Inhibition of LPS-inducible COX-2 expression by butein. (A) COX-2 protein expression. COX-2 expression was measured in RAW 264.7 cells cultured with 200 ng/ml LPS with or without various concentrations of butein for 12 h. Control cells were incubated with vehicle alone. Equal loading of proteins was verified by actin immunoblotting. One of two representative experiments is shown. (B) The level of COX-2 mRNA. COX-2 mRNA was assessed by Northern blot analysis in RAW 264.7 cells exposed to LPS with or without butein. The amount of RNA loaded in each lane was confirmed by rehybridization of the stripped membrane with a ^{32}P-labeled probe complementary to GAPDH. One of two representative experiments is shown.

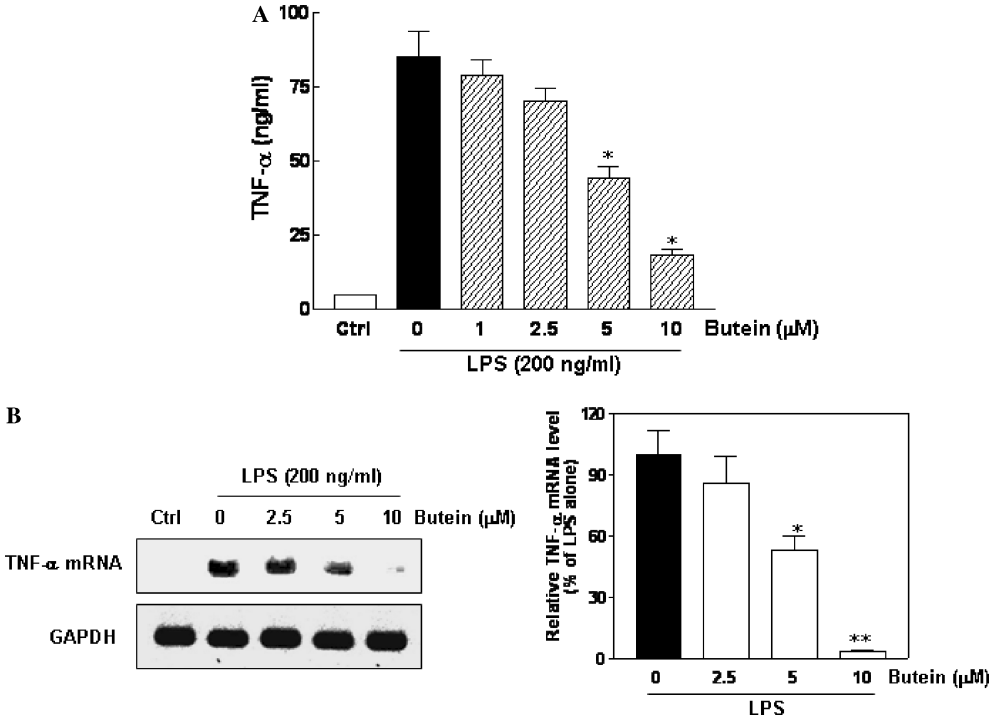

Fig. 2. The effect of butein on LPS-inducible TNF-α expression. (A) Levels of TNF-α. Production of TNF-α was measured in the medium of RAW 264.7 cells cultured with LPS (200 ng/ml) in the presence or absence of butein for 6 h. Control cells were incubated with vehicle alone. Each column shows the mean ± SD of triplicate determinations. Statistical significance: *$p < 0.001$ vs. LPS alone, as analyzed by one-way ANOVA and Tukey's multiple comparison tests. (B) Expression of TNF-α mRNA. TNF-α mRNA was monitored by Northern blot analysis in cells cultured with LPS (200 ng/ml) in the presence or absence of butein for 3 h. The amount of RNA loaded in each lane was confirmed by rehybridization of the stripped membrane with a ^{32}P-labeled probe complementary to GAPDH. The results presented are representative of three independent experiments. Results are means ± SD. Statistical significance: *$p < 0.05$ and **$p < 0.001$ vs. LPS alone, as analyzed by one-way ANOVA and Tukey's multiple comparison tests.

findings suggest that inhibition of TNF-α production by butein is due to the suppression of LPS-induced expression of TNF-α mRNA.

Inhibition of LPS-inducible COX-2 expression

COX-2 is induced by LPS, certain serum factors, cytokines, and growth factors, and is a predominant cyclooxygenase at sites of inflammation. The development of COX-2 inhibitors represents a major advance in treating the inflammatory process and their use includes prevention or treatment of disorders associated with the induction of this enzyme [24]. Recent findings suggest that COX-2 may play an important role in the pathogenesis of diseases such as colon carcinoma, Alzheimer's disease, heart failure, and hypertension [25–27]. Therefore, there is an increasing interest in the usefulness of COX-2 inhibitors. We further evaluated the effect of butein on LPS-inducible COX-2 gene expression in macrophages. The expression of COX-2 protein was monitored in RAW 264.7 cells exposed to LPS (200 ng/ml) for 12 h. Butein effectively suppressed the induction of COX-2 by LPS (Fig. 3A). LPS (200 ng/ml) also increased the COX-2 mRNA, which was inhibited in the presence of butein (Fig. 3B). Hence, butein actively suppressed the expression of genes implicated in inflammation.

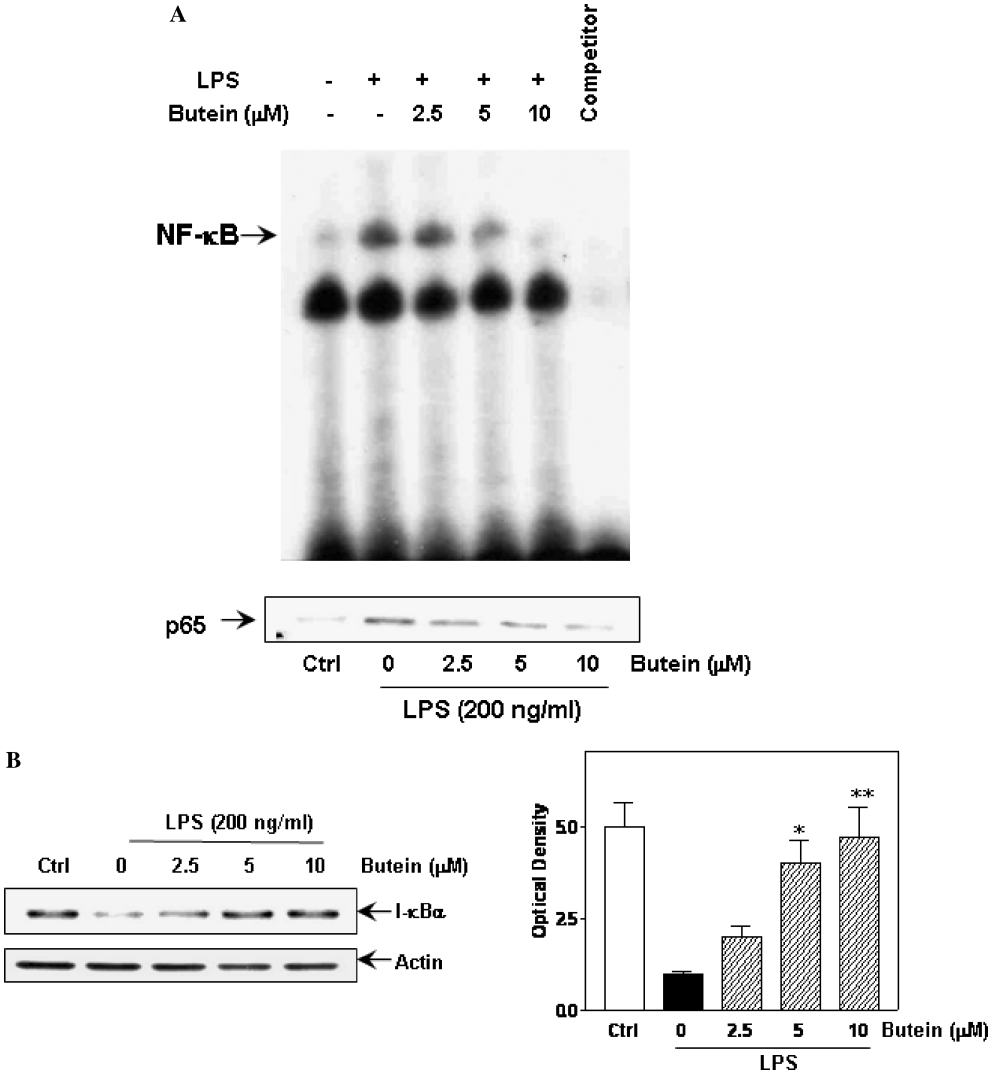

Fig. 4. Inhibition of LPS-inducible NF-κB activation and I-κBα degradation by butein. (A) Gel shift analysis of nuclear extracts using the consensus sequence of NF-κB. Nuclear extracts were isolated 1 h after LPS treatment (200 ng/ml) of RAW 264.7 cells and subjected to gel shift analysis. Each lane contained 5 μg of nuclear extracts (upper panel). RAW 264.7 cells were treated with 200 ng/ml LPS for 1 h and p65 protein was immunochemically detected using anti-p65 antibody. LPS caused the p65 protein to migrate to the nucleus at 1 h. Butein prevented LPS-induced nuclear translocation of p65 protein (lower panel). (B) Effect of butein on LPS-induced I-κBα degradation. The effect of LPS on I-κBα degradation was immunochemically assessed in RAW 264.7 cells. Degradation of I-κBα protein was significantly inhibited by treatment of cells with butein. Equal loading of proteins was verified by actin immunoblotting. The results presented are representative of three independent experiments. Results are means ± SD. Statistical significance: $*p < 0.05$ and $**p < 0.01$ vs. LPS alone, as analyzed by one-way ANOVA and Tukey's multiple comparison tests.

Effect of butein on LPS-inducible NF-κB activation

Gene expression of iNOS is regulated mainly at the transcriptional level in macrophages and its major transcriptional regulators are the NF-κB family of transcription factors, which are also key regulators of a variety of genes involved in immune and inflammatory responses [28]. Importantly, the NF-κB binding site has been identified on the murine iNOS promoter and plays a role in the LPS-mediated induction of iNOS in RAW 264.7 cells [29].

To investigate whether the transcription factor NF-κB is an important target for the action of butein in RAW 264.7 cells, we performed an electrophoretic mobility shift assay. Treatment of RAW 264.7 cells with LPS (200 ng/ml) caused a significant increase in the DNA binding activity of NF-κB. In the presence of butein, LPS-induced NF-κB binding was markedly suppressed in a concentration-dependent manner (Fig. 4A, upper panel).

Since p65 was the major component of NF-κB activated by LPS in macrophages, we determined the translocation of p65 into the nucleus by immunoblotting (Fig. 4A, lower panel). RAW 264.7 cells were incubated with LPS in the presence or absence of butein for 1 h. Negligible levels of p65 protein were detected in the nucleus of control cells. In contrast, p65 protein moved into the nucleus 1 h after LPS treatment. The p65 protein decreased in the nucleus of cells exposed to LPS in combination with butein, which verified that butein inhibited nuclear localization of the p65 protein.

The nuclear translocation and DNA binding of the NF-κB transcription factor are preceded by the degradation of inhibitory factor-κBα (I-κBα) [30]. To determine whether the inhibition of NF-κB DNA binding by butein is due to an effect on I-κBα degradation, cytoplasmic levels of I-κBα were examined by Western blot analysis. Pretreatment of RAW 264.7 cells with butein blocked LPS-induced I-κBα degradation (Fig. 4B). Since NF-κB is activated by I-κBα degradation following phosphorylation of I-κBα at serine residues [31], the recovery of I-κBα protein in RAW 264.7 cells provided strong evidence that butein inhibited the activation of NF-κB.

Effect of butein on phosphorylation of Erk1/2, SAPK/JNK, and p38 MAP kinase

The mitogen-activated protein (MAP) kinases play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stresses. Moreover, they are also known to be important for the activation of NF-κB [32]. To investigate whether the inhibition of NF-κB activation by butein is mediated through the MAP kinase pathway, we examined the effect of butein on the LPS-stimulated phosphorylation of Erk1/2, SAPK/JNK, and p38 MAP kinase in RAW 264.7 cells using Western blot analysis. As shown in Fig. 5, butein suppressed LPS-induced activation of Erk1/2 MAP kinase in a dose-dependent manner. The amount of non-phosphorylated Erk was unaffected by either LPS or butein treatment. Butein (5 and 10 μM) markedly inhibited Erk1/2 MAP kinase activation, while phosphorylation of the SAPK/JNK and p38 MAP kinase was unaffected by butein treatment. These results suggest that phosphorylation of Erk1/2 is involved in the inhibitory effect of butein on LPS-stimulated NF-κB binding in RAW 264.7 cells.

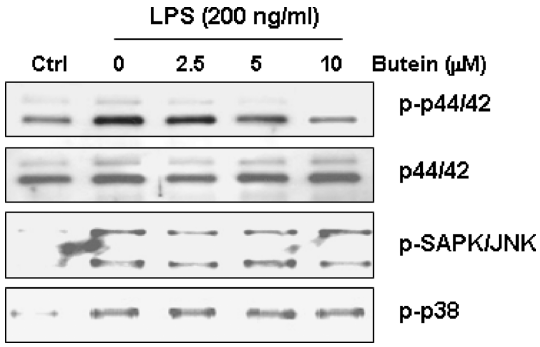

Fig. 5. Effect of butein on LPS-induced phosphorylation of Erk1/2, SAPK/JNK, and p38 MAP kinase in RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of butein before being incubated with LPS (200 ng/ml) for 30 min. Whole-cell lysates were analyzed by Western blot analysis. The results presented are representative of three independent experiments.

Effects of butein on OPN protein and RAW 264.7 cell surface expression of αvβ3 integrin

Osteopontin (OPN) production has been reported to be increased in a number of pathological conditions, including inflammation, atherosclerosis, nephritis, and malignancy, as well as in normal situations of morphogenesis [33]. Interestingly, OPN and iNOS are induced in response to many of the same agents, such as TNF-α, interleukin-1β, interferon-γ, and LPS [9,34]. Recently, Hwang et al. [35] demonstrated that endogenous OPN can inhibit induction of iNOS and that OPN is an important regulator of the NO signaling pathway and NO-mediated cytoregulatory processes. Guo et al. [36] reported that OPN promoter activity and gene transcription are significantly up-regulated in the presence of LPS-mediated NO production and that OPN inhibits NO production in the presence of endotoxin stimulation.

In the present study, Western blot analysis was performed to determine the effect of butein on OPN protein expression in LPS-treated cells (Fig. 6A). OPN protein was detected in the cell culture medium as described previously [36]. No secreted OPN protein was found in the LPS-unstimulated control, whereas OPN was detectable in the culture media of LPS-stimulated

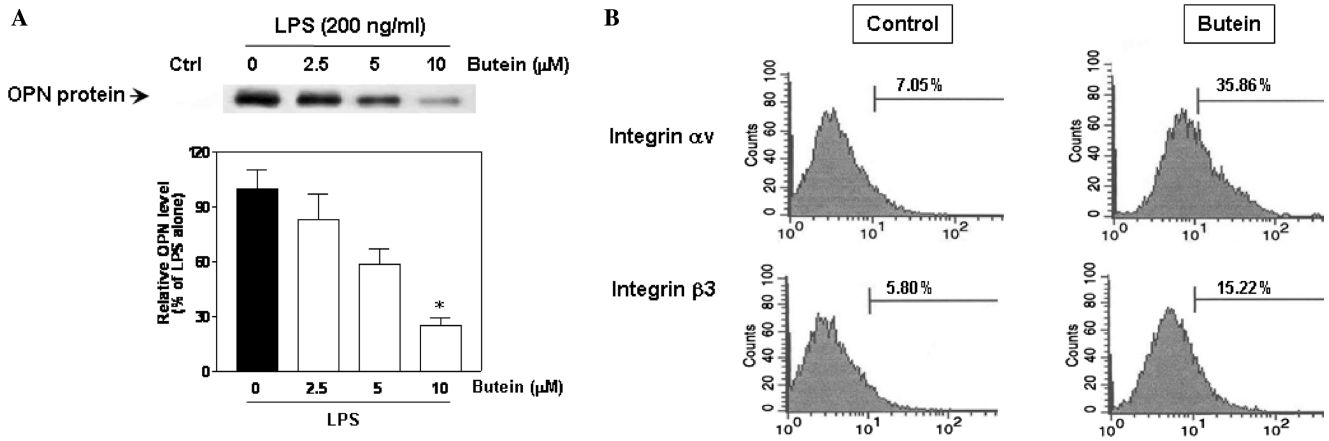

Fig. 6. Effects of butein on secreted OPN protein and RAW 264.7 cell surface expression of integrin αvβ3. (A) Western blot analysis of secreted OPN protein in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were cultured with LPS (200 ng/ml) in the presence or absence of butein for 12 h. The results presented are representative of three independent experiments. Results are means ± SD. Statistical significance: $*p < 0.01$ vs. LPS alone, as analyzed by one-way ANOVA and Tukey's multiple comparison tests. (B) Enhancement of cell surface expression of integrin αvβ3 by butein. RAW 264.7 cells were cultured for 24 h in medium containing the control vehicle or butein (10 μM). Cells were labeled with biotin conjugated anti-integrin αv or anti-integrin β3 followed by incubation with streptavidin–FITC. After washing, cells were subjected to flow cytometry.

cells. Interestingly, treatment with butein showed a concentration-dependent reduction of secreted OPN protein in LPS-stimulated RAW 264.7 cells.

Guo et al. [36] also demonstrated that addition of a competitive inhibitor of OPN receptor binding results in a significant concentration- and time-dependent increase in LPS-induced NO synthesis. That is, the αvβ3 integrin receptor appears to transduce an inhibitory signal for the down-regulation of LPS-induced NO synthesis. Therefore, we next determined whether there may be a functional significance to the NO-inhibitory effect of butein by investigating the cell surface expression of αvβ3 integrin protein complexes. RAW 264.7 cells were cultured for 24 h in the presence of butein and cell surface expression of αv and β3 integrins was analyzed by flow cytometry with specific antibodies. Butein increased the cell surface expression of both αv and β3 integrin proteins (Fig. 6B). These data indicate that butein exerts its effect on the induction of αvβ3 integrin expression at the cell surface, resulting in decreases in secreted OPN protein. Therefore, it is possible that the increased binding of the OPN αvβ3 integrin receptor by butein results in the inhibition of LPS-mediated NO production in RAW 264.7 cells.

In summary, this study demonstrates that butein inhibits LPS-induced NO production and iNOS gene expression in macrophages and that these effects are mediated by inhibition of the activity of NF-κB and the phosphorylation of Erk1/2. Our results also suggest that the inhibition of NO production by butein is mediated by enhancing the expression of αvβ3 integrin. In view of the fact that NO plays an important role in mediating inflammatory responses, the inhibitory effect of butein on iNOS gene expression might be responsible for its anti-inflammatory action.

Acknowledgments

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