Sasa quelpaertensis Phenylpropanoid Derivative Suppresses Lipopolysaccharide-induced Nitric Oxide Synthase and Cyclo-oxygenase-2 Expressions in RAW 264.7 Cells

Ji-Young Moon,[a,b] Eun-Jin Yang,[a] Sang Suk Kim,[b] Ji-Yong Kang,[a]
Gi-Ok Kim,[a] Nam Ho Lee,[*,b,#] and Chang-Gu Hyun[a,#]

[a]Bio-Convergence Center, Jeju Technopark, Jeju 690–121, Korea, and [b]Department of Chemistry,
Jeju National University, Jeju 690–756, Korea

3-O-p-Coumaroyl-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-O-β-D-gulcopyranosylpropanol (ESQ10) is a naturally occurring phenylpropanoid derivative isolated from *Sasa quelpaertensis* (Gramineae). In the present study, we discovered that ESQ10 inhibits nitric oxide (NO) and prostaglandin E_2 (PGE_2) production in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages. ESQ10 attenuated LPS-induced synthesis of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in parallel and inhibited LPS-induced interleukin-6 production, as determined by an enzyme-linked immunosorbent assay in the macrophages. The mechanism of the antiinflammatory action of ESQ10, *i.e.*, suppression of nuclear factor (NF)-κB and mitogen-activated protein kinase activation, has been documented. However, ESQ10 could not influence LPS-mediated IκB-α degradation and extracellular signal-regulated kinase/c-Jun amino-terminal kinase phosphorylation at concentrations of up to 373 μM. To test the potential application of ESQ10 as a topical material, we also conducted a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on human HaCaT keratinocytes as well as human dermal fibroblast cells. In this assay, ESQ10 did not induce cytotoxicity. Taken together, the results suggest that ESQ10 may be considered an antiinflammatory candidate for treating inflammatory and skin diseases.

Key words——cyclooxygenase-2 (COX-2); inducible nitric oxide synthase (iNOS); inflammation; phenylpropanoid; *Sasa quelpaertensis*

INTRODUCTION

Inflammation is one of the most important host defense mechanisms against tissue injuries and invading pathogens. However, although acute inflammation is a beneficial process, particularly in response to infectious pathogens, chronic inflammation is an undesirable phenomenon that can ultimately lead to inflammatory diseases[1] such as asthma,[2,3] rheumatoid arthritis,[4,5] inflammatory bowel disease,[6] psoriasis,[7] bronchitis,[8,9] gastritis,[10] and multiple sclerosis.[11] Inflammation is a central feature in many pathologic conditions and is mediated by a variety of soluble factors and cellular signalling events such as nuclear transcription factor kappa-B (NF-κB) and mitogen-activated protein kinases (MAPKs) pathways. NF-κB regulates various genes involved in cell survival as well as immune and acute-phase inflammatory responses. In response to proinflammatory stimuli, NF-κB is activated and the IKK signalosome complex

rapidly phosphorylates IκBs. The resulting free NF-κB then translocates to the nucleus, where it binds to κB-binding sites in the promoter regions of target genes and induces the transcription of proinflammatory mediators such as inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), interleukin-6 (IL-6), tumour necrosis factor-alpha (TNF-α), IL-1β, and IL-8.[12–14] The MAPKs [(extracellular signal-regulated kinase (ERK), p38, and c-Jun amino-terminal kinase (JNK)] react to extracellular stimuli and control various cellular activities, including gene expression, mitosis, differentiation, and cell survival/apoptosis. MAPKs regulate inflammatory and immune responses, and their signalling pathways are involved in lipopolysaccharide (LPS)-induced COX-2 and iNOS expression in macrophages.[15,16] Thus NF-κB, iNOS, COX-2, and IL-6 have been exploited as molecular targets in the discovery and development of drugs for inflammatory-related diseases.[17–19]

There have been many attempts to derive new antiinflammatory and anticancer agents from natural compounds. Traditional remedies derived from herbs or medicinal plants have been considered safe, less

toxic, and readily available, even though their mechanisms of action are mostly still ambiguous. Thus uncovering the molecular mechanism underlying the antiinflammatory function of natural products may be a good strategy for identifying new therapeutic agents. Previously, our research group documented the antiinflammatory properties of various medicinal plants.[20–24]

During our ongoing screening program designed to identify the antiinflammatory potential of natural compounds, we isolated 3-O-p-coumaroyl-1-(4-hydroxy-3,5-dimethoxyphenyl)-1-O-β-D-gulcopyranosylpropanol (ESQ10) from the leaves of *Sasa quelpaertensis* using activity-directed fractionation and characterized its structural identity using spectroscopy (^{1}H- and ^{13}NMR, IR, MS), as published previously.[25] However, there has been no report on its biological activity or mechanism of action, although we previously reported that ESQ10 significantly inhibits mushroom tyrosinase.[25] Therefore, as a prelude to revealing the mechanisms underlying the antiinflammatory effects of ESQ10, we evaluated variations in inflammatory proteins and cytokine expression *in vitro* in the presence of ESQ10. Moreover, we determined how ESQ10 exhibits antiinflammatory activity.

MATERIALS AND METHODS

Chemicals and Reagents   ESQ10 was isolated essentially as described previously.[25] Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen-Gibco (Grand Island, NY, USA). Enzyme-linked immunosorbent assay (ELISA) kits for prostaglandin E$_2$ (PGE$_2$) and IL-6 were purchased from R&D Systems, Inc. (St. Louis, MO, USA) and BD Biosciences (San Diego, CA, USA). Anti-IκB-α, anti-phosphorylated IκB-α (anti-p-IκB-α), anti-JNK, anti-phosphorylated JNK (anti-p-JNK), anti-ERK1/2, and anti-phosphorylated ERK1/2 (anti-p-ERK1/2) mouse or rabbit antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). PD98059 (a specific inhibitor of ERK1/2), SP600125 (a specific inhibitor of JNK), and pyrollidine dithiocarbamate (PDTC, a specific inhibitor of NF-κB) were purchased from Calbiochem (San Diego, CA, USA). All other reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). ESQ10 was prepared as in a previous study.[25]

RAW 264.7 Cell Culture   RAW 264.7 cells were obtained from the Korean Cell Line Bank (KCLB; Seoul, Korea) and maintained at subconfluence in a 95% air, 5% CO$_2$ humidified atmosphere at 37℃. The medium for routine subcultivation was DMEM supplemented with FBS (10%), penicillin (100 units/ml), and streptomycin (100 μg/ml). Cells were counted with a hemocytometer, and the number of viable cells was determined by trypan blue dye exclusion.

Nitric Oxide Determination   Nitric oxide accumulation was used as an indicator of NO production in the cell culture medium and was determined in the Griess reaction. The culture supernatant (100 μl) was mixed with the same volume of Griess reagent [1% sulphanilamide and 0.1% N-(1-naphthyl)-ethylenediamine dihydrochloride in 5% phosphoric acid] for 10 min, and the absorbance was measured at 540 nm. Sodium nitrite (NaNO$_2$) was used for the standard curve (1–100 μM).

Detection of PGE$_2$ and IL-6 in Supernatant   Sandwich ELISA was used to determine the inhibitory effects of ESQ10 47 μM, 94 μM, 186 μM, and 373 μM on the production of the cytokines PGE$_2$ and IL-6 in LPS-treated RAW 264.7 cells. RAW 264.7 cells were stimulated for 24 h before the supernatant was harvested and assayed according to the manufacturer's protocol for the relevant ELISA kit. Results from 3 independent experiments were used for statistical analysis.

Western Blot Analysis   The RAW 264.7 cells (1.5×10^5 cells/ml) were preincubated for 18 h and then treated with LPS (1 μg/ml) plus aliquot sample for 24 h. After incubation, the cells were washed twice with cold PBS. Whole-cell lysates (25 μg) were separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electrotransferred to a polyvinylidene fluoride (PVDF) membrane (BIO-RAD, HC). The membrane was incubated for 24 h with 5% skim milk and then incubated with a specific primary antibody (1 : 2500) at room temperature for 2 h. The membrane was washed 4 times with TTBS and incubated for 30 min with a peroxidase-conjugated secondary antibody (1 : 5000) at room temperature. Finally, the proteins on the membrane were detected using the WESTZOL Western Blot Detection System (iNtRON, Gyeonggi, Korea).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazo-

lium Bromide Assay for Cell Viability   Cell viability was determined in the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 cells were cultured in 96-well plates for 18 h, followed by treatment with LPS (1 μg/ml) in the presence of various concentrations of the sample. After 24-h incubation, MTT was added to the medium for 4 h. Finally, the supernatant was removed, and the formazan crystals were dissolved in DMSO. Absorbance was measured at 540 nm. The percentage of cells showing cytotoxicity relative to the control group was determined.

Statistical Analysis   Student's *t*-test and one-way ANOVA were used to determine statistically significant differences between the values in the various experimental and control groups. Data are expressed as mean±standard error (S.E.M.), and the results are taken from at least three independent experiments performed in triplicate.

RESULTS

ESQ10 Reduces NO Production and Inhibits iNOS Formation   It is well established that RAW 264.7 cells, a mouse macrophage cell line, induce the expression of COX-2 and iNOS, which produce large amounts of PGE_2 and NO, respectively, when activated with LPS. Therefore the modulation of macrophage-mediated inflammatory responses is emerging as a promising new therapeutic approach against inflammatory diseases.[26–29] LPS, a major cell wall component of Gram-negative bacteria, induces a strong innate and adaptive immune response and was used to elicit increased NO synthesis. Since the half-life of NO is very short, we used nitrite production as an indicator of NO released by LPS-activated macrophages. Compared with normal macrophages, NO production increased >15-fold in LPS-activated macrophages, to 17.1±0.7 *vs.* 1.10±0.05, $p<0.01$ (Fig. 1A). ESQ10 reduced LPS-induced NO production in a dose-dependent manner. At ESQ10 concentrations of 47 μM, 94 μM, 186 μM, and 373 μM, the production of NO by LPS-treated macrophages decreased by 31.6% (11.8±2.4, $p<0.01$), 43.7% (9.63±2.5, $p<0.05$), 55.3% (7.73±0.9, $p<0.01$), and 76.2% (4.12±2.3, $p<0.01$), respectively, as compared with LPS-treated macrophages not treated with ESQ10 (Fig. 1A). DMSO, the vehicle control, had no effect on NO production, reconfirming its immunologic inertness. Furthermore, ESQ10 itself caused minimal change in NO production in resting macrophages. In parallel, the potential cytotoxicity of ESQ10 was evaluated in the MTT assay after incubating cells for 24 h in the presence and absence of LPS. However, cell viability was negligibly affected at the concentrations used (47 μM, 94 μM, 186 μM, and 373 μM) to inhibit NO (Fig. 1A). Thus the inhibitory effects of ESQ10 were not attributable to cytotoxicity.

To elucidate further the mechanisms by which ESQ10 inhibited NO production in LPS-activated macrophages, we analyzed its effect on LPS-induced iNOS gene expression in macrophages. Under normal conditions, RAW 264.7 cells expressed undetectable levels of iNOS mRNA, but iNOS mRNA levels increased markedly after 24 h of LPS stimulation (Fig. 1B). With the addition of ESQ10 (47 μM–373 μM), dose-dependent inhibition of iNOS expression was observed, indicating that ESQ10 modulates iNOS expression.

ESQ10 Reduces the Release of Inflammatory Mediators and Proinflammatory Cytokines in Macrophages PGE_2, the most important inflammatory product of COX-2, was quantified in the supernatants of LPS-treated RAW 264.7 macrophages. Cells were preincubated with ESQ10 for 1 h, after which they were stimulated with LPS 1 μg/ml for 24 h. Neither LPS nor the sample were treated in the control group. Cell culture media were collected, and PGE_2 levels determined. Compared with unstimulated macrophages, the PGE_2 level increased dramatically by 4-fold in LPS-stimulated macrophages. With the addition of ESQ10 47 μM, 94 μM, 186 μM, and 373 μM, a dose-dependent reduction in PGE_2 was observed to 4.7±3.5%, 13.2±3.2%, 27.7±7.4%, and 36.7±5.7%, respectively (Fig. 2A). To determine the mechanism by which ESQ10 reduces LPS-induced PGE_2 production, we studied the ability of ESQ10 to influence the LPS-induced expression of COX-2. The addition of LPS resulted in a clearly defined increase in COX-2 expression which was markedly attenuated in a dose-dependent fashion when treated with ESQ10 (Fig. 2B), corroborating that ESQ10 induces a decrease in COX-2, which translates into a dramatic decrease in PGE_2. As expected, the reference compounds, dexamethasone (COX-2 inhibitor) and 2-amino-4-methyl pyridine (iNOS inhibitor) potently inhibited PGE_2 and NO production at 20 μM, respectively.
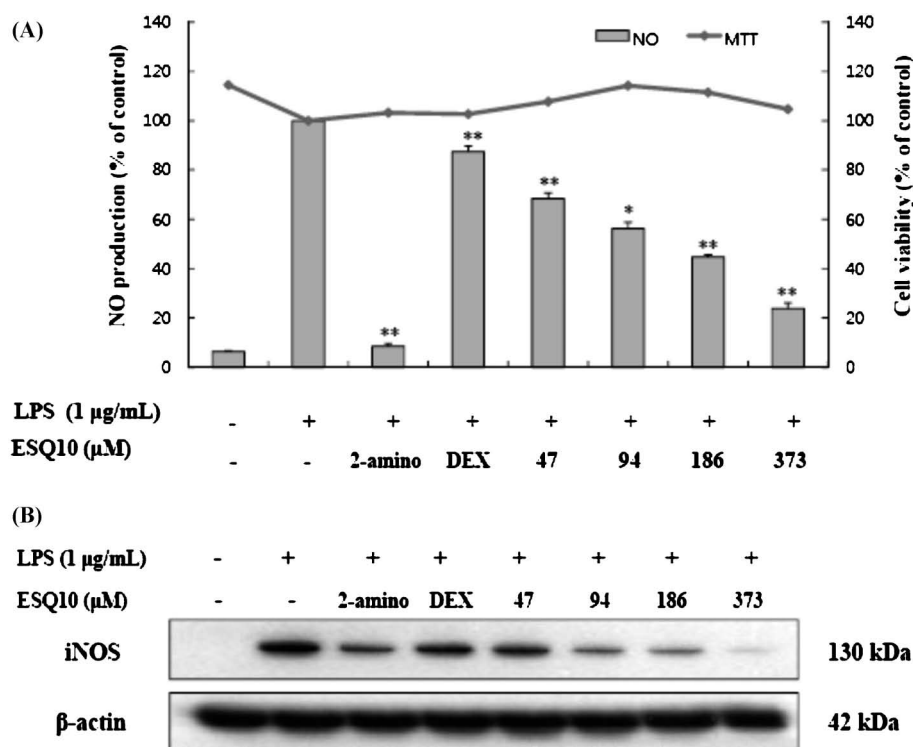
When macrophages are stimulated with LPS, they

Fig. 1.   Effects of ESQ10 on Nitric Oxide (A) Production and Levels of iNOS Protein (B) in LPS-stimulated RAW 264.7 Cells

The cells were stimulated with 1 μg/ml of LPS alone or with LPS plus various concentrations (47 μM, 94 μM, 186 μM, and 373 μM) of ESQ10, 2-amono-4-methylpyridine (2-amino; 20 μM), and dexamethasone (DEX; 20 μM) for 24 h. Nitric oxide production was determined using the Griess reagent method. Cell viability was determined in the 24-h culture of cells stimulated with LPS (1 μg/ml) in the presence of ESQ10. Whole-cell lysates (25 μg) were prepared, and the protein was subjected to 10% SDS-PAGE. Expression of iNOS and β-actin were determined by Western blotting. β-Actin was a loading control. The data represent the mean ± S.D. of triplicate experiments. Cytotoxicity was determined using the MTT method. Values are presented as the mean ± S.E.M. of triplicate experiments. $*p < 0.05$; $**p < 0.01$.

secrete several proinflammatory cytokines, including IL-1β, IL-6, and TNF-α. Accordingly, we examined the effect of ESQ10 on the LPS-induced expression of IL-6. LPS induced a dramatic increase in IL-6 expression, which ESQ10 decreased in a dose-dependent manner (Fig. 3).

ESQ10 Treatment Has No Effect on LPS-induced IκB and MAPK Phosphorylation   When resting, in unstimulated macrophages, the heterodimeric NF-κB complex is sequestered in the cytoplasm as an inactive precursor complexed with an inhibitory protein, IκB. LPS-mediated activation of NF-κB correlates with the hyperphosphorylation of IκB-α and its subsequent degradation.[30] Therefore we examined the levels of phosphorylated IκB using immunoblot analysis. As expected, treatment with LPS enhanced the phosphorylation of IκB, as evidenced by the increased binding of phosphospecific IκB antibody as compared with that in controls (Fig. 4). However, Fig. 4 shows that LPS-induced IκB-α degradation is not significantly blocked by pretreatment with ESQ10.

MAPKs play a critical role in the regulation of cell growth and differentiation, and they control cellular responses to cytokines and stress. In addition, MAPKs play a critical role in the modulation of NF-κB activity. To investigate the molecular mechanism of NF-κB inhibition by ESQ10 in LPS-stimulated RAW 264.7 cells, we studied the inhibition of the phosphorylation of ERK1/2 and JNK. RAW 264.7 cells were pretreated with ESQ10 at the indicated concentrations for 30 min and then stimulated with LPS 1 μg/ml for 30 min. The total cell lysates were then probed with phosphospecific antibodies for ERK1/2 and JNK. Phosphorylation of ERK1/2 and JNK increased in cells treated with LPS alone. No changes in the expression of phosphorylated and nonphosphorylated ERK and JNK were observed in cells treated with LPS or LPS and ESQ10 (Fig. 5). These results suggest that the suppression of phosphorylation of MAPKs might not be involved in the inhibitory effect of ESQ10 on LPS-stimulated NF-κB activation in RAW 264.7 cells.
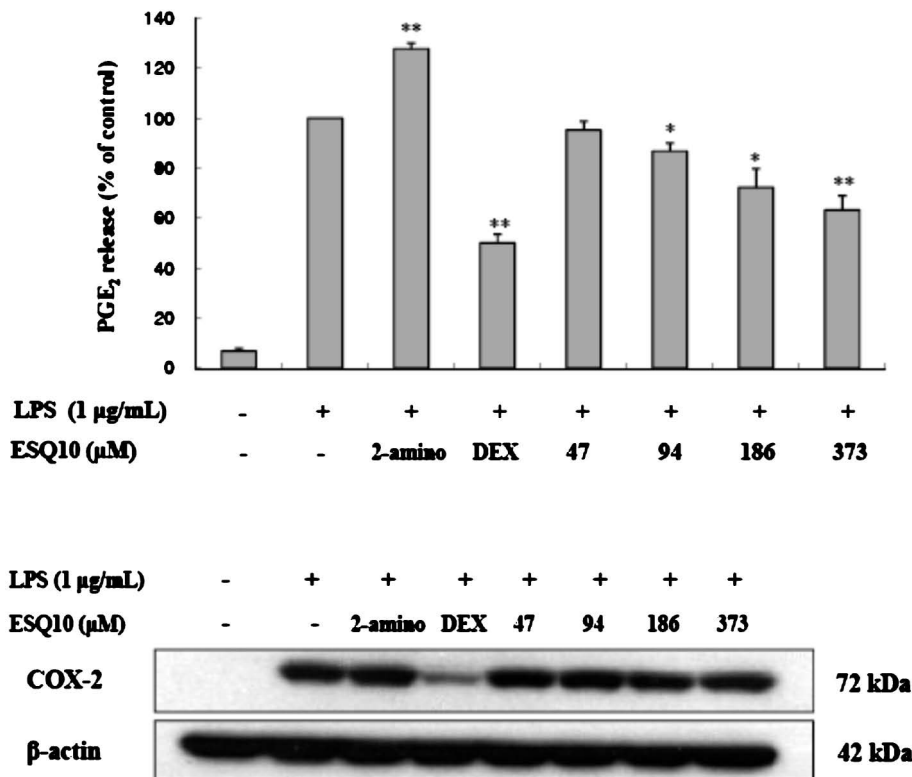
Fig. 2. Effects of ESQ10 on PGE₂ Production and Level of COX-2 Protein in LPS-stimulated RAW 264.7 Cells

The cells were stimulated with 1 μg/ml of LPS alone or with LPS plus various concentrations (47 μM, 94 μM, 186 μM, and 373 μM) of ESQ10, 2-amono-4-methylpyridine (2-amino; 20 μM), and dexamethasone (DEX; 20 μM) for 24 h. PGE₂ produced and released into the culture medium was assayed using the EIA method. The data represent the mean$\pm$S.D. of triplicate experiments. Whole-cell lysates (25 μg) were prepared, and the protein was subjected to 10% SDS-PAGE. Expression of COX-2 and β-actin were determined by Western blotting. β-Actin was a loading control. Values are presented as the mean$\pm$S.E.M. of triplicate experiments. *$p<$0.05; **$p<$0.01.
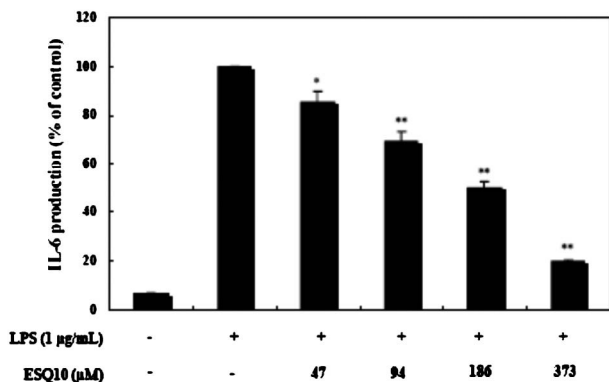

Fig. 3. Effects of ESQ10 on IL-6 Production in LPS-stimulated RAW 264.7 Cells

The cells were stimulated with 1 μg/ml of LPS alone or with LPS plus various concentrations (47 μM, 94 μM, 186 μM, and 373 μM) of ESQ10 for 24 h. IL-6 produced and released into the culture medium was assayed using the ELISA method.

Fig. 4. Effects of ESQ10 on the Degradation of IκB-α in LPS-stimulated RAW 264.7 Cells

RAW 264.7 cells (1.0$\times$10⁶ cells/ml) were stimulated with LPS (1 μg/ml) in the presence of ESQ10 (47 μM, 94 μM, 186 μM, and 373 μM) or PDTC (40 μM) for 15 min. Whole-cell lysates (30 μg) were prepared, and the protein was subjected to 12% SDS-PAGE. Expression of IκB-α and β-actin was determined by Western blotting. β-Actin antibody was the loading control.

DISCUSSION

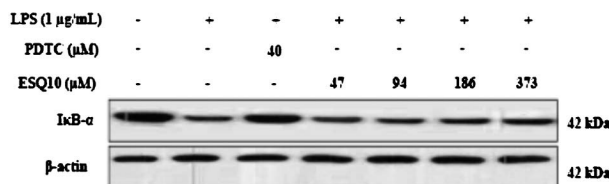Inflammatory processes play a critical role in the pathogenesis of many human diseases. Overproduction of inflammatory mediators by macrophages, such as cytokines and NO, has been implicated in inflammatory diseases such as asthma,[2,3] rheumatoid arthritis,[4,5] and inflammatory bowel disease.[6] In this study, we investigated the inhibitory effects of ESQ10 on LPS-induced NO production and expression of iNOS and COX-2 in RAW 264.7 cells. ESQ10 significantly inhibited LPS-induced NO production in a dose-dependent manner. ESQ10 strongly inhibited
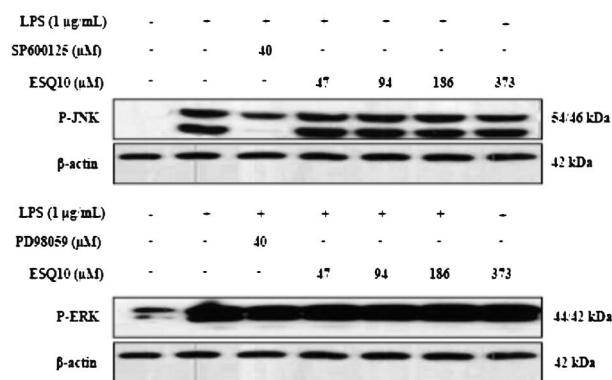
Fig. 5. Effects of ESQ10 on the Phosphorylation of p-JNK and p-ERK in LPS-stimulated RAW 264.7 Cells

RAW 264.7 cells $(1.0 \times 10^6$ cells/ml) were stimulated with LPS $(1 \mu g/$ ml$)$ in the presence of ESQ10 $(47 \mu M, 94 \mu M, 186 \mu M, $ and $373 \mu M)$, SP600125 $(40 \mu M$ for 30 min$)$, or PD98059 $(40 \mu M$ for 20 min$)$. Whole-cell lysates $(30 \mu g)$ were prepared, and the protein was subjected to 12% SDS-PAGE. Expression of p-JNK, p-ERK, and β-actin were determined by Western blotting. β-Actin antibody was the loading control.

LPS-induced NO production in RAW 264.7 cells, with an IC_{50} value of $175.0 \mu M$. These inhibitory effects may not be related to their cytotoxic effects since no effects were observed on the viability of RAW 264.7 cells at concentrations of up to $373 \mu M$. This inhibitory effect of NO production could be related to the gene expression of iNOS, since ESQ10 inhibited iNOS protein in RAW 264.7 cells. ESQ10 also inhibited LPS-induced COX-2 expression. These results show that ESQ10 could interfere with LPS-induced signalling involving the production of proinflammatory molecules. However, our data showed that the expression of COX-2 was less sensitive than that of iNOS to ESQ10. This could account for the higher sensitivity of iNOS gene transcription to ESQ10 compared with that of COX-2.

Since LPS-induced iNOS and COX-2 expression is primarily regulated by NF-κB, we partially examined the effect of ESQ10 on the LPS-induced activation of NF-κB. Several studies showed that antiinflammatory agents inhibit the activation of NF-κB by preventing IκB degradation. IκBα specifically binds and masks the nuclear translocation signals of p50 and p65, thereby preventing the nuclear translocation of the NF-κB heterodimer.[12,30] Reddy and Reddanna[31] showed that chebulagic acid prevents the induced degradation of IκBα by diverse stimuli and therefore interferes with a common step in the signalling cascade leading to the activation of NF-κB and MAPK. Lee et al.[32] have also demonstrated that preventing IκB degradation by carabrol contributes to the inacti-
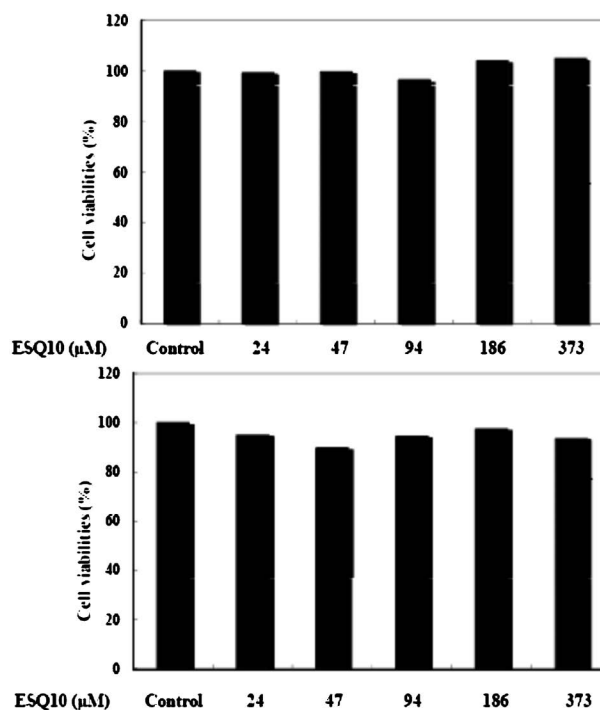

Fig. 6. Assessment of ESQ10 Cytotoxicity in Human Dermal Fibroblasts (A) and HaCaT Keratinocytes (B)

An MTT assay was performed after incubating human dermal fibroblasts and HaCaT keratinocytes. HaCaT cells were treated with various concentrations of ESQ10 for 24 h at 37°C in a 5% CO_2 atmosphere. Absorbance was measured at 570 nm using a spectrophotometer (Power Wave; Bio-Tek, Winooski, VT, USA).

vation of NF-κB (p50) in the antiinflammatory reaction in RAW 264.7 cells. However, ESQ10 did not inhibit the expression of iNOS/COX-2 and NO and PGE$_2$ production through the prevention of IκB degradation. MAPKs play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and stressors. Moreover, MAPKs are involved in the LPS-induced signalling pathway via which iNOS is expressed. In the present study, we also assessed whether the phosphorylation of MAPKs can be induced by ESQ10. However, treatment with ESQ10 had no effect on LPS-induced JNK and ERK phosphorylation. This result suggests that JNK and ERK are not involved in the inhibition by ESQ10 of LPS-stimulated NF-κB binding in RAW 264.7 cells.

Finally, to test the use of ESQ10 as a topical material, we performed an MTT assay with human cell lines, HaCaT keratinocytes and dermal fibroblasts. In these assays, ESQ10 did not induce any severe adverse reactions in the human cell lines (Fig. 6). Therefore we conclude that ESQ10 can be used as a therapeutic agent for inflammatory diseases via

IκB, a JNK- and ERK-independent pathway.

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