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Anti-inflammatory functions of purpurogallin in LPS-activated human endothelial cells

Tae Hoon Kim[1,#], Sae-Kwang Ku[2,#], In-Chul Lee[3] & Jong-Sup Bae[4,]*

[1]Department of Herbal Medicinal Pharmacology, College of Herbal Bio-Industry, [2]Department of Anatomy and Histology, College of Oriental Medicine, Daegu Haany University, Gyeongsan 712-715, [3]Senior Industry Cluster Agency, Youngdong University, Youngdong 370-701, [4]College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 702-701, Korea

Enzymatic oxidation of commercially available pyrogallol was efficiently transformed to an oxidative product, purpurogallin. Purpurogallin plays an important role in inhibiting glutathione S-transferase, xanthine oxidase, catechol O-methyltransferase activities and is effective in the cell protection of several cell types. However, the anti-inflammatory functions of purpurogallin are not well studied. Here, we determined the effects of purpurogallin on lipopolysaccharide (LPS)-mediated proinflammatory responses. The results showed that purpurogallin inhibited LPS-mediated barrier hyper-permeability, monocyte adhesion and migration and such inhibitory effects were significantly correlated with the inhibitory functions of purpurogallin on LPS-mediated cell adhesion molecules (vascular cell adhesion molecules, intracellular cell adhesion molecule, E-selectin). Furthermore, LPS-mediated nuclear factor-κB (NF-κB) and tumor necrosis factor-α (TNF-α) releases from HUVECs were inhibited by purpurogallin. Given these results, purpurogallin showed its anti-inflammatory activities and could be a candidate as a therapeutic agent for various systemic inflammatory diseases. [BMB reports 2012; 45(3): 200-205]

INTRODUCTION

The vascular endothelium is the center of events leading to the development of inflammatory lesions (1). Endothelial dysfunction is related especially with leukocyte recruitment during the formation of inflammatory lesions (2). Binding and recruitment of circulating leukocytes to the vascular endothelium and further migration into the subendothelial spaces are major processes in the development of inflammatory lesions and are mediated through a diverse family of cell adhesion molecules (CAMs) that are expressed on the surface of vascular endothelial cells (2). Among the identified adhesion molecules, the expression and biological properties of vascular cell adhesion molecule-1 (VCAM-1), endothelial-leukocyte adhesion molecule-1 (E-selectin), and intracellular cell adhesion molecule-1 (ICAM-1) are well characterized (3-6). The accumulated data imply that selectins mediate initial rolling of leukocytes along the endothelium and that VCAM-1 and ICAM-1 play important roles in the firm attachment and transendothelial migration of leukocytes (2-6). These molecules have been observed consistently within the milieu of the inflammatory relesions (3-6). The level of adhesion of circulating leukocytes to resting vascular endothelial cells normally is low (1, 7). However, upon challenge by injury or infection, released inflammatory mediators markedly increase the adhesive property of leukocytes and vascular endothelium toward each other (1, 7). The increased adhesiveness is the result, in part, of increased expression of adhesion proteins on the surface of endothelial cells as well as a change in affinity of leukocyte proteins for endothelial cell ligands (7). Therefore, inhibition of neutrophil rolling and attachment to vascular endothelium as a therapeutic approach is an attractive way to potentially prevent early inflammatory injury.

Blood capillaries are lined by continuous endothelial cells which form a semi-permeable barrier (8). This barrier is very important in regulating and controlling the macromolecules and fluid passing (8). Altered permeability of endothelial barrier is a characteristic hallmark of inflammatory responses and contributes to the morbidity and mortality in several inflammatory diseases such as sepsis, acute lung injury and anaphylaxis (9, 10). Therefore, there is great interest in the mediators for maintaining barrier integrity of endothelial cells and this mediator could be a target for preventing inflammatory injury.

Purpurogallin (Fig. 1A) is a benzotropolone containing natural product, which occurs in the nut gall of *Quercus spp.* (11). It can also be obtained by the oxidation of pyrogallol (Fig. 1B) (12). Purpurogallin has been reported to inhibit glutathione S-transferase, xanthine oxidase and catechol O-methyltransfer-

*Corresponding author. Tel: +82-53-950-8570; Fax: +82-53-950-8557; E-mail: baejs@knu.ac.kr
#These authors equally contributed to this work.
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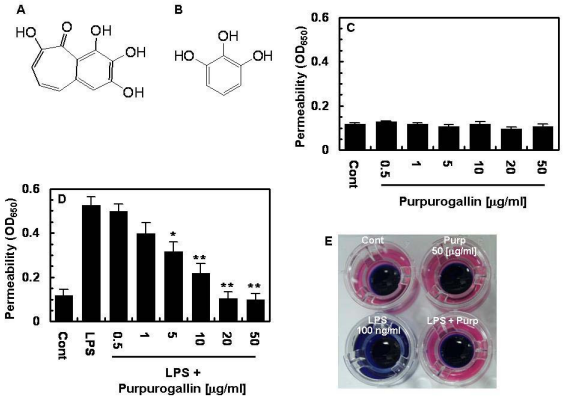

Fig. 1. The chemical structure of purpurogallin (A), pyrogallol (B) and effect of purpurogallin on LPS-mediated hyper-permeability. (C) Effect of various concentrations of purpurogallin on the endothelial permeability was monitored from the flux of Evans blue-bound albumin across HUVECs as described under "Experimental Procedures". (D) The same as (C) except that HUVECs were incubated with LPS (100 ng/ml, 4 h) to induce permeability after treating with purpurogallin. (E) Pictures of permeability assay. All results are shown as the means ± SD of different three experiments. *P < 0.05 and **P < 0.01 as compared to LPS.

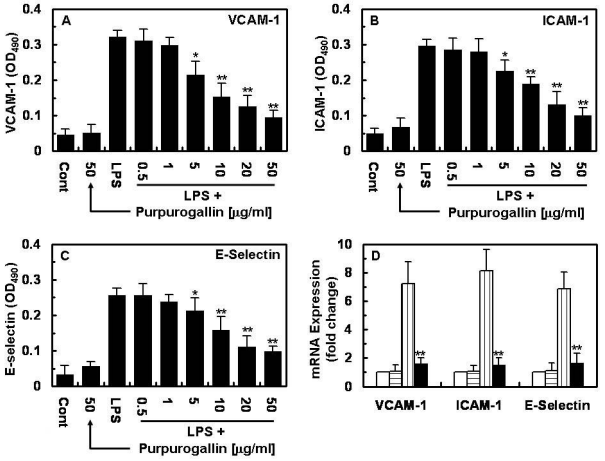

Fig. 2. Effect of purpurogallin on LPS-mediated CAMs expression. LPS-mediated (100 ng/ml) expression of VCAM-1, ICAM-1 and E-selectin at protein (ELISA, A-C) and transcription (mRNA; RT-PCR, D) levels in HUVECs was analyzed after treating with purpurogallin as described under "Experimental Procedures". Control (□), purpurogallin (50 μg/ml) (▤), LPS (100 ng/ml) (▥), LPS (100 ng/ml) + purpurogallin (50 μg/ml) (■). All results are shown as the means ± SD of different three experiments. *P < 0.05 and **P < 0.01 as compared to LPS.

ase (13-15). And it is also effective in the cytoprotection of hepatocytes (16), kidney cells (14), cardiac cells (17). Furthermore, purpurogallin showed antibacterial activity against gram-positive bacteria and phytotoxicity against all plants tested (18). However, no work has yet been done on the inhibitory activity of purpurogallin on LPS-mediated inflammatory responses in human endothelial cells. In the present study, anti-inflammatory responses of purpurogallin on the LPS-activated human endothelial cells and its mechanisms were carried out.

RESULTS

Barrier protective effect of purpurogallin in LPS-mediated HUVECs

To determine the effects of purpurogallin on barrier integrity in LPS-stimulated HUVECs, permeability assay was introduced. Purpurogallin alone did not alter barrier integrity (Fig. 1C, E). LPS is known to cleave and disrupt barrier integrity (19, 20). HUVECs were treated with various concentrations of purpurogallin for 6 h before adding LPS (100 ng/ml). As shown in Fig. 1D, purpurogallin decreased LPS-mediated membrane disruption in dose-dependent.

Purpurogallin inhibits the expression of CAMs in LPS-mediated endothelial cells

LPS treatment of human endothelial cells is associated with upregulation of several cell adhesion molecules (CAMs) such as VCAM-1, ICAM-1 and E-selectin (3-6). To determine the effect of purpurogallin on the expression of CAMs, the expression of VCAM-1, ICAM-1 and E-selectin in LPS-activated HUVECs which were pretreated with purpurogallin was measured. As demonstrated in Fig. 2, purpurogallin significantly suppressed VCAM-1, ICAM-1 and E-selectin expression at both protein (Fig. 2A-C) and transcription (mRNA expression) (Fig. 2D) levels. However, purpurogallin alone did not alter the expression of CAMs (Fig. 2).

Effects of purpurogallin on the adhesion of THP-1 cells to LPS-mediated endothelial cells and transendothelial migration (TEM)

The adhesion and migration of circulating leukocytes to the vascular endothelium is a fundamental step during inflammation (2). The cell adhesion molecules such as VCAM-1, ICAM-1 and E-selectin mediate these processes (3-6). Therefore it is very crucial question whether purpurogallin could inhibit the adhesion and migration of monocytes to LPS-mediated HUVECs. The result presented in Fig. 3 demonstrated that purpurogallin effectively inhibited the adhesion (A, B) and migration (C, D) of monocytes to the LPS-stimulated endothelial cells. And purpurogallin alone did not change the adhesion and TEM of HUVECs (Fig. 3).

To exclude the possibility that the inhibition of hyper-permeability, adhesion and migration by LPS were due to the cytotoxicity caused by purpurogallin, cellular viability assays were performed in HUVECs and THP-1 cells treated with pur-

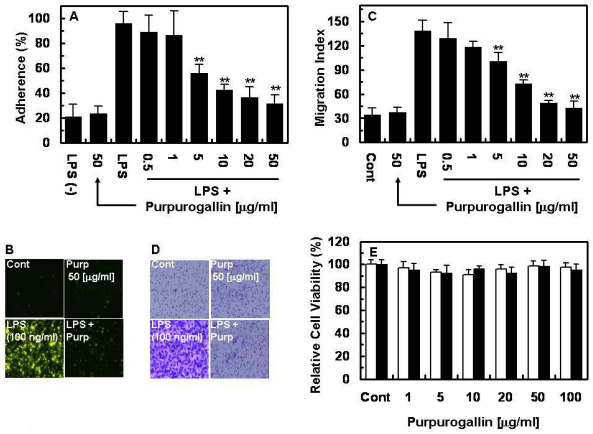

Fig. 3. Effect of purpurogallin on LPS-mediated monocytes adhesion and TEM. (A) LPS-mediated (100 ng/ml) adherence of monocytes to HUVEC monolayers was analyzed after treating HUVECs with purpurogallin as described under "Experimental Procedures". (B) Pictures of monocyte adhesion to HUVECs. (C) LPS-mediated migration of monocytes through HUVEC monolayers was analyzed after treating HUVECs with purpurogallin as described under "Experimental Procedures". (D) Pictures of TEM. (E) Effect of purpurogallin on the cellular viability in HUVECs (□) or THP-1 cells (■) was measured by MTT assay as described under "Materials and methods". All results are shown as means ± SD of five different experiments. All results are shown as the means ± SD of different three experiments. *P < 0.05 and **P < 0.01 as compared to LPS.

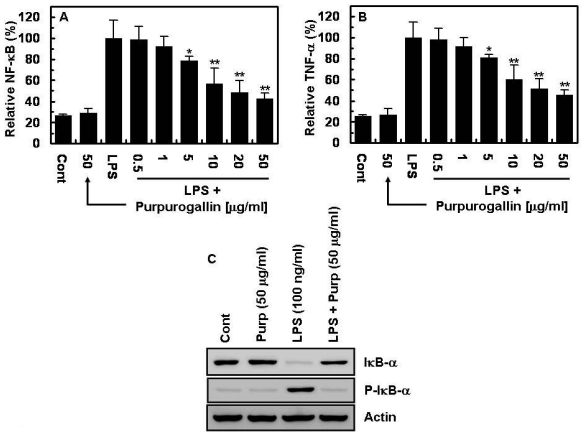

Fig. 4. Effect of purpurogallin on LPS-mediated NF-κB expression and TNF-α production. LPS-mediated (100 ng/ml) mediated NF-κB activation (A) or TNF-α production (B) in HUVECs was analyzed after treating cells with indicated concentrations of purpurogallin as described under "Experimental Procedures". (C) Inhibitory effect of purpurogallin on LPS-induced IkB-α degradation. Cells were pretreated with purpurogallin and then stimulated with or without LPS. Cytosolic proteins were prepared and analyzed by Western blot. Actin is used as a loading control. All results are shown as means ± SD of five different experiments. *P < 0.05 and **P < 0.01 as compared to LPS.

purogallin for 24 h. At the concentrations used (upto 100 μg/ml), purpurogallin did not effect cell viability (Fig. 3E).

Effect of purpurogallin on LPS-mediated NF-κB activation and TNF-α production

It is well known that activation of NF-κB is necessary for the pro-inflammatory responses and the two most important factors that provide inflammatory signals to endothelial cells are NF-κB and pro-inflammatory cytokine TNF-α (21-23). Thus, it was tested determine whether pretreatment of purpurogallin could inhibit LPS-mediated NF-κB activation and TNF-α production in HUVECs, and data showed that the levels of pro-inflammatory cytokine TNF-α and activation of NF-κB were increased by LPS and this increase was significantly decreased by purpurogallin (Fig. 4A, B). Also, since it is known that LPS activates NF-κB via IκB phosphorylation and degradation, followed by a p65 translocation (24, 25), the effect of purpurogallin on this pathway was looked at. Fig. 4C showed that LPS treatment caused a large increase in the phosphor-IκB-α. However, when treated with purpurogallin, the pathway is inhibited and phosphorylation of IκB-α was affected. Therefore, these results indicate that purpurogallin could regulate two most important signals which induce pro-inflammatory responses in human endothelial cells.

DISCUSSION

Purpurogallin is a phenolic compound extracted from various nutgalls as aglycone of several glycosides and is prepared commercially by the oxidation of pyrogall (12). It protects ventricular myocytes and hepatocytes against oxyradicals produced by xanthine oxidase and hypoxanthine (16, 26). However, the effects of purpurogallin on the modulation of endothelial barrier integrity are not studied yet. The aim of this study was to investigate whether purpurogallin could modulate the vascular permeability and affects the expression of VCAM-1, ICAM-1 and E-selectin involved in the multistep process of monocyte adhesion and migration through endothelial cells. LPS is an inflammatory mediator that elevates vascular permeability, expression of CAMs and leads to edema formation in several inflammations (27).

The endothelium lining the vasculature forms the size-selective barrier that controls the exchange of macromolecules and fluid between the blood and interstitial tissue (8, 10, 28). Disruption of endothelial barrier function leads to protein-rich tissue edema (8, 10, 28). Therefore, plasma leakage is a characteristic feature of inflammatory responses and endothelial barrier integrity allows the cells to from tight barrier between the vessel lumen and the stroma. Based on our results that purpurogallin attenuated LPS induced endothelial permeability, we conclude that purpurogallin has barrier protective activity.

The inflammatory response includes the transcriptional acti-

vation of several pro-inflammatory genes, which leads to the release of pro-inflammatory cytokines, chemokines, and cell adhesion molecules (2, 5). In the inflammatory process one of the earliest events and is the adhesion of circulating monocytes to intact endothelial cells (1, 2, 7). It occurs through a multistep process in which leukocytes interact with the endothelium (1, 7). This process involves sequential capture on, rolling along and firm adhesion to the microvascular endothelium, followed by transmigration through the vessel wall and further migration in extravascular tissue (29). All the steps in the recruitment cascade are orchestrated by cell adhesion molecules (CAMs) on both leukocytes and endothelial cells, and different subsets of CAMs are responsible for the different steps in extravasation (2-6). Local accumulation of leukocytes in the vascular wall includes initial marginalization and rolling of the leukocytes along the endothelium, a process mediated by the selectins, then attachment to endothelial cells and transmigration into the intimal spaces, a process mediated by the adhesive molecules (VCAM and ICAM) expressed by activated endothelium (2). Noting that purpurogallin inhibited expression of VCAM-1, ICAM-1 and E-selectin which mediated the monocyte firm adhesion and transendothelial migration and it also inhibited monocytes adhesion toward endothelial cells and migration across endothelial cells, we conclude that the inhibitory functions of purpurogallin in the function of key molecules in the multistep recruitment cascade presents promising strategies for therapeutic intervention in inflammatory disorders.

Evidence suggests that pro-inflammatory cytokine TNF-α and NF-κB are involved in various signal transduction pathways of the expression of CAMs, mediation of adhesion, migration and endothelial permeability (21-23). Therefore, in this study we further tested the effects of purpurogallin on the production of pro-inflammatory cytokine TNF-α and NF-κB to gain further insight into the underlying mechanism of inhibitory effects of purpurogallin on the inhibition of adhesion molecules and endothelial permeability. Data showed that purpurogallin effectively suppressed the LPS-induced pro-inflammatory cytokine TNF-α expression and NF-κB activation and indicated that purpurogallin could modulate the anti-inflammatory effects such as inhibition of vascular permeability, monocyte adhesion, migration and expression of CAMs by inhibiting two important molecules. The limitation of this study was the inability to determine the *in vivo* effects of purpurogallin on vascular inflammatory disease model. Thus, additional work will be required to elucidate whether purpurogallin has beneficial effects on inflammatory disease models *in vivo*.

In conclusion, the results in this study showed that purpurogallin could inhibit LPS-mediated inflammatory responses by increasing barrier integrity, inhibiting CAMs expression, blocking monocytes adhesion and migration. And purpurogallin could inhibit LPS mediated NF-κB activation and pro-inflammatory cytokine TNF-α production which are the most important inflammatory mediator. Therefore purpurogallin might be used for the drug candidate for inflammatory diseases.

MATERIALS AND METHODS

Reagents

Bacterial lipopolysaccharide, evans blue, crystal violet, MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) and pyrogallol were purchased from Sigma (St. Louis, MO, USA). Vybrant DiD (used at 5 μM) was purchased from Invitogen (Carlsbad, CA, USA). Anti-(IκB-α) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibody against phospho- IκB-α (Ser32) was obtained from Cell Signaling Technology (Beverly, MA, USA).

Plant materials, extraction, isolation of purpurogallin

Please see supplementary documents.

Cell culture

Primary HUVECs were obtained from Cambrex Bio Science (Charles City, IA, USA) and maintained as described before (30). Briefly, the cells were cultured to confluency at 37°C and 5% CO_2 in EBM-2 basal media supplemented with growth supplements (Cambrex Bio Science). Human monocyte cell line, THP-1 cells, was maintained at a density of 2×10^5 to 1×10^6 cells/ml in RPMI 1,640 with L-glutamine and 10% heat-inactivated FBS supplemented with 2-mercaptoethanol (55 μM), and antibiotics (penicillin G and streptomycin).

Cell viability assay

MTT was used as an indicator of cell viability. The cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and then treated with purpurogallin. After 48 h incubation, cells were rewashed and 100 μl of MTT (1 mg/ml) was added and incubated for 4 h. Finally, DMSO (150 μl) was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the OD at 540 nm using an microplate reader (Tecan Austria GmbH, Austria). The data were expressed as mean from at least three independent.

Permeability assay

Permeability was measured by the flux of Evans blue-bound albumin across functional HUVEC monolayers as previously described (30). Briefly, HUVECs were plated in transwell for 3 days. The confluent monolayers were incubated with purpurogallin for 6 h followed by LPS (100 ng/ml) for 4 h. Inserts were washed with PBS before adding 0.5 ml Evans blue (0.67 mg/ml) diluted in growth medium containing BSA. Fresh growth medium was added to the lower chamber, and the medium in the upper chamber was replaced with Evans blue/BSA. After 10 min the optical density at 650 nm was measured in the lower chamber.

Cell-Cell adhesion assay

THP-1 cell adherence to endothelial cells was evaluated by fluorescent labeling of THP-1 as described (31). Briefly, THP-1 cells were labeled with Vybrant DiD for 20 min at 37°C in phenol red-free RPMI containing 5% FBS. Following twice washing of THP-1 cells (1.5×10^6/ml, 200 μl/well), they were resuspended in adhesion medium (RPMI containing 2% FBS and 20 mM HEPES) and added to confluent monolayers of HUVECs in 96-well plates which were treated for 6 h with purpurogallin followed by LPS (100 ng/ml for 4 h). Non-adherent THP-1 cells were washed off and the fluorescence of the adherent cells were measured. The percentage of adherent THP-1 cells was calculated by the formula: % adherence = (adherent signal/total signal) × 100 as described (31).

Migration assay

Migration assays were performed in transwell plates. HUVECs were cultured for three days to obtain confluent endothelial monolayers. Before adding THP-1 to the upper compartment, the cell monolayers were treated for 6 h with purpurogallin followed by LPS for 4 h and THP-1 cells were added to the upper compartment. After transwell plates were incubated for 2 h, THP-1 cells on the lower side of the filter were fixed with 8% glutaraldehyde and stained with 0.25% crystal violet in 20% methanol (w/v). Each experiment was repeated in duplicate wells and, within each well, nine randomly selected microscopic high power fields (HPF, ×200) were counted and expressed as migration index.

Expression of cell adhesion molecules (CAMs)

The expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1) and E-selectin on HUVECs was determined by a whole-cell ELISA as described (32). Briefly, confluent monolayers of HUVECs were treated with purpurogallin for 6 h followed by LPS (100 ng/ml) for 4 h. After washing, mouse anti-human monoclonal antibodies (VCAM-1, ICAM-1, E-selectin, Temecula, CA, USA, 1 : 50 each) were added. After washing, peroxidase-conjugated anti-mouse IgG antibodies (Sigma, Saint Louis, MO) were added for 1 h. Colorimetric analysis was performed by measuring absorbance at 490 nm.

RNA isolation and real time-PCR

HUVECs were grown in six-well plates and were incubated with purpurogallin for 6 h followed by LPS (100 ng/ml) for 4 h. RNA was isolated by using TRI-Reagent (Invitrogen) according to the manufacturer's suggested protocol. An aliquot (5 μg) of extract RNA was reverse transcribed into first-strand cDNA with a PX2 Thermal Cycler (Thermo Scientific) using 200 U/μl M-MLV reverse-transcriptase (Invitrogen) and 0.5 mg/μl of oligo(dT)-adapter primer (Invitrogen) in a 20-μl reaction mixture. Real-time PCR for VCAM-1, ICAM-1, E-selectin, and α-actin was carried out with a Mini Opticon Real-Time PCR System (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA). The primers had the following sequences: for VCAM-1, sense 5′-TGGAGGAAATGGGCATAAAG-3′ and antisense 5′-CAGGATTTTGGGAGCTGGTA-3′; for ICAM-1, sense 5′-CGAAGGTTCTTCTGAGC-3′ and antisense 5′-GTCTGCTGAGACCCCTCTTG-3′; for E-selectin, sense 5′-TCTGGACCTTTCCAAAATGG-3′ and antisense 5′-TGCAAGCTAAAGCCCTCATT-3′; and for α-actin, sense 5′-TGAGAGGGAAATCGTGCGTG-3′ and antisense 5′-TTGCTGATCCACATCTGCTGG-3′. The PCR settings were as follows: initial denaturation at 95°C was followed by 35 cycles of amplification for 15 s at 95°C and 20 s at 60°C, with subsequent melting curve analysis, increasing the temperature from 72 to 98°C. Quantification of gene expression was calculated relative to α-actin.

ELISA for NF-κB and TNF-α

The concentrations of NF-κB (from Cell Signaling Technology, Danvers, MA, USA) in nuclear lysates and TNF-α (both from R&D Systems, Minneapolis, MN, USA) in cell culture supernatants were determined by ELISA system according to the manufacturer's protocol. The values were measured using an ELISA reader (Tecan, Austria GmbH, Austria)

Western blot analysis

Cells were washed twice in ice-cold phosphate-buffered saline (PBS), and then solubilized in buffer containing (mM): Tris-HCl 20 (pH 7.5), EGTA 0.5, EDTA 2, dithiothreitol 2, p-methylsulphonyl fluoride 0.5, and 10 μg/m leupeptin. Samples of equal amounts of protein (100 μg) were subjected to SDS-PAGE on 10% polyacrylamide gels, then transferred onto a nitrocellulose membrane, which was then incubated in TBST buffer (150 mM NaCl, 20 mM Tris-HCl, 0.02% Tween 20, pH 7.4) containing 1% nonfat milk. The IκB-α band was visualized by immunoblotting with a specific antibody. Protein bands were detected by SUPEX Kit (Neuronex, South Korea) according to the manufacturer's instructions.

Statistical analysis

Data are expressed as the means ± standard deviations of at least three independent experiments. Statistical significance between 2 groups was determined by Student's *t*-test. The significance level was set at $P < 0.05$.

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