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Tetramethylpyrazine suppresses interleukin-8 expression in LPS-stimulated human umbilical vein endothelial cell by blocking ERK, p38 and nulear factor-κB signaling pathways

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ABSTRACT

Aim of the study: To determine the anti-inflammatory effects of Tetramethylpyrazine (TMP) and to investigate the inhibitory effect of TMP on IL-8 production in human umbilical vein endothelial cells (HUVECs) induced by LPS might be mediated by inhibiting p38, ERK and NF-κB signaling pathways.
Materials and methods: HUVECs were treated with or without TMP for 24 h before exposure to LPS for 4 h. IL-8 gene and protein expressions were determined by RT-PCR and ELISA. Cell viability was determined by methyl thiazoyltetrazolium (MTT) assay. Phosphorylation of ERK1/2 and p38 were examined by western blotting.
Results: TMP inhibits LPS-induced IL-8 production in HUVECs at both the protein and mRNA levels, suggesting that TMP has an antiinflammatory effect on endothelial cells. TMP also inhibited U937 monocyte adhesion to HUVECs stimulated by LPS. LPS-induced phosphorylation of ERK1/2 and p38 were inhibited by TMP. The inhibitory effect of TMP on NF-κB (p65) activity was mediated by blocking the consequent translocation of p65 into the nucleus.
Conclusions: The inhibitory effect of TMP on the LPS–induced IL-8 production is mediated by the NF-κB–dependent pathway, and TMP also separately affects the ERK and p38 MAPK pathway. TMP may be beneficial in the treatment of cardiovascular disorders such as atherosclerosis.

1. Introduction

Vascular inflammatory process has been suggested to play a key role in the initiation and progression of atherosclerosis (Ross, 1999). Several epidemiological studies have postulated an association between infectious agents and chronic cardiovascular disease such as atherosclerosis (Fong, 2000; Ostos et al., 2002). Lipopolysaccharide (LPS), a glycolipid that constitutes the major portion of the outermost membrane of gram-negative bacteria (Raetz, 1990), is infectious agent. Exposure of endothelial cells to LPS results in a complex activation of endothelial cells in vivo and in vitro. This has been demonstrated in animal models where the administration of LPS is found to increase the atherosclerotic lesion size (Ostos et al., 2002). Endothelial cells are a crucial source of inflammatory cytokines contributing to atherosclerosis. Endothelial cells respond to LPS in a TLR4-dependent manner leading to the subsequent release of inflammatory cytokines and MCP-1 and enhanced expression of adhesion molecules including ICAM and VCAM (Zeuke et al., 2002). An important endothelial cell-derived cytokine, the CXC-chemokine IL-8 (Baggiolini et al., 1997) is a pro-inflammatory cytokine that might have atherogenic properties through its multiple actions. Those actions include recruitment of neutrophils and T lymphocytes into the subendothelial space, monocyte adhesion to endothelium (Matsushima et al., 1992; Gerszten et al., 1999) and migration of vascular smooth muscle cells (Yue et al., 1994; Temaru et al., 1997). Macrophage-derived human foam cells contain high amounts of IL-8 (Apostolopoulos et al., 1996; Wang et al., 1996), which can also increase the instability of atherosclerotic plaque through inhibition of tissue inhibitor of metalloproteinase expression (Moreau et al., 1999). The production of some LPS-responsive cytokines are commonly mediated by transcription factors such as NF-κB and AP-1. Indeed, the binding sites for NF-κB and AP-1 are found in the promoter region of the IL-8 gene (Yasumoto et al., 1992). Of the signaling pathways activated by LPS, the MAP kinase pathways have both been shown to be directly involved in the production of cytokines (Adams et al., 2001). In particular, the central role of p38 MAP kinase-dependent signaling in LPS-stimulated endothelium has been highlighted in

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recent studies (Anand et al., 2008). Elucidation of how LPS signals through cell-surface receptors to induce chemokine production is of prime importance.

Tetramethylpyrazine (TMP) is a biologically active ingredient first extracted from the Chinese medicinal plant *Ligusticum wallichil franchat*. TMP has been synthesized and used widely in oriental medicine to effectively treat several cardiovascular complications, including angina pectoris, cerebrovascular and thrombotic vascular diseases (Guo et al., 1983; Dai and Bache, 1985; Zeng et al., 1998), due to its biological activities including vasodilation (Dai and Bache, 1985) and antiplatelet aggregation (Liu and Sylvester, 1990). In recent years, several pharmacological actions produced by TMP have been performed. The oral administration of TMP significantly inhibited the hindpaw edema induced by carrageen in rats (Ozaki, 1991). TMP can cause coronary vasodilation in the dog (Dai and Bache, 1985; Lin et al., 1993) and pig (Shan et al., 2003). Moreover, TMP possesses antiplatelet activity in rat thrombotic model (Liu and Sylvester, 1990, 1994), which can result in improvement of microcirculation by attenuation of microembolism. These data suggest that TMP has antiinflammatory properties and that TMP might regulate inflammatory responses at atherosclerotic lesions. However, no data elucidate the effects of TMP on synthesis of IL-8 in human endothelial cells.

In the present study, we questioned whether TMP can inhibit the IL-8 production that we observed upon LPS stimulation. Some observations that MAPK pathways (Hippenstiel et al., 2000; Fan et al., 2007) affect IL-8 expression, prompted us to study the effects of TMP via specific MAPK on the stimulation of IL-8 production by LPS in human umbilical vein endothelial cells.

2. Materials and methods

2.1. Chemicals and reagents

2′,7′-Bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) Nuclear and Cytoplasmic Protein Extraction Kit, and BeyoECL Plus Western blotting detection reagent were obtained from Beyotime Institute of Biotechnology (Jiangsu, China). Dulbecco's modified Eagle's medium F12 (DMEM-F12), fetal bovine serum (FBS), antibiotics (penicillin/streptomycin), glutamine and collagenase were supplied by Gibco-BRL (Rockville, MD, USA). Trizol was obtained from Sangon Biological Engineering Technology & Services (Shanghai, China). SB203580, PD98059 were obtained from Calbiochem (San Diego, CA, USA). Anti-NF-κB p65 and anti-p-ERK 1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p-p38 antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Human IL-8 ELISA Assay kits were purchased from Neobioscience Technology Company (Beijing, China). All other chemicals, including LPS, TMP, endothelial cell growth supplements (ECGS) and heparin, were supplied by Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture and treatment

HUVECs were isolated from the vein of normal human umbilical cord as described previously (Jaffe et al., 1973). The cells at passages 5–8 were cultured in DMEM-F12 supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 5 U/ml heparin, 100 IU/ml penicillin, 10 μg/ml streptomycin and 50 μg/ml ECGS. Cells were cultured in 100 mm dishes and grown in a humidified 5% CO_2 incubator. HUVECs were plated at a density of 1×10^7 cells per 100 mm dish. When the cells reached sub-confluence, they were pretreated for 24 h with culture medium containing different concentrations of TMP (30, 60 and 120 μg/ml) that were tested in the experiments. Subsequently, after washing twice with phosphate buffered saline (PBS, pH 7.4), the cells were exposed to LPS (100 ng/ml) diluted in culture medium for 4 h at 37 °C.

U937 human monocyte was obtained from Shanghai Institute of Cell Biology (Shanghai, China) and grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 25 mM HEPES, 25 mM $NaHCO_3$, 100 IU/ml penicillin, and 10 μg/ml streptomycin.

2.3. Cell viability assay

HUVECs were seeded at density of 7.5×10^3 cells/well in 96-well plates and the cell viability was determined by methyl thiazoyltetrazolium (MTT) assay (Mosmann, 1983). Briefly, at the indicated time after the treatment with or without TMP before exposure to LPS for 4 h, the culture supernatant was removed. The cells were washed with PBS and incubated with 100 μl of MTT (1 mg/ml) in culture medium at 37 °C for 4 h. Then culture medium with dye was removed and 150 μl of DMSO per well was added for formazan solubilization. The absorbance of converted dye was measured at a wavelength of 490 nm using a Sunrise Remote Microplate Reader (Grodig, Austria). The viability of HUVECs in each well was presented as percentage of control (0.1% DMSO medium) cells.

2.4. Measurement of IL-8 in the media

Supernatants of HUVECs were collected after treatment with TMP and exposure to LPS, centrifuged at 13,000 × *g* to remove cellular debris, and were analyzed for IL-8 synthesis using a standard enzymelinked immunosorbent assay (ELISA) according to the manufacturer's instructions. The absorbance was measured at 450 nm (ref. 570 nm). The concentrations of IL-8 in the experimental samples were extrapolated from a standard curve.

2.5. Preparations of RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from 1×10^6 cells according to the manufacture's instructions (Chomczynski and Sacchi, 1987). Briefly, Trizol was added to the plates to lyses the cells, and then the cells were transferred to a microcentrifuge tube. Chloroform was added and total RNA was collected in the aqueous phase after centrifugation. Finally, RNA was precipitated by isopropyl alcohol, and then redissolved in diethyl pyrocarbonate (DEPC)-treated water. The OD_{260} and $OD_{260/280}$ values were measured with a spectrophotometer to determine the RNA concentrations. Reverse transcription was performed at 42 °C for 60 min and followed by incubation at 95 °C for 5 min. The reaction mixture (50 μl of total volume) consisted of 5 μg of total RNA, 5 mM of $MgCl_2$, 10 mM of Tris–HCl, pH 9.0, 50 mM of KCl, 0.1% Triton X-100, 1 mM of dNTP Mixtures, 1 units/μl recombinant RNasin ribonuclese inhibitor, 15 U/μg of avian myeloblastosis virus (AMV) reverse transcriptase and 0.5 μg of oligo(dT) 18 primer. DNA samples were analyzed for the specific cDNA of IL-8 and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) by PCR amplification using specific primers. The primers used for PCR were as follows: for IL-8 (forward: 5′-TCT CAG CCC TCT TCA AAA ACT TCT C-3′; reverse: 5′-ATG ACT TCC AAG CTG GCC GTG GCT-3′) (Devaraj et al., 2004), and GAPDH (forward: 5′-CTC TCT GCT CCT CCT GTT CGA CAG-3′; reverse: 5′-GTG GAA TCA TAT TGG AAC ATG T-3′). 5 μl of cDNA was added to 50 μl of PCR mixture containing 33.5 μl of H_2O, 1 μl of 5′ primer (10 pM), 1 μl of 3′ primers, 4 μl of dNTPs (2.5 mM), 5 μl of 10× PCR buffer, 0.5 μl of Taq DNA polymerase (5 U/μl). The following conditions were used for PCR amplification: 30 cycles × 30 s 94 °C, 30 s at 60 °C, and 1 min at 72 °C for IL-8; 35 cycles × 30 s 94 °C, 30 s at 54 °C, and 1 min at 72 °C for GAPDH. 5 μl of PCR product from each sample were electrophoresed on a 1.2% agarose gel containing 0.1 μg/ml dye (Gold view, SBS Genetech, Beijing, China). Gels were visualized

and photographed by a Gel-Doc image analyzer (Bio-Rad, Hercules, CA, USA). The housekeeping gene GAPDH was used for normalization. The ratios of the emissions incorporated into the PCR products of the tested gene to the GAPDH products were calculated to evaluate relative changes in the mRNA expression levels of the tested genes.

2.6. Preparation of nuclear extracts and Western blot analysis

HUVECs were washed twice with ice-cold PBS and scraped in 1 ml of the same buffer. After centrifugation at 10,000 × *g*, the cell pellet was suspended in ice-cold hypotonic lysis buffer (10 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 0.2 mM KCl 0.2 mM phenylmethylsulphonylfluoride, 0.5 mM dithiothreitol), vortexed for 10 s and then centrifuged at 10,000 × *g* for 5 min. The packed cells were suspended in ice-cold hypotonic lysis buffer in the presence of 50 μl of 10% Nonidet P-40 and then kept on ice for 25 min. The nuclear fraction was precipitated by centrifugation at 10,000 × *g* for 15 min. The supernatants, corresponding to the cytosolic fraction, were transferred to fresh tubes and assayed for protein content by the Bradford method (Bradford, 1976). The nuclei pellet was resuspened in 50–100 μl of low salt extraction buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 20 mM KCl 0.2 mM, EDTA 0.2 mM phenylmethylsulphonylfluoride, 0.5 mM dithothreitol) and added to an equal volume of high salt extraction buffer (20 mM HEPES pH 7.9, 1.5 mM $MgCl_2$, 25% glycerol, 80 mM KCl, 0.2 mM EDTA, 0.2 mM henylmethylsulphonylfluoride, 0.5 mM dithothreitol) in a dropwise fashion, and then incubated under continuous shaking at 4 °C for 45 min. The sample was centrifuged for 20 min at 10,000 × *g*. The nuclear extract was aliquoted and store at −80 °C. Protein samples (50 μg) were electrophoretically fractionated with a discontinuous system consisting of 10% polyacrylamide resolving gels and 5% stacking gels, and then transferred to nitrocellulose membranes (Amersham, Buckinghamshire, England) at 20 V and 100 mA (current constant) overnight. The membranes were washed, blocked, and then incubated with primary antibodies (1:2000 dilution) against NF-κB p65 and β-actin proteins or phospho-specific antibodies recognizing ERK(p44/42) and p38, respectively. The bound horseradish peroxidase-conjugated secondary antibody was detected by an enhanced chemiluminescence procedure. Protein expression levels were determined by analyzing the signals captured on the nitrocellulose membranes using a Chemi-doc image analyzer (Bio-Rad, Hercules, CA, USA).

2.7. Adhesion of U937 mononuclear cells to HUVEC monolayers

The cell adhesion assay was modified as described (Yang et al., 2004). Briefly, U937 cells were labeled with BCECF-AM (10 mg/ml) for 30 min at 37 °C, washed, and resuspended in serum free media. HUVECs were cultured and incubated with reagents on a 24-well culture plate, then cocultured with BCECF-AM-labeled U937 cells for 30 min at 37 °C. Nonadhering U937 cells were removed by gentle aspiration, and wells were washed with PBS. Each adhesion assay was performed 4 h after treatment with LPS (100 ng/ml). The effect of TMP on U937 adhesion was assessed by preincubation with HUVECs for 24 h before adding LPS for 4 h. U937 cells bound to HUVECs were lysed with 0.1% SDS and fluorescence was measured on a spectrofluorometer at 485 nm excitation and 530 nm emission wavelength.

2.8. Statistical analyses

The results were expressed as mean ± S.E.M. of at least three independent experiments performed in triplicate. Treatment groups were compared using one-way analysis of variance (ANOVA) and the Newman–Keuls test was used to locate any significant differences identified in the ANOVA. $P < 0.05$ or $P < 0.01$ was accepted as significant.

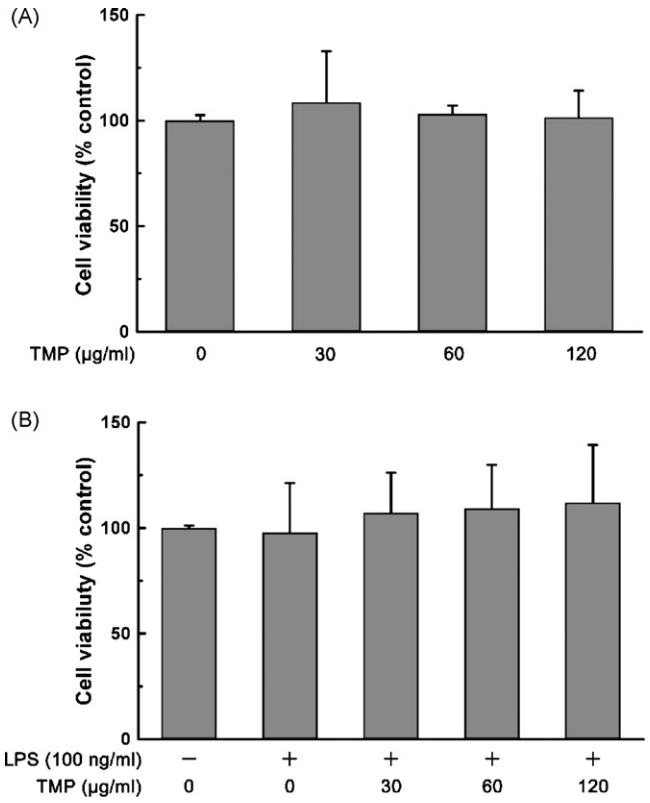

Fig. 1. Effect of TMP on cell viability measured by MTT assay in human umbilical vein endothelial cells (HUVECs). (A) Cells were treated with 0.1% DMSO or TMP (30, 60 and 120 μg/ml) for 24 h. (B) Cells were pre-treated with TMP (30, 60 and 120 μg/ml) for 24 h and then exposed to 100 ng/ml of LPS for 4 h viabilities were determined by MTT assay. Values are means ± S.E.M. of three independent experiments.

3. Results

3.1. TMP preconditioning reduced LPS-induced HUVECs injury

As cell viability is the most direct indicator to show the cell state, the effects of TMP on viability of LPS-induced HUVECs were performed. The results in Fig. 1B shows that, the survival rate of HUVECs was about 95.9 ± 3.5% after exposure to 100 ng/ml of LPS for 4 h. However, pre-incubation of HUVECs with various concentrations of TMP (30, 60 and 120 μg/ml) for 24 h markedly increased the viability of LPS-induced HUVECs. In addition, no difference was seen in cell viability between cells treated with TMP (30–120 μg/ml) alone and controls (Fig. 1A). Apparently, TMP were effective for the protection of HUVECs against LPS-induced injury. It is suggested that TMP underlie the concentration (30–120 μg/ml) has no cytotoxicity in HUVECs.

3.2. Effect of TMP on IL-8 production induced by LPS in HUVECs

To investigate whether TMP could exert an anti-inflammatory effect on endothelial cells, the expression of IL-8, one of several proinflammatory factors expressed on endothelial cells in response to several inflammatory stimuli, was determined after stimulation of HUVECs with LPS. The cells were pretreated with various concentrations of TMP (30, 60 and 120 μg/ml) for 24 h and then treated with LPS (100 ng/ml) for 4 h. The secretion of IL-8 into culture medium induced by LPS were significantly reduced (69.4–41.6%, $P < 0.05$) as the concentration of TMP increased (Fig. 2A). Enhanced

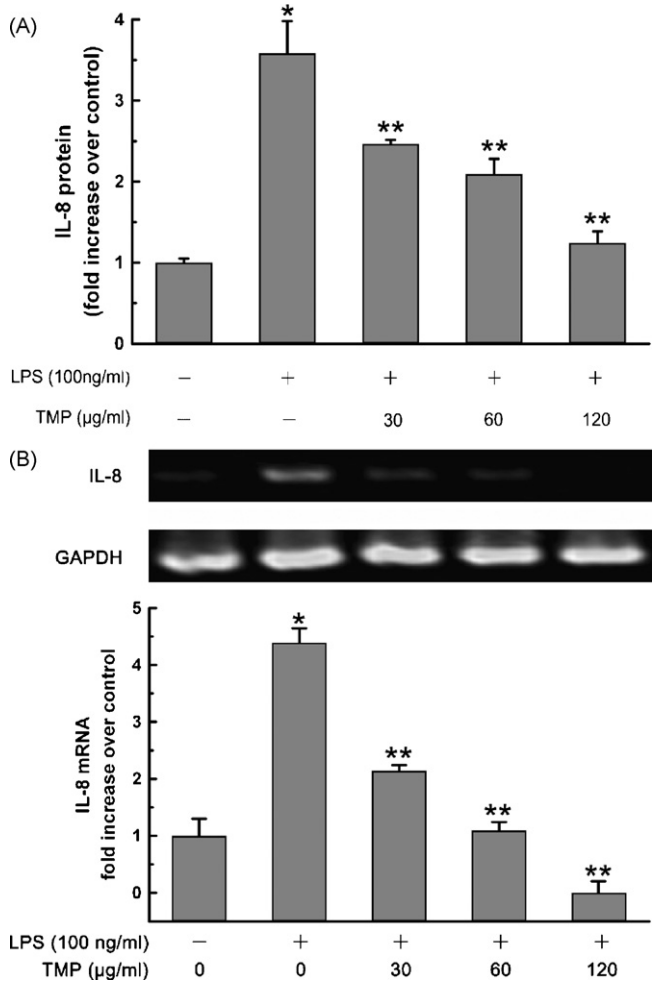

Fig. 2. Effect of TMP on IL-8 production and IL-8 mRNA expression induced by LPS in HUVECs. (A) Effect of TMP on IL-8 production. HUVECs were cultured in medium in 24-well plates without or with the indicated concentration of TMP (30–120 μg/ml) for 24 h. The cells were then stimulated with LPS (100 ng/ml) for 4 h. The protein levels of IL-8 in the medium were measured by ELISA as described in Section 2. (B) LPS-induced IL-8 mRNA expression in HUVECs treated with TMP. HUVECs were cultured in medium in 25 cm^2 culture flasks without or with the indicated concentration of TMP (30–120 μg/ml) for 24 h. After stimulation with LPS (100 ng/ml) for 4 h, total RNA was extracted and cDNA was synthesized from total RNA. Specific cDNA for IL-8 and glyceraldehyde-3-phosphate dehydrogenase were amplified by reverse transcription-PCR. The data are representative of three experiments (means ± S.E.M.). $^{*}P < 0.05$, compared to the vehicle-treated control group. $^{**}P < 0.05$, compared to the LPS-treated group.

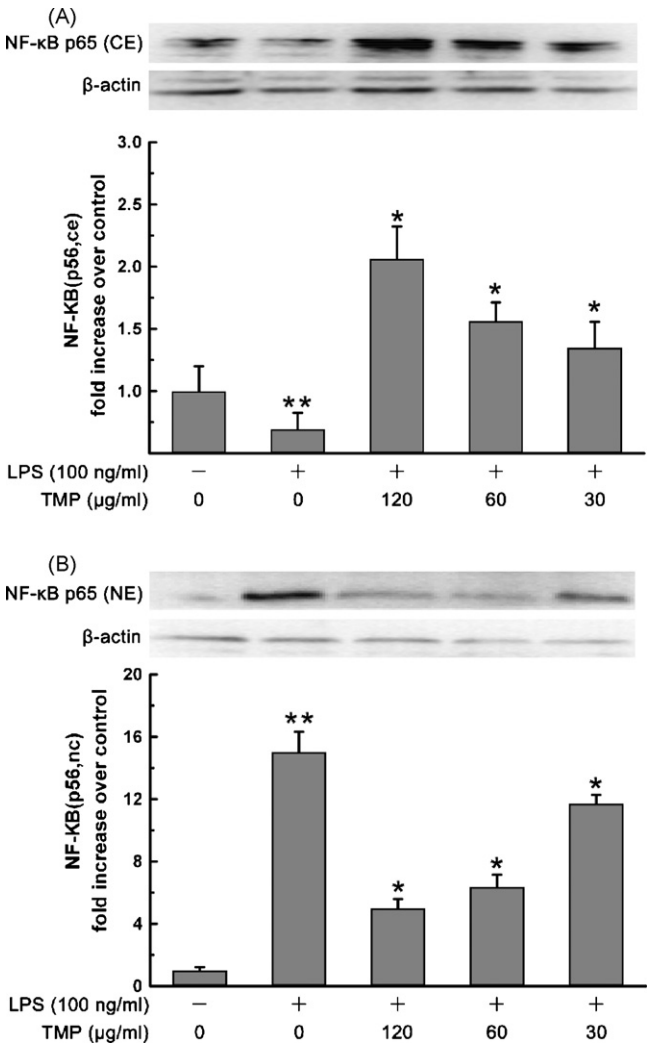

Fig. 3. Effect of TMP on LPS-induced NF-κB activation in HUVECs. HUVECs were cultured in medium in 25 cm^2 culture flasks pretreated with TMP (30, 60 and 120 μg/ml) for 24 h and then treated with LPS (100 ng/ml) for 4 h. After treatment, nuclear and cytoplasmic fractions were analyzed for the detection of NF-κB translocation from the cytosol into the nucleus. The data are representative of three independent experiments (means ± S.E.M.). $^{**}P < 0.05$ compared with control and $^{*}P < 0.05$ compared with LPS alone.

expressions of the mRNA of IL-8 by LPS were reduced by TMP pretreatment in a statistically significant and dose-dependent manner (Fig. 2B).

3.3. Effect of TMP on NF-κB activation induced by LPS in HUVECs

To understand the mechanism of action of TMP for its improved bioactivity, we examined its effect on the NF-κB signaling pathway, which is suggested to be targeted by TMP (Sue et al., 2009). NF-κB is a well-known transcription factor involved in the progress of inflammation. TMP was examined to see whether or not it exerted an inhibitory effect on this progress. To monitor the activation status of NF-κB, we used a western blot approach to visualize the dynamic movement of the NF-κB p65 subunit between the cytoplasm and nucleus under various experimental conditions. To examine whether TMP has any effect on the NF-κB pathway, HUVECs were pretreated with TMP (30, 60 and 120 μg/ml) for 24 h before LPS (100 ng/ml) was added to cause predominant nuclear translocation of NF-κB. Strikingly, TMP pretreatment retained the NF-κB in the cytoplasm even when the amount of LPS used completely relocated the p65 subunit to the nucleus (Fig. 3A and B). There are 4-fold difference in blocking the NF-κB translocation activity between TMP (120 μg/ml) and LPS. It is likely that TMP exerted an antiinflammation in part through interfering with the NF-κB-mediated signaling.

3.4. Effect of TMP on phosphorylation of ERK, p38 MAPK induced by LPS in HUVECs

To clarify whether TMP affects LPS-induced MAPK activation, we examined the effects of various concentrations of TMP on LPS-induced ERK1/2 and p38 activation in HUVECs. The cells were pretreated with TMP (30–120 μg/ml) for 24 h before the addition of LPS (100 ng/ml) for 30 min for ERK1/2 activation and 10 min for p38 activation. LPS-induced phosphorylation of ERK1/2 (Fig. 4A) and p38 were inhibited by TMP (Fig. 4B). These findings suggest that phosphorylation of ERK1/2 and p38 are involved in IL-8 expression induced by LPS, and that these pathways can be regulated by TMP.

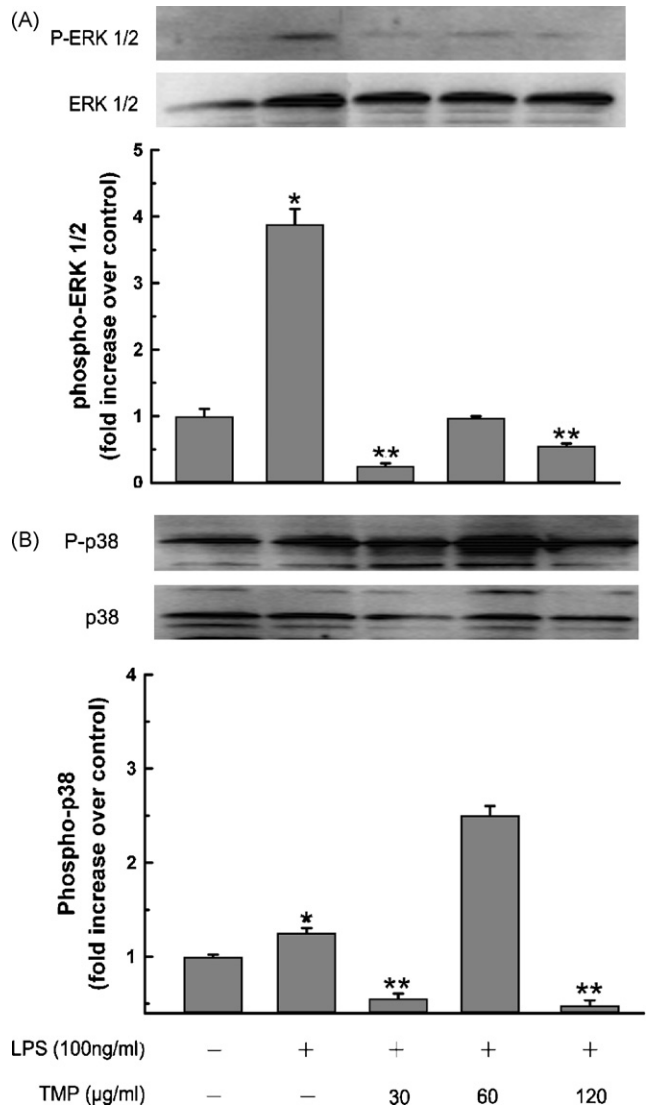

Fig. 4. Effect of TMP on MAPK phosphorylation induced by LPS in HUVECs. HUVECs were cultured in 25 cm^2 flasks without or with TMP (30–120 μg/ml) for 24 h respectively. Cells were then stimulated with LPS (100 ng/ml) for 10 min (p-p38) or 30 min (p-ERK1/2). The phosphorylation of p38 and ERK1/2 induced by LPS was analyzed by Western blotting as described in Section 2. The intensity of the band of each p-ERK1/2 (A), p-p38 MAPK (B) was corrected with that of total ERK and p38 MAPK respectively. Values are representative of three independent experiments (means ± S.E.M.).*$P < 0.05$ compared with control. **$P < 0.05$ compared with 100 ng/ml LPS.

3.5. Effect of ERK, p38 inhibitors on IL-8 protein expression induced by LPS in HUVECs

It has been suggested that activation of MAPKs might be involved in the NF-κB signaling pathways that induce IL-8 gene expression. We investigated the signal-transduction pathway mediating the LPS-induced increase in IL-8 expression. After LPS treatment, the IL-8 protein level decreased significantly after the addition of SB203580 (25 μM), a specific p38 inhibitor, and PD98059 (30 μM), a specific ERK inhibitor (Fig. 5). These results indicate that MAPKs regulate LPS-induced IL-8 expression, which means that p38 and ERK are involved in the LPS-induction of IL-8 protein production.

3.6. Effect of ERK, p38 inhibitors on the activation of NF-κB induced by LPS in HUVECs

To determine whether NF-κB activated by LPS is regulated by ERK and p38 MAPK, we investigated the effect of MAPK inhibitors

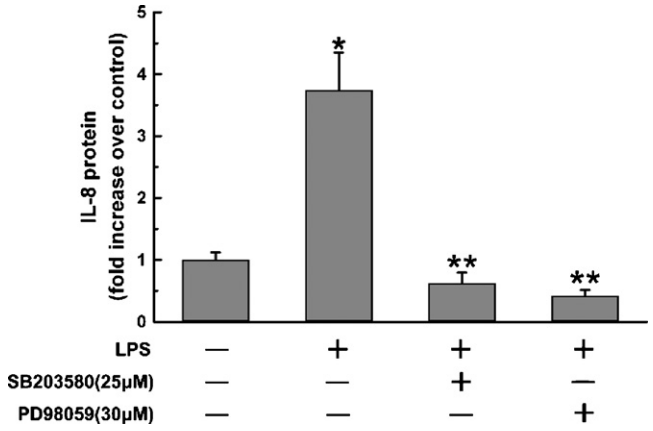

Fig. 5. Effect of ERK, p38 inhibitors (PD98059, SB203580) on IL-8 protein expression induced by LPS in HUVECs. HUVECs were cultured in the 25 cm^2 culture flasks, cells were then treated for 1 h with PD98059 (30 μM), and SB203580 (25 μM). After stimulation with LPS (100 ng/ml) for 4 h, the expression of IL-8 protein was assayed by ELISA method, as described in Section 2. The data are representative of three independent experiments (means ± S.E.M.). *$P < 0.01$ and **$P < 0.01$ compared with control (LPS alone).

PD98059 and SB203580 on the activity of NF-κB in HUVECs. Results shown that both PD98059 and SB203580 inhibited the activation of NF-κB by LPS significantly (Fig. 6). It is indicated that NF-κB may be an important downstream molecule of ERK and p38, and TMP attenuated NF-κB translocation possibly through inhibiting phosphorylation of ERK and p38 MAPK.

3.7. Effect of TMP on U937 cell adhesion in HUVECs

Mononuclear cell adhesion, stimulated by cytokines such as IL-8, plays a crucial role in vascular inflammation and atherosclerosis. Therefore, we investigated the effect of TMP on the adhesion of monocytes to HUVECs after stimulation with LPS. Adhesion of U937 cells to HUVECs increased four times after stimulation with LPS at 100 ng/ml for 4 h compared with unstimulated cells. In contrast, HUVECs pretreated with TMP for 24 h before stimulation with LPS showed significant reduction of adherent cells to HUVECs from fourfold to baseline (Fig. 7). Thus, TMP effectively inhibited the adhesion of monocytes to HUVECs after stimulation with LPS.

4. Discussion

In this study, we demonstrated for the first time to our knowledge that TMP inhibits LPS-induced IL-8 production in HUVECs at both the protein and mRNA levels, suggesting that TMP has an anti-inflammatory effect on endothelial cells. We also provide novel evidence that the mechanism of the inhibitory effect of TMP on the LPS-induced IL-8 production is mediated by the NF-κB-dependent pathway, and that TMP also separately affects the ERK and p38 MAPK pathway.

It is well-known that key pathogenic events in atherosclerosis can be described as inflammatory processes (Ross, 1999). IL-8, a member of the CXC chemokine family, has been shown to be expressed by plaque macrophages in humans and to play an crucial role in the pathogenesis of atherosclerosis (Apostolopoulos et al., 1996; Wang et al., 1996). It has been shown to be critical for chemotaxis and firm adhesion of monocytes to endothelial cells, a pivotal step in early stages of atherosclerosis (Gerszten et al., 1999). The IL-8 expression level was elevated in atherosclerotic lesions in carotid arteries (Simonini et al., 2000). Those data suggest that IL-8 has a key role in progression of atherosclerotic lesions. Therefore, the inhibitory effect of TMP on the IL-8 production in HUVECs might be an important effect of antiatherogenesis. In addition, LPS

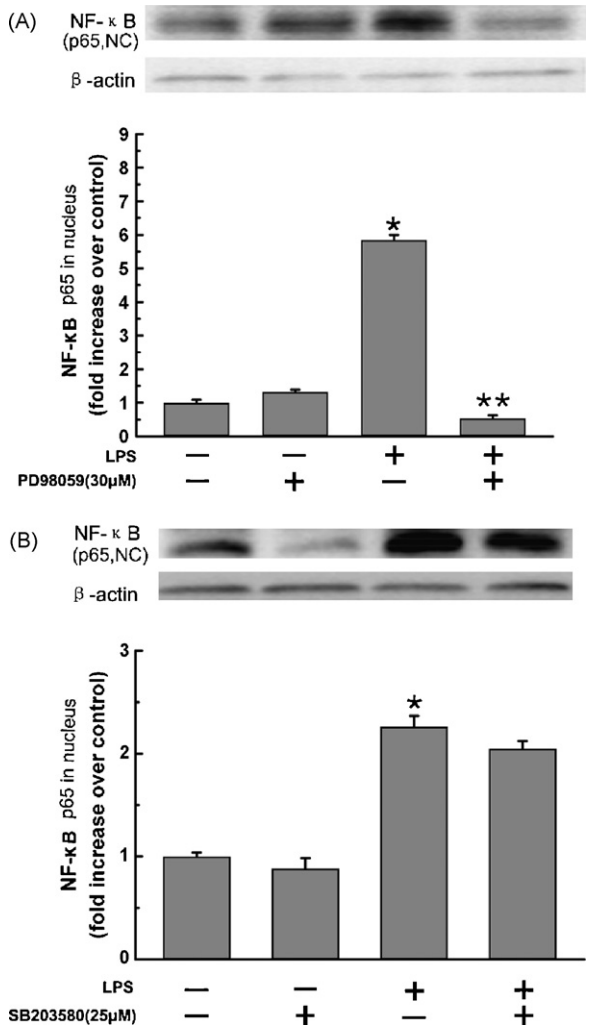

Fig. 6. Effect of ERK, p38 inhibitors on the activation of NF-κB induced by LPS in HUVECs. HUVECs were seeded into 25 cm^2 culture flasks and pretreated with inhibitors (PD98059, 30 μM; SB203580, 25 μM) for 1 h at 37 °C. The cells were then stimulated with LPS (100 ng/ml) for 4 h. The cells were lysed and analyzed by Western blotting as described in Section 2. (A) Effect of PD98059 on the activation of NF-κB induced by LPS in HUVECs. (B) Effect of SB203580 on the activation of NF-κB induced by LPS in HUVECs. Values are representative of three independent experiments (means ± S.E.M.). $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with control (LPS alone).

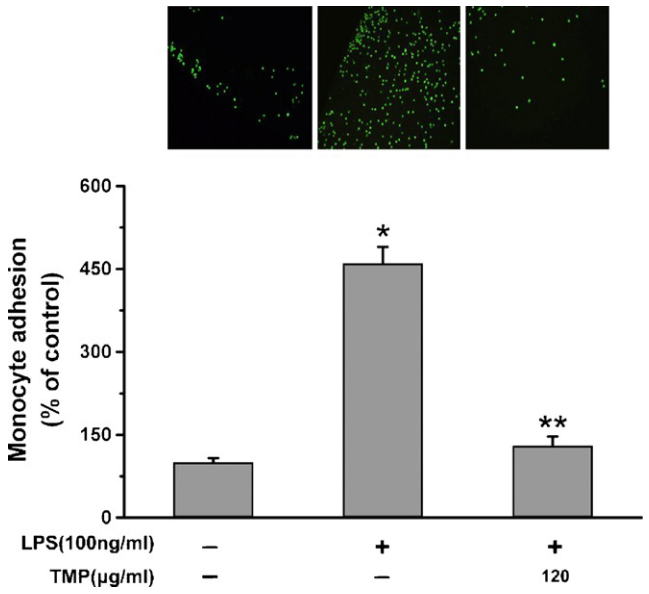

Fig. 7. Inhibition by TMP on binding of U937 monocytes to HUVECs. HUVECs were pretreated with or without 120 μg/ml of TMP for 24 h and exposured to LPS for 4 h. After co-incubation of fluorescent-labeled U937 monocytes with HUVEC for 30 min at 37 °C, monocyte adhesion is presented as a percentage of U937 cells bound to LPS-untreated cells (0.1% DMSO control). Data represent means ± S.E.M. of three separate experiments. (Significance compared with control, $^{*}P < 0.01$. Significance compared with LPS, $^{**}P < 0.01$.)

is putatively an important regulator of atherogenesis and thrombogenesis in vascular endothelial cells (Kisseleva et al., 2006). It is also known that LPS can induce IL-8 production strongly in endothelial cells. Taken together, we hypothesized that antiatherogenic effects of TMP are involved in IL-8 production induced by LPS. We found here that LPS caused transcriptional activation of NF-κB, which was inhibited by TMP in a concentration-dependent manner. Further studies revealed that the inhibitory effect of TMP on NF-κB (p65) activity was mediated by blocking the consequent translocation of p65 into the nucleus. Consistent with the inhibition of transcriptional activity of NF-κB, TMP also inhibited LPS-induced IL-8 expression in HUVECs at both protein and mRNA levels.

In the intracellular signal transduction pathways inducing IL-8 production by LPS, it is as yet unclear. LPS binds to surface receptors TLR4 and activates several signal transduction pathways such as ERK1/2 and p38 MAP kinase and induces translocation of the NF-κB subunits to the nucleus (Ross, 1999). Thus, here we investigated the effects of specific inhibitors of MAPK on the expression of IL-8 protein, to determine what signaling pathway might be involved in the activation of IL-8 by LPS in HUVECs. We observed that each inhibitor of ERK1/2, p38 MAPK respectively inhibited the LPS-induced IL-8 synthesis in HUVECs, suggesting that those kinase-dependent pathways is involved in the inhibitory effect of TMP. LPS-induced IL-8 production in human endothelial cells is associated with activation of transcription factors including NF-κB. Therefore, a potential mechanism whereby TMP can modulate inflammatory responses to LPS is blocking of the activation of those signal transduction pathways. Our results suggest that p38 and ERK are upstream regulators of NF-κB activation in LPS-stimulated HUVECs and the inhibitory effect of TMP on NF-κB activity is mediated, at least in part, by inhibiting ERK and p38 activation.

TMP, an active ingredient of a Chinese herbal medicine, has been widely used in China for treatment of angina pectoris and cerebral ischaemic syndromes (Lu et al., 1978). Increasing evidence suggested that TMP can reduce arterial resistance, increase coronary (Dai and Bache, 1985) and cerebral blood flow in dogs, has inhibitory effects on platelet function in vivo and in vitro, improves the microcirculation (Dai and Bache, 1985) and reduces capillary permeability (Tuttle et al., 1989). In addition, a substantial body of evidence has shown that TMP can provide early cardioprotection against ischaemia and reperfusion injury (Feng et al., 1999) and induce delayed cardioprotective effects by activation of protein kinase C and ERK1/2 signalling pathways in rat neonatal cardiomyocytes (Chen et al., 2007). Although there is considerable interest in various health promoting benefits of TMP such as its potential anti-inflammatory effects, reduction of the risk of coronary heart disease and prevention of some chronic diseases, its effects on endothelial cell function have not been clearly elucidated. Thus, here we investigated the effects of specific inhibitors of MAPK on the expression of IL-8 protein, to determine what signaling pathway might be involved in the activation of TMP in HUVECs. Our data indicated that LPS-induced phosphorylation of ERK1/2 and p38 were inhibited by TMP. These findings clearly show that TMP inhibits LPS-induced NF-κB activation through ERK1/2 and p38 signaling pathways.

Because cardiovascular disease is the major cause of mortality. Targeting the MAPK-NF-κB signaling pathway might ultimately engender novel approaches to the management of cardiovas-

cular risk in those individuals. As shown in this study, TMP inhibits LPS-induced IL-8 production in HUVECs at both protein and mRNA levels. These antiinflammatory effects of TMP seem to be extremely important for prevention of coronary artery disease in patients. Taken together, results of this study suggest that TMP exerts atheroprotective effects on endothelial cells by attenuating IL-8 expression; these results also provide insight into some mechanisms in endothelial cells that underlie the vascular protective properties of TMP, highlighting its effects on IL-8 production.

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