Available online at www.sciencedirect.com

SCIENCE DIRECT

European Journal of Pharmacology 498 (2004) 303–314

www.elsevier.com/locate/ejphar

Effects of benidipine, a dihydropyridine-Ca^{2+} channel blocker, on expression of cytokine-induced adhesion molecules and chemoattractants in human aortic endothelial cells

Masahiro Matsubara*, Kazuhide Hasegawa

Department of Pharmaceutical Research Institute, Kyowa Hakko Kogyo Co., Ltd., 1188 Shimotogari, Nagaizumi-cho, Sunto-gun, Shizuoka 411-8731, Japan

Received 12 February 2004; received in revised form 12 July 2004; accepted 15 July 2004
Available online 20 August 2004

Abstract

Benidipine hydrochloride (benidipine) is a dihydropyridine-Ca^{2+} channel blocker with antioxidant properties. We examined the effects of benidipine on cytokine-induced expression of adhesion molecules and chemokines, which play important roles in the adhesion of monocytes to endothelium. Pretreatment of human aortic endothelial cells (HAECs) with benidipine (0.3–10 μmol/l) for 24 h significantly suppressed cytokine-induced vascular cell adhesion molecule-1 (VCAM-1) and intracellular cell adhesion molecule-1 (ICAM-1) mRNA and protein expression, resulting in reduced adhesion of THP-1 monocytes. Benidipine also suppressed induction of monocyte chemoattractant protein (MCP)-1 and interleukin-8. Benidipine inhibited redox-sensitive transcriptional nuclear factor-κB (NF-κB) pathway, as determined by Western blotting of inhibitory κB (IκB) phosphorylation and luciferase reporter assay. Results of analysis using optical isomers of benidipine and antioxidants suggested that these inhibitory effects were dependent on pharmacological effects other than Ca^{2+} antagonism such as antioxidant effects. Benidipine may thus have anti-inflammatory properties and benefits for in the treatment of atherosclerosis.

Keywords: Benidipine; Antioxidant; Endothelium; VCAM-1; Atherosclerosis

1. Introduction

Early in atherosclerosis, monocyte-derived macrophages invade the vascular wall and accumulate cholesterol, resulting in the formation of foam cells (Lusis, 2000). Attraction and adhesion of monocytes to endothelium initiate the development of atherosclerosis (Bevilacqua et al., 1994). Vascular cell adhesion molecule-1 (VCAM-1, CD104) and intracellular cell adhesion molecule-1 (ICAM-1, CD54) are adhesion molecules on endothelium (Bevilacqua et al., 1994; Lusis, 2000; Ross, 1989). Previous studies with experimental animals and humans have revealed increased expression of VCAM-1 and ICAM-1 in atherosclerotic plaques (Davies et al., 1993; Iiyama et al., 1999; Li et al., 1993; Nakashima et al., 1998; Richardson et al., 1994; Van der Wal et al., 1992). Chemokines such as interleukin-8 and monocyte chemoattractant protein (MCP)-1 act in combination with surface adhesion molecules to attract monocytes to sites of inflammation (Lukacs et al., 1995). MCP-1 and interleukin-8 are also highly expressed in atherosclerotic plaque (Nelken et al., 1991; Rus et al., 1996; Takeya et al., 1993). Studies using atherosclerosis-prone mice, which are deficient in VCAM-1 gene expression and deficient in or overexpress the MCP-1 gene, have indicated the important roles of these genes in atherogenesis (Aiello et al., 1999; Cybulsky et al., 2001; Dansky et al., 2001; Gosling et al., 1999). Therefore, normalization of interactions between endothelium and monocytes should be effective for the prevention and treatment of atherosclerosis.

The levels of expression of several adhesion molecules and chemokines in endothelial cells are regulated, at least in part, by redox-sensitive signal transduction. Intracellular generation of reactive oxygen species in response to tumor necrosis factor (TNF)-α, interleukin-1β and other stimuli serve as a signaling event for activation of the transcrip-

* Corresponding author. Tel.: +81 55 989 2039; fax: +81 559 86 7430.
E-mail address: masahiro.matsubara@kyowa.co.jp (M. Matsubara).

0014-2999/$ - see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2004.07.086

tional nuclear factor-κB (NF-κB) (Collins and Cybulsky, 2001). NF-κB is located in cytoplasm in an inactivated form associated with its inhibitors, the inhibitory κB (IκB) proteins. In response to several stimuli, IκBs are phosphorylated by IκB kinases (IKKs), ubiquitinated, and proteolytically degraded, allowing NF-κB to translocate to the nucleus (Collins and Cybulsky, 2001). Antioxidants suppress cytokine-induced VCAM-1 expression via inhibition of NF-κB activation in cultured endothelial cells (Marui et al., 1993). Moreover, some antioxidants have been found to inhibit progression of atherosclerosis in experimental animal models and in clinical trials (Gotto, 2003; Sundell et al., 2003; Tardif et al., 2003; Weber et al., 1994).

Benidipine, a dihydropyridine-Ca^{2+} antagonist, has pharmacological effects distinct from L-type calcium channel blockade (Ikeda et al., 2000; Jesmin et al., 2002; Kobayashi et al., 1999; Sanada et al., 2003; Yamashita et al., 2001; Yao et al., 2000, 2003). One of the pleiotropic effects reported for benidipine is antioxidative activity (Yao et al., 2000, 2003). In addition, interest in the ability of some dihydropyridine-Ca^{2+} channel blockers to prevent atherosclerosis in experimental animal models and in clinical trials (Fleckenstein-Grun et al., 1992; Henry, 1985; Jorgensen and Thaulow, 2003; Nayler, 1999; Pitt et al., 2000) suggests the usefulness of examining the effects of benidipine on expression of surface adhesion molecules and chemokines in human aortic endothelial cells (HAECs).

2. Materials and methods

2.1. Materials

Benidipine [(±)-α] ((3*RS*)-1-Benzylpiperidin-3-yl methyl (4*RS*)-1,4-dihydro-2, 6-dimethyl-4-(3-nitrophenyl)pyridine-3, 5-dicarboxylate monohydrochloride), its optical isomers, KF9210 [(+)-α] ((3*S*)-1-Benzylpiperidin-3-yl methyl (4*S*)-1,4-dihydro-2, 6-dimethyl-4-(3-nitrophenyl)pyridine-3, 5-dicarboxylate monohydrochloride) and KF9211 [(−)-α] ((3*R*)-1-Benzylpiperidin-3-yl methyl (4*R*)-1,4-dihydro-2, 6-dimethyl-4-(3-nitrophenyl)pyridine-3, 5-dicarboxylate monohydrochloride), and amlodipine were synthesized in our laboratories. The following materials were purchased from the indicated commercial source: Nifedipine (Sigma); Nitrendipine (Wako); recombinant human TNF-α (Strathmann Biotec Ag); recombinant human interleukin-1β (R&D system); Probucol (Wako), *N*-acetyl-L-cysteine (NAC, Sigma), pyrrolidine dithiocarbamate (PDTC, Sigma) and (±)-α-tocopherol (Sigma). All dihydroprydine-calcium channel blockers were dissolved in dimethyl sulfoxide at a concentration of 0.1 mol/l and stored at −20 °C before use.

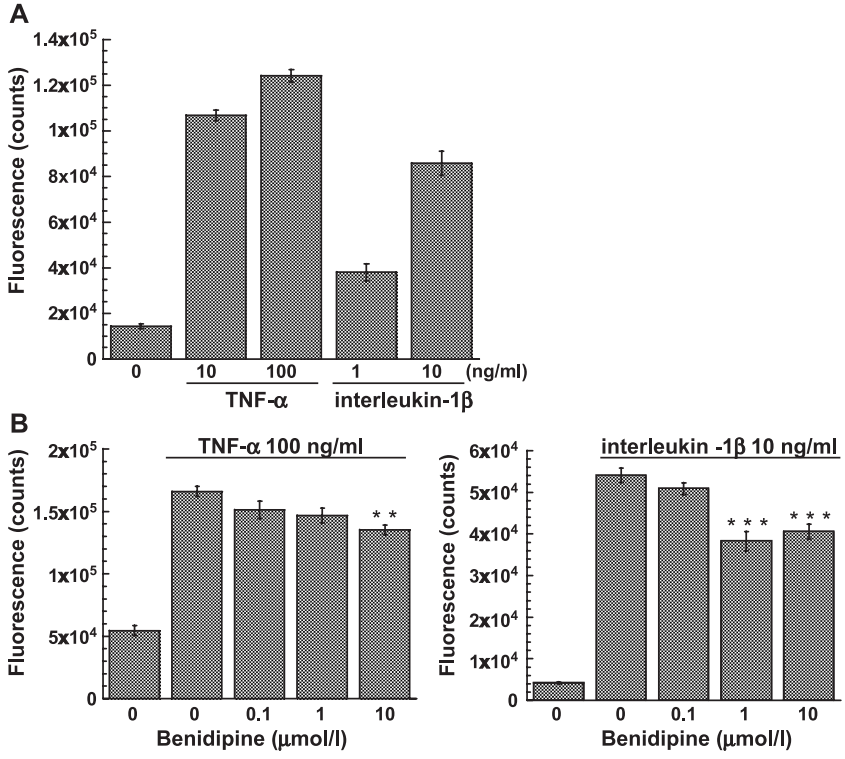

Fig. 1. Effects of benidipine on FITC-labeled THP-1 adhesion to cytokine-stimulated human aortic endothelial cells (HAECs). (A) HAECs were stimulated with the indicated concentrations of TNF-α or interleukin-1β for 16 h. FITC-labeled THP-1 cells were added and incubated for 30 min. After incubation, non-adherent THP-1 cells were washed out. Adhesion of THP-1 to HAECs was determined by measuring fluorescence of FITC. (B) HAECs were pretreated with the indicated concentrations of benidipine for 24 h prior to stimulation with 100 ng/ml TNF-α or 10 ng/ml interleukin-1β for 16 h. Values represent means±S.E. of six determinations. $^{**}P<0.01$, $^{***}P<0.001$ compared with the control group (Dunnett).

The final concentration of dimethyl sulfoxide in the assay system described below was 0.1 v/v%.

2.2. Cell culture

HAECs and human umbilical vein endothelial cells (HUVECs) were purchased from Bio Whittaker and grown in HuMedia-EG2 (Kurabo) medium containing of 2 v/v% fetal bovine serum, 10 ng/ml human epidermal growth factor, 5 ng/ml fibroblast growth factor, 10 μg/ml heparin, 1 μg/ml hydrocortisone, 50 μg/ml gentamycin and 50 ng/ml amphotericin B according to the manufacturer's instruction. THP-1 cells (American Type Tissue Culture Collection) were grown in Roswell Park Memorial Institute (RPMI)-1640 (Sigma) containing 10 v/v% fetal bovine serum (JRH Biosciences), 2 mmol/l L-glutamate (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen). Cultures were maintained in 5% CO_2 at 37 °C in tissue culture flasks. HAECs were detached with trypsin–EDTA solution (Invtirogen). After the cells were washed, they were resuspended in medium and used for subsequent experiments. HAECs between passages 3 and 7 were used. The agents used within the concentrations applied in our studies did not significantly affect cell viability.

2.3. Adhesion assay

THP-1 cells, a human monocytic cell line, were used for monocyte-endothelial adhesion assay. THP-1 cells were labeled with 60 mg/ml fluorescein isothiocyanate (FITC, Dojindo) in labeling medium (RPMI-1640, 10 v/v% fetal serum albumin, 20 mmol/l HEPES, pH 7.0) for 30 min. After incubation, the FITC-labeled THP-1 cells were washed and the labeling medium was replaced with adhesion assay medium (RPMI-1640, 1 v/v% bovine serum albumin, 20 mmol/l HEPES, pH 7.4). Confluent HAECs in 96-well plates were cultured in HuMedia-EG2 medium. The cells were treated with the indicated concentrations of benidipine for 24 h. The cells were stimulated with the indicated concentration of TNF-α or interleukin-1β for 16 h. After incubation, the medium was removed. The FITC-labeled THP-1 cells (5×10^5/well) were added and incubated for 30 min. Plates then were gently washed four times to remove non-adherent THP-1 cells. The fluorescence of the FTIC-labeled THP-1 cells adherent to HAECs was measured by spectrofluorometery on an automatic microtiter plate reader (ARVO SX 1420 Multilabel Counter, Wallac) at an excitation wavelength of 355 nm and emission wavelength of 450 nm.

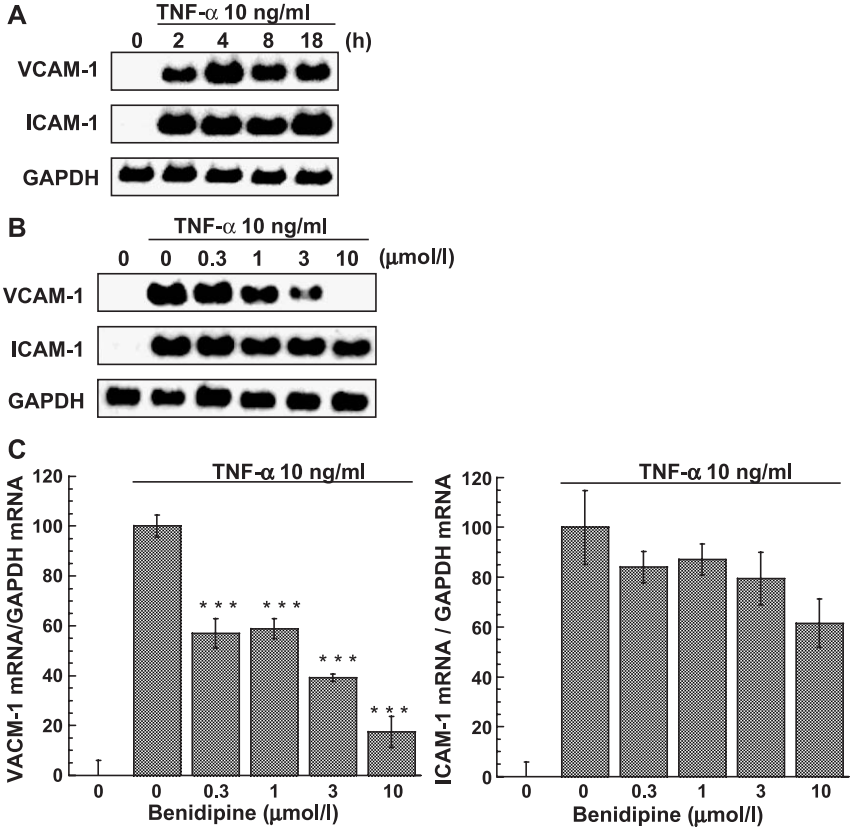

Fig. 2. Effects of benidipine on TNF-α-stimulated VCAM-1 or ICAM-1 mRNA expression in human aortic endothelial cells (HAECs). (A) HAECs were stimulated with 10 ng/ml TNF-α. The time course of VCAM-1 or ICAM-1 mRNA levels was analyzed with the semi-quantitative RT-PCR method. (B) HAECs were pretreated with the indicated concentrations of benidipine for 24 h prior to stimulation with 10 ng/ml TNF-α for 4 h. One representative result of three independent experiments is shown. (C) Results of densitometric analyses are shown as % of control (ratio of VCAM-1 or ICAM-1 mRNA/GAPDH mRNA). Values represent means±S.E. of three determinations. ***P<0.001 compared with the control group (Dunnett).

2.4. Cell-surface enzyme-linked immunosorbent assay (ELISA) for determination of VCAM-1 and ICAM-1

Confluent HAECs in 96-well plates were cultured in HuMedia-EG2 medium. The cells were treated with the indicated concentrations of benidipine for 24 h, and stimulated with 100 ng/ml of TNF-α or interleukin-1β for 12 h. After incubation, the medium was removed and the cells were fixed with 3.7 % formaldehyde on ice for 30 min. The plates were then washed with phosphate-buffered saline (PBS) and blocked with 1 w/v % bovine serum albumin on ice for 1 h. A primary monoclonal antibody to either VCAM-1 (clone IE5, Cymbus Biotechnology) or ICAM-1 (clone 6.5B5, Cymbus Biotechnology) was added at 1:100 dilution and the plates were incubated on ice for 1 h. The plates were then washed and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibody (Amersham Bioscience) at 1:1000 dilution on ice for 1 h. After incubation, the plates were washed again. The antibodies were detected with Super Signal West Pico Chemiluminescent Substrate (Pierce) using Microplate Scintillation and Luminescence Counter TopCount NXT (Packard).

2.5. Measurement of MCP-1 and interleukin-8

Confluent HAECs in 96-well plates were cultured in HuMedia-EG2 medium. The cells were treated with the indicated concentrations of benidipine for 24 h, and stimulated with 10 ng/ml of TNF-α or interleukin-1β for 12 h. MCP-1 or interleukin-8 levels in the supernatant were determined using commercially available ELISA (OptEIA Human MCP-1 Set, Pharamingen or Human interleukin-8 Immunoassay Kit, BioSource International) according to the manufacturer's instructions.

2.6. Semi-quantification of mRNA by reverse transcription-polymerase chain reaction (RT-PCR)

Confluent HAECs in 12-well plates were cultured in HuMedia-EG2 medium. The cells were treated with the indicated concentrations of benidipine for 24 h in the HuMedia-EG2 medium, and then stimulated with 10 ng/ml TNF-α. After incubation, the cells were washed twice with ice-cold PBS. Total RNA was isolated with RNeasy according to the manufacturer's instruction (Qiagen). First-strand cDNA synthesis was performed from 0.3 μg of total RNA in 20-μl volumes with oligo (dT) priming using the Superscript First-strand Synthesis System (Invitrogen). PCR was performed from 1 μl of the generated cDNA using 1 U of ExTaq polymerase (Takara). Based on previous reports (Koga et al., 2002; Lee et al., 2003; Zhang et al., 2002), the following primer pairs (including PCR product sizes in parentheses) were synthesized by Invitrogen: VCAM-1, 5′-ATG ACA TGC TTG AGC CAG G-3′

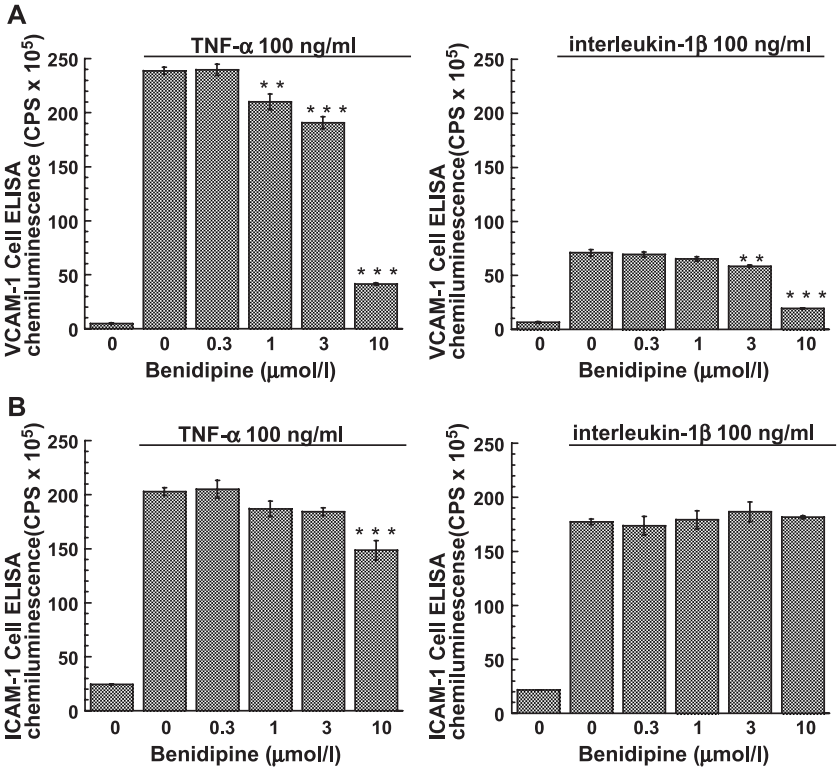

Fig. 3. Effects of benidipine on cytokine-stimulated expression of VCAM-1 or ICAM-1 protein on the surface of human aortic endothelial cells (HAECs). HAECs were pretreated with the indicated concentrations of benidipine for 24 h prior to stimulation with 100 ng/ml TNF-α (left panels) or interleukin-1β (right panels) for 12 h. Expression of VCAM-1 (A) or ICAM-1 (B) protein on the surface of cells was determined by the cell-surface ELISA method. Values represent means±S.E. of three determinations. $^{**}P<0.01$, $^{***}P<0.001$ compared with the control group (Dunnett).

and 5′-GTG TCT CCT TCT TTG ACA CT-3′ (260 bp); ICAM-1, 5′-TAT GGC AAC GAC TCC TTC T-3′ and 5′-CAT TCA GCG TCA CCT TGG-3′ (238 bp); MCP-1, 5′-CAG CCA GAT GCA ATC AAT GC-3′ and 5′-GTG GTC CAT GGA ATC CTG AA-3′ (198 bp); GAPDH, 5′-CCC ATC ACC ATC TTC CAG GAG-3′ and 5′-TTC ACC ACC TTC TTG ATG TCA-3′ (572 bp). The denaturation, annealing, and extension conditions were 94 °C for 60 s, 60 °C for 45 s and 72 °C for 45 s, respectively. The PCR cycle conditions were 22 cycles for VCAM-1, 22 cycles ICAM-1, 19 cycles for MCP-1 and 19 cycles for GAPDH. PCR reaction was performed with a GeneAmp PCR system 9700 (Applied Biosystems). The PCR products were electrophoresed in 2 w/v% agarose gels and stained with SYBR Green I (Molecular Probes). The detection and densitometric analysis of bands were performed with a Lumino Image Analyzer LAS-1000 plus (Fujifilm). The sizes of bands were confirmed with reference to molecular size markers (100 bp DNA Ladder Marker, Takara). The value of VCAM-1, ICAM-1 or MCP-1 mRNA was normalized to the amount of GAPDH mRNA, which was utilized as a housekeeping gene for each experimental condition.

2.7. Transfection and luciferase assay

Transient transfections of HUVEC were performed using Lipofectamine plus reagent (Invitrogen) in OPTI-MEM medium (Invitrogen). Confluent HUVECs in 96-well plates were transfected with original NF-κB reporter plasmid encoding the triple consensus NF-κB binding sites (gggaaattcc) upstream of the luciferase gene for 3 h. After incubation, cultures were maintained in normal growth medium HuMedia-EG2 for 24 h. The transfected cells were pretreated with the indicated concentrations of benidipine for 24 h prior to stimulation with 100 ng/ml TNF-α for 6 h. Luciferase activity was detected with the Steady-Glo luciferase assay system (Promega) using Microplate Scintillation and Luminescence Counter Top-Count NXT.

2.8. Western blot analysis

Confluent HAECs in 6-well plates were cultured in HuMedia-EG2 medium. The cells were treated with the indicated concentrations of benidipine for 24 h in the HuMedia-EG2 medium, and then stimulated with 10 ng/ml TNF-α. After incubation, the cells were washed with ice-cold PBS. The cells were lysed with ice-cold lysis buffer (150 mmol/l NaCl, 1 mmol/l Na_2EDTA, 1 mmol/l EGTA, 1 v/v% Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, 1 μg/ml leupeptin, 10 mmol/l NaF, 1 mmol/l phenylmethylsulfonyl fluoride, 20 mmol/l Tris–HCl, pH 7.5) for 30 min on ice. Insoluble material was removed by centrifugation and the supernatant was boiled with Laemmli's buffer containing dithiothreitol for 3 min. Protein concentrations were determined using a Micro bicinchoninic acid protein assay Kit (Pierce). Equivalent contents of protein in each sample (4 μg/lane) were separated by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted onto a PVDF membrane (Immobilon, Millipore). The membranes were probed with polyclonal anti-phospho-IκB-α (Ser32) antibody (Cell Signaling Technology) and subsequently incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Amersham Bioscience). The antibodies were detected with Super Signal West Pico Chemiluminescent Substrate and imaged using a Lumino Image Analyzer LAS-1000 plus (Fujifilm). The effects of treatment on phosphorylation of IkB-α are reported as % of control.

2.9. Statistical analysis

Statistical analysis was performed using the Dunnett test. P values <0.05 were considered significant.

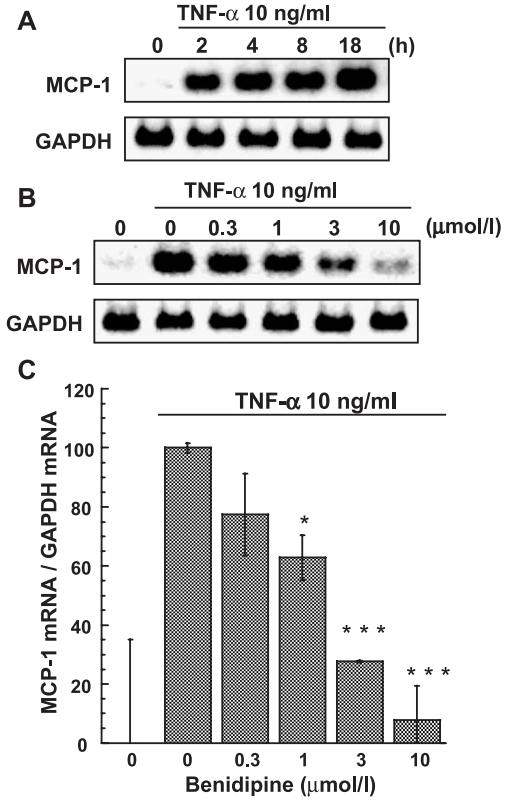

Fig. 4. Effects of benidipine on TNF-α-stimulated MCP-1 mRNA expression in human aortic endothelial cells (HAECs). (A) HAECs were stimulated with 10 ng/ml TNF-α. The time course of MCP-1 mRNA levels was analyzed with the semi-quantitative RT-PCR method. (B) HAECs were pretreated with the indicated concentrations of benidipine for 24 h prior to stimulation with 10 ng/ml TNF-α for 4h. One representative result of three independent experiments is shown. (C) Results of densitometric analysis are shown as % of control (ratio of MCP-1 mRNA/GAPDH mRNA). Values represent means±S.E. of three determinations. *P<0.05, ***P<0.001 compared with the control group (Dunnett).

3. Results

3.1. Effects of benidipine on THP-1 adhesion to TNF-α- or interleukin-1β-stimulated HAECs

To explore the effects of benidipine on endothelial cell–monocyte interaction, we examined the adhesion of THP-1 monocytic cells to cytokine-activated HAECs. Control confluent HAECs exhibited minimal binding to THP1 cells, whereas stimulation of HAEC with TNF-α or interleukin-1β for 16 h substantially increased adhesion of THP-1 monocytic cells (Fig. 1A). Pretreatment with benidipine (0.1–10 μmol/l) for 24 h significantly reduced adhesion of THP-1 cells to TNF-α- or interleukin-1β-stimulated HAECs (Fig. 1B).

3.2. Effects of benidipine on induction of VCAM-1 and ICAM-1 in HAECs stimulated with TNF-α or interleukin-1β

Since adhesion molecules such as VCAM-1 and ICAM-1 are essential for the interaction of endothelial cells with monocytes, the effects of benidipine on induction of VCAM-1 and ICAM-1 expression were investigated. Stimulation of HAECs with TNF-α resulted in a sustained increase in mRNA expression of VCAM-1 and ICAM-1 for 18 h (Fig. 2A). Pretreatment with benidipine significantly suppressed the increase in VCAM-1 mRNA in a concentration-dependent manner (0.3–10 μmol/l), whereas benidipine marginally inhibited expression of ICAM-1 mRNA (Fig. 2B and C) up to 10 μmol/l. Next, we developed a cell-surface ELISA for VCAM-1 and ICAM-1, and confirmed that protein levels were also increased by TNF-α and by interleukin-1β. Pretreatment with benidipine (0.3–10 μmol/l) concentration-dependently prevented the increase in VCAM-1 cell-surface expression (Fig. 3A). On the other hand, benidipine partially inhibited the induction of ICAM-1 cell-surface expression (Fig. 3B). In view of the above results, benidipine might regulate the expression of VCAM-1 at level of transcription.

3.3. Effects of benidipine on MCP-1 and interleukin-8 production by HAECs stimulated with TNF-α or interleukin-1β

Expression of MCP-1 mRNA was also induced by TNF-α stimulation of HAECs (Fig. 4A). Benidipine (0.3–10 μmol/l) concentration dependently suppressed the expression of MCP-1 mRNA (Fig. 4B and C) In addition, pretreatment with benidipine (0.1–10 μmol/l) also significantly prevented MCP-1 and IL-8 production from HAECs stimulated with TNF-α or interleukin-1β (Fig. 5).

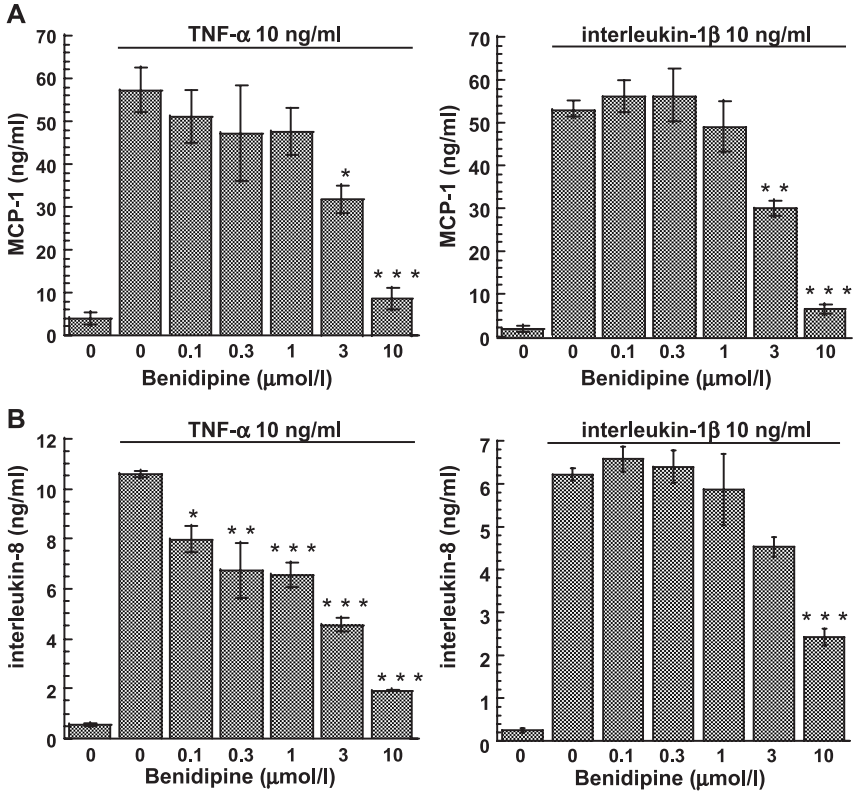

Fig. 5. Effects of benidipine on production of MCP-1 or interleukin-8 induced by cytokines in human aortic endothelial cells (HAECs). HAECs were pretreated with the indicated concentrations of benidipine for 24 h prior to stimulation with 10 ng/ml TNF-α (left panels) or interleukin-1β (right panels) for 16 h. The content of MCP-1 (A) or IL-8 (B) in supernatant was determined by ELISA method according to the manufacturer's manual. Values represent means±S.E. of three determinations. *P<0.05, **P<0.01, ***P<0.001 compared with the control group (Dunnett).

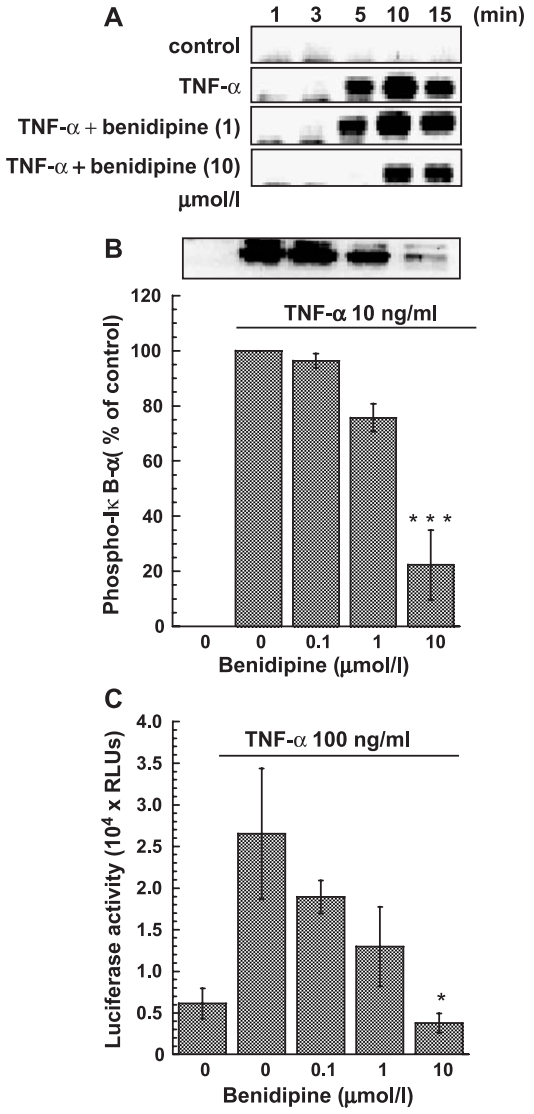

Fig. 6. Effects of benidipine on NF-κB activation in human endothelial cells. (A) HAECs were pretreated with the indicated concentrations of benidipine for 24 h prior to stimulation with 10 ng/ml TNF-α. The time courses of change in phosphorylated IκB-α level were analyzed by Western blotting with a phospho-IκB-α specific antibody. (B) Levels of phosphorylation of IκB-α after 5 min of TNF-α stimulation were analyzed. One representative result of three independent experiments is shown in the upper panel. The intensity of the band was determined by densitometry. Results of densitometric analysis are shown as % of control in the lower panel. Values represent means±S.E. of three determinations. ***$P<0.001$ compared with the control group (Dunnett). (C) HUVECs were transiently transfected with NF-κB reporter plasmid. After transfection, cells were pretreated with the indicated concentrations of benidipine for 24 h and stimulated with 10 ng/ml TNF-α for 6 h. Then, luciferase activity was determined. Values represent means±S.E. of six determinations. *$P<0.05$ compared with the control group (Dunnett).

3.4. Effects of benidipine on TNF-α induced NF-κB activation in endothelial cells

Since activation of NF-κB at least is required for the transcriptional induction of adhesion molecules and chemokines, such as VCAM-1 and MCP-1 (Bevilacqua et al., 1994; Collins and Cybulsky, 2001; Lusis, 2000; Ross, 1989), effects of benidipine on TNF-α-activated NF-κB pathway in endothelial cells were investigated. First, we evaluated effects of benidipine on the phosphorylation of IκB-α induced by TNF-α using Western blot analysis. Pretreatment with benidipine (0.1–10 μmol/l) concentration dependently suppressed the phosphorylation of IκB-α in HAECs (Fig. 6A and B). However, the inhibitory effect of benidipine on IκB-α phosphorylation was transient. Nevertheless, we confirmed the inhibitory effects of benidipine (0.1–10 μmol/l) on NF-κB transcriptional activity by luciferase gene reporter assay using HUVECs (Fig. 6C). These results suggested that benidipine may suppress cytokine-induced VCAM-1 and MCP-1 mRNA expression via in part inhibition of IκB-α phosphorylation upstream of NF-κB activation.

3.5. Effects of optical isomers of benidipine on TNF-α-induced expression of VCAM-1 and ICAM-1 in HAECs

To clarify the involvement of calcium antagonism in the inhibition by benidipine of VCAM-1 and ICAM-1 expression, we compared the effects of KF9210 [(+)-α] and KF9211 [(−)-α], the optical isomers of benidipine [(±)-α], on VCAM-1 and ICAM-1 expression. The optical isomers also exhibited selective inhibition of VCAM-1 (Fig. 7). KF9210 has been demonstrated to exhibit antihypertensive activity about 10 times stronger than that of KF9211 (Ishii et al., 1988; Muto et al., 1998). However, KF9210 inhibited VCAM-1 expression as potently as KF9211, suggesting that

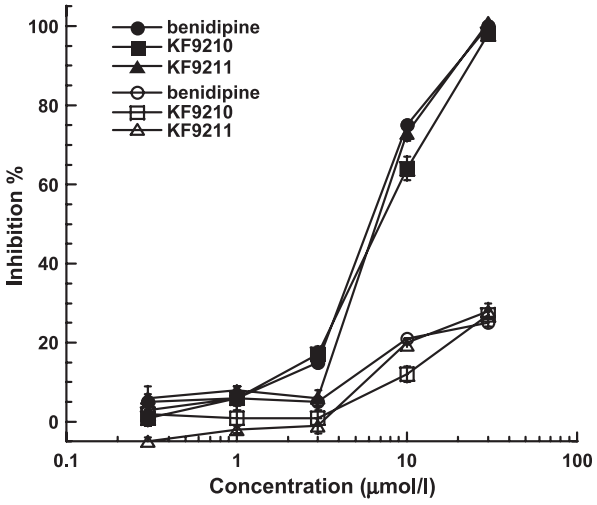

Fig. 7. Effects of optical isomers of benidipine on expression of VCAM-1 or ICAM-1 protein induced by TNF-α on the surface of human aortic endothelial cells (HAECs). HAECs were pretreated with the indicated concentrations of KF9210 [(+)-α] or KF9211 [(−)-α], the optical isomers of benidipine [(±)-α] for 24 h prior to stimulation with 100 ng/ml TNF-α for 12 h. Expression of VCAM-1 (closed symbols) or ICAM-1 (open symbols) on the cell surface was determined by the cell-surface ELISA method. The effect of each compound on expression of adhesion molecules is shown as %inhibition. Values represent means±S.E. of three determinations.

pharmacological effects other than Ca^{2+} antagonism are involved in the inhibition of VCAM-1 expression. The IC_{50} values estimated in this study were 6.1 μmol/l for benidipine, 6.5 μmol/l for KF9210 and 7.0 μmol/l for KF9211.

3.6. Effects of several antioxidants on TNF-α-induced expression of VCAM-1 and ICAM-1 in HAECs

Because antioxidants have been reported to inhibit NF-κB activation and induction of VCAM-1 (Marui et al., 1993), the suppressive effects of benidipine on activation of HAECs by inflammatory cytokines may be mediated by the antioxidative activity of benidipine. We therefore applied antioxidants including NAC (1–10 mmol/l) (Marui et al., 1993), PDTC (30–100 μmol/l) (Marui et al., 1993), α-tocopherol (30–300 μmol/l) (Wu et al., 1999) and probucol (30–300 μmol/l) (Zapolska-Downar et al., 2001) to the cell-surface ELISA for VCAM-1 or ICAM-1. All antioxidants tested inhibited VCAM-1 expression more effectively than ICAM-1 expression (Fig. 8), in agreement with results of the previous study. These suppressive effects were similar to those of benidipine.

3.7. Effects of dihydropyridine derivatives on TNF-α-induced expression of VCAM-1 and ICAM-1 in HAECs

Effects of other dihydropyridine derivatives, amlodipine, nifedipine and nitrendipine on TNF-α-induced VCAM-1 and ICAM-1 expression were also examined (Fig. 9). Benidipine and amlodipine significantly inhibited VCAM-1 expression, whereas nifedipine and nitrendipine did not. Only benidipine significantly inhibited ICAM-1 expression among these dihydropyridine derivatives.

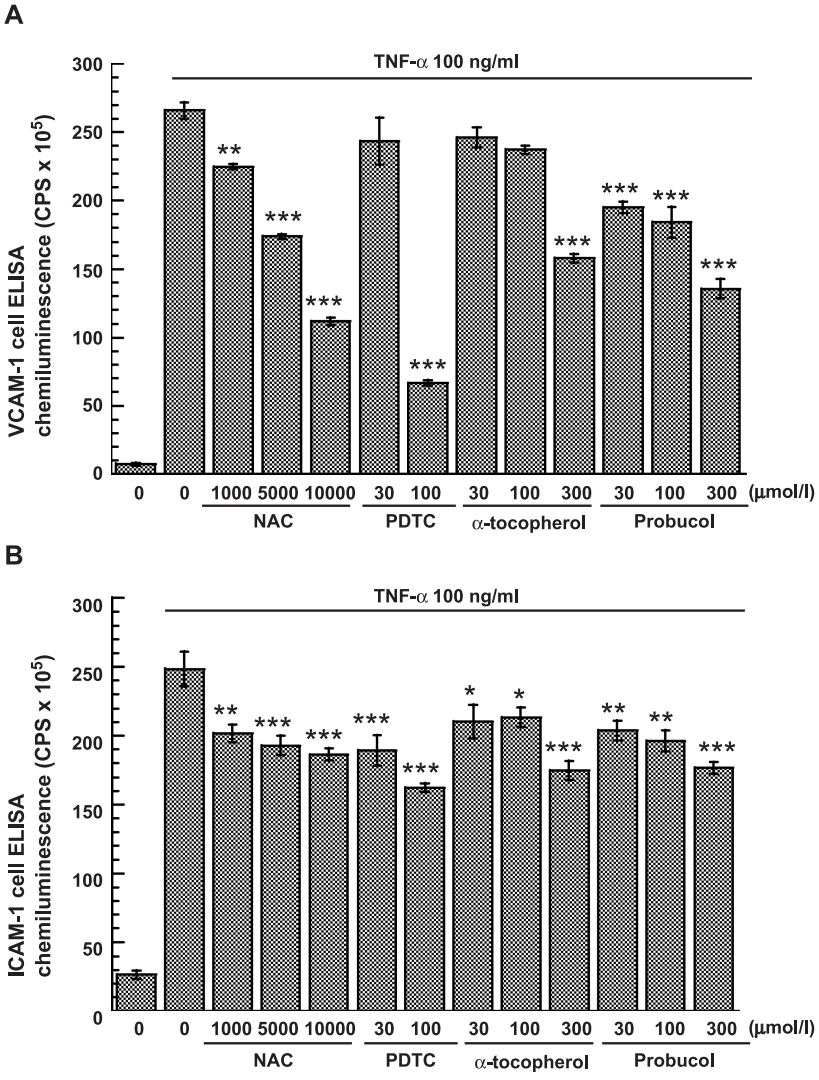

Fig. 8. Effects of antioxidants on expression VCAM-1 or ICAM-1 protein induced by TNF-α on the surface of human aortic endothelial cells (HAECs). HAECs were pretreated with the indicated concentrations of antioxidants for 24 h prior to stimulation with 100 ng/ml TNF-α for 12 h. Expression of VCAM-1 (A) or ICAM-1 (B) on the surface of cells was determined by the cell-surface ELISA method. The method used is described in Materials and methods. Values represent means±S.E. of three determinations. *P<0.05, **P<0.01, ***P<0.001 compared with the control group (Dunnett).

4. Discussion

Activated endothelial cells express a number of adhesion molecules on their surface and produce several chemoattractants, causing the recruitment and adherence of leukocytes (Bevilacqua et al., 1994; Ross, 1989). Adherence of monocytes to endothelium is one of the events triggering the development of atherosclerosis (Lusis, 2000). In the present study, pretreatment with benidipine, a dihydroprydine-Ca^{2+} channel blocker, partially suppressed the adhesion of THP-1 monocytic cells to cytokine-activated HAECs. In addition, we found that cell-surface expression of VCAM-1 and production of MCP-1 were inhibited by benidipine in a concentration-dependent manner. Benidipine selectively inhibited the induction of VCAM-1 expression in cytokine-stimulated HAECs to a greater extent than ICAM-1. The partial suppression of THP-1 adherence by benidipine may be due to selective inhibition of expression of adhesion molecules. Moreover, benidipine inhibited cytokine-induced VCAM-1 and MCP-1 mRNA as well as protein expression. Thus, benidipine may interfere with some common pathways responsible for the transcriptional expression of these genes.

We set applied incubation time for each experiment up as the plateau phase of its reaction based on our preliminary investigations. We found differences in the potency of benidipine among experiments. Probably, this pharmacological effect may be due to the difference of incubation time for exposure to the stimulant TNF-α. Although there are some differences in experimental conditions, we confirmed anti-inflammatory effects of benidipine in several assays, as described above.

The inhibition of VCAM-1 and MCP-1 expression by benidipine may play roles in mediating the pharmacological effects other than its Ca^{2+} antagonism. Since endothelial cells do not express L-type voltage-dependent Ca^{2+} channels, in contrast to their principal pharmacological effects in vascular smooth muscle cells, dihydropyrdine-Ca^{2+} channel blockers may not influence intracellular Ca^{2+} in endothelial cells. In addition, KF9210 [(+)-α], an optical isomer of benidipine has binding affinity for L-type voltage-dependent Ca^{2+} channels about 10 times that of KF9211 [(−)-α], using [^{3}H] nitrendipine with rat heart membrane

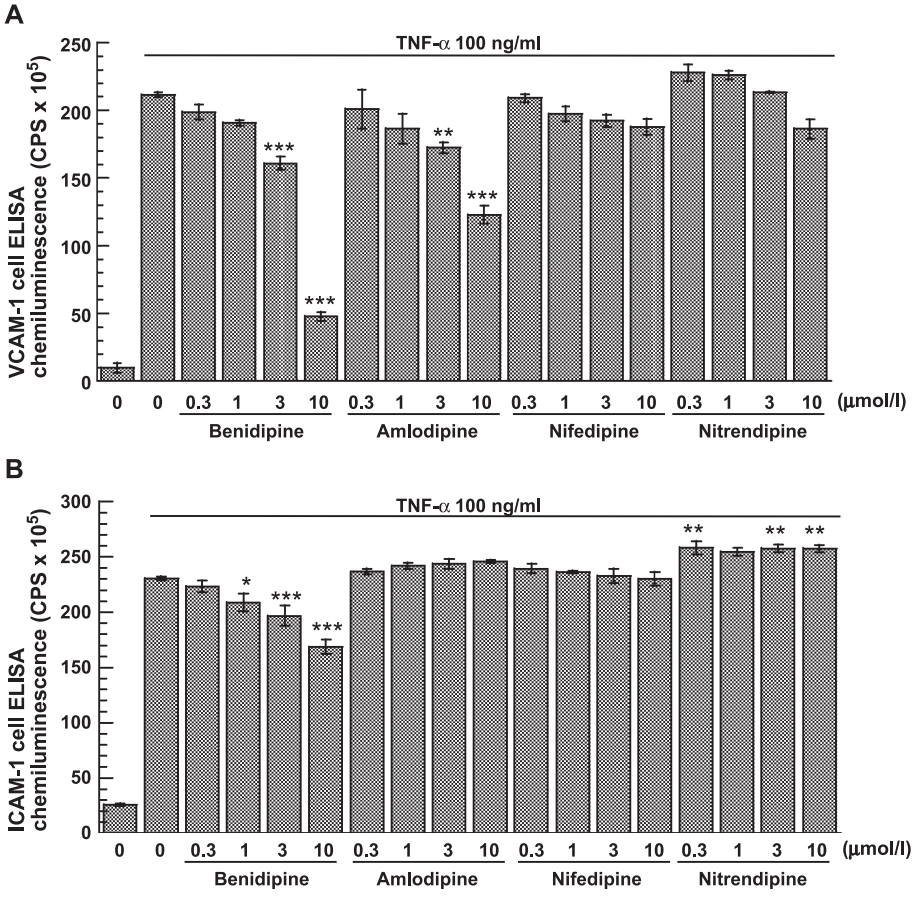

Fig. 9. Effects of dihydropyridine derivatives on expression of VCAM-1 or ICAM-1 protein induced by TNF-α on the surface of human aortic endothelial cells (HAECs). HAECs were pretreated with the indicated concentrations of each dihydropyridine for 24 h prior to stimulation with 100 ng/ml TNF-α for 12 h. Expression of VCAM-1 (A) or ICAM-1 (B) on the surface of cells was determined by the cell-surface ELISA method. The method used is described in Materials and methods. Values represent means±S.E. of three determinations. *P<0.05, **P<0.01, ***P<0.001 compared with the control group (Dunnett).

fractions (K_i value of 0.08 nmol/l for KF9210 versus 0.96 nmol/l of KF9211) (Ishii et al., 1988; Muto et al., 1998). In this study, however, KF9210 inhibited VCAM-1 expression as potently as KF9211 (IC_{50} of 6.5 μmol/l for KF9210 versus IC_{50} of 7.0 μmol/l for KF9211). Therefore, the inhibitory effects of benidipine on the induction of adhesion molecules and chemokines may not be due to Ca^{2+} antagonism.

NF-κB is known to be responsible for up-regulation of expression of VCAM-1 and MCP-1 (Bevilacqua et al., 1994; Collins and Cybulsky, 2001). Benidipine inhibited the phosphorylation of IκB-α and suppressed the NF-κB inducible reporter activities in TNF-α-induced endothelial cells. Although the inhibitory effect of benidipine on IκB-α phosphorylation was transient, benidpine significantly inhibited the reporter activities and VCAM-1 and MCP-1 expression. Thus, it is likely that benidipine may also inhibit other signaling pathways leading to NF-κB activation, such as its translocation into the nucleus and its DNA binding affinities. From these aspects, the mechanism of inhibition of VCAM-1 and MCP-1 expression by benidipine calls for further research.

NF-κB has been proposed to be a redox-sensitive transcription factor, because reactive oxygen species play an important role in the signaling events leading to NF-κB activation in endothelial cells (Collins and Cybulsky, 2001). This notion was supported by the observations that the antioxidants, NAC and PDTC, suppressed NF-κB activation, resulting in the inhibition of VCAM-1 expression in endothelial cells (Marui et al., 1993). Furthermore, other antioxidants, such as α-tocopherol (Zapolska-Downar et al., 2000) and probucol (Zapolska-Downar et al., 2001), also inhibit VCAM-1 expression. MCP-1 expression is also sensitive to antioxidants. Moreover, NAC is reported to suppress the activation IKKs upstream of IκB-α, suggesting that IKKs are subject to redox regulation (Oka et al., 2000). In the present study, antioxidants including NAC and PDTC suppressed VCAM-1 expression more potently than ICAM-1 expression, in agreement with findings of another study (Marui et al., 1993).

The pharmacological profiles of benidipine on the expression of these two adhesion molecules were similar to those of the antioxidants. Antioxidant activity has been shown to be one of the pharmacological effects of benidipine other than Ca^{2+} antagonism (Yao et al., 2000, 2003). From these circumstantial data, we supposed that the antioxidant activity of benidipine, at least in part, might be involved in the inhibition of VCAM-1 and MCP-1 expression via suppression of some pathways leading to NF-κB activation.

However, we cannot exclude mechanisms of action other than antioxidant activity, as described below. In the previous studies on the antioxidant properties of Ca^{2+} channel blockers, nifedipine inhibited lipid peroxidation more potently than the other Ca^{2+} channel blockers (Yao et al., 2000), whereas benidipine was found to be the drug most potent in inhibiting cell-surface expression of VCAM-1 on HAECs among dihydropyridine-Ca^{2+} antagonists tested. In addition, the inhibition of VCAM-1 protein expression by benidipine (6.1 μmol/l) was more effective almost 12 times than the suppression of lipid peroxidation (71.2 μmol/l). Thus, further studies are necessary for clarification of the mechanism of benidipine on the expression of VCAM-1.

TNF-α and interleukin-1β each induced concurrent expression of VCAM-1 and ICAM-1. Expression of ICAM-1 as well as VCAM-1 is regulated by NF-κB and has been demonstrated to be redox-sensitive in endothelial cells (Omori et al., 2002). Nevertheless, benidipine and antioxidants slightly inhibited ICAM-1 expression compared to VCAM-1 expression. The precise molecular mechanism(s) of expression of these two adhesion molecules is also still unclear.

In conclusion, benidipine, a dihydropyridine-Ca^{2+} channel blocker, inhibited cytokine-induced VCAM-1 and MCP-1 expression, suggesting that this drug may have protective or therapeutic effects not only for hypertension but also atherosclerosis. In fact, dihydropyridine-Ca^{2+} antagonists have been reported to reduce the progression of atherosclerosis in experimental animal models (Fleckenstein-Grun et al., 1992; Henry, 1985; Nayler, 1999). In addition, these drugs have been demonstrated to slow the development of carotid atherosclerosis in clinical trials (Jorgensen and Thaulow, 2003; Pitt et al., 2000). Taken together, the findings of this study should shed light on the anti-atherosclerotic mechanism of some dihydropyridine-Ca^{2+} channel blockers.

References

Aiello, R.J., Bourassa, P.A., Lindsey, S., Weng, W., Natoli, E., Rollins, B.J., Milos, P.M., 1999. Monocyte chemoattractant protein-1 accelerates atherosclerosis in apolipoprotein E-deficient mice. Arterioscler. Thromb. Vasc. Biol. 19, 1518–1525.

Bevilacqua, M.P., Nelson, R.M., Mannori, G., Cecconi, O., 1994. Endothelial–leukocyte adhesion molecules in human disease. Annu. Rev. Med. 45, 361–378.

Collins, T., Cybulsky, M.I., 2001. NF-kappaB: pivotal mediator or innocent bystander in atherogenesis? J. Clin. Invest. 107, 255–264.

Cybulsky, M.I., Iiyama, K., Li, H., Zhu, S., Chen, M., Iiyama, M., Davis, V., Gutierrez-Ramos, J.C., Connelly, P.W., Milstone, D.S., 2001. A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J. Clin. Invest. 107, 1255–1262.

Dansky, H.M., Barlow, C.B., Lominska, C., Sikes, J.L., Kao, C., Weinsaft, J., Cybulsky, M.I., Smith, J.D., 2001. Adhesion of monocytes to arterial endothelium and initiation of atherosclerosis are critically dependent on vascular cell adhesion molecule-1 gene dosage. Arterioscler. Thromb. Vasc. Biol. 21, 1662–1667.

Davies, M.J., Gordon, J.L., Gearing, A.J., Pigott, R., Woolf, N., Katz, D., Kyriakopoulos, A., 1993. The expression of the adhesion molecules ICAM-1, VCAM-1, PECAM, and E-selectin in human atherosclerosis. J. Pathol. 171, 223–229.

Fleckenstein-Grun, G., Frey, M., Thimm, F., Fleckenstein, A., 1992. Protective effects of various calcium antagonists against experimental arteriosclerosis. J. Hum. Hypertens. 6 (Suppl. 1), S13–S18.

Gosling, J., Slaymaker, S., Gu, L., Tseng, S., Zlot, C.H., Young, S.G., Rollins, B.J., Charo, I.F., 1999. MCP-1 deficiency reduces susceptibility to atherosclerosis in mice that overexpress human apolipoprotein B. J. Clin. Invest. 103, 773–778.

Gotto, A.M., 2003. Antioxidants, statins, and atherosclerosis. J. Am. Coll. Cardiol. 41, 1205–1210.

Henry, P.D., 1985. Atherosclerosis, calcium, and calcium antagonists. Circulation 72, 456–459.

Iiyama, K., Hajra, L., Iiyama, M., Li, H., DiChiara, M., Medoff, B.D., Cybulsky, M.I., 1999. Patterns of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 expression in rabbit and mouse atherosclerotic lesions and at sites predisposed to lesion formation. Circ. Res. 85, 199–207.

Ikeda, K., Tojo, K., Tokudome, G., Akashi, T., Hosoya, T., Harada, M., Nakagawa, O., Nakao, K., 2000. Possible involvement of endothelin-1 in cardioprotective effects of benidipine. Hypertens. Res. 23, 491–496.

Ishii, A., Nishida, K., Oka, T., Nakamizo, N., 1988. Receptor binding properties of the new calcium antagonist benidipine hydrochloride. Arzneimittelforschung 38, 1677–1680.

Jesmin, S., Sakuma, I., Hattori, Y., Fujii, S., Kitabatake, A., 2002. Long-acting calcium channel blocker benidipine suppresses expression of angiogenic growth factors and prevents cardiac remodelling in a Type II diabetic rat model. Diabetologia 45, 402–415.

Jorgensen, B., Thaulow, E., 2003. Coronary Angioplasty Amlodipine Restenosis Study. Effects of amlodipine on ischemia after percutaneous transluminal coronary angioplasty: secondary results of the Coronary Angioplasty Amlodipine Restenosis (CAPARES) Study. Am. Heart. J. 145, 1030–1035.

Kobayashi, N., Kobayashi, K., Hara, K., Higashi, T., Yanaka, H., Yagi, S., Matsuoka, H., 1999. Benidipine stimulates nitric oxide synthase and improves coronary circulation in hypertensive rats. Am. J. Hypertens. 12, 483–491.

Koga, T., Claycombe, K., Meydani, M., 2002. Homocysteine increases monocyte and T-cell adhesion to human aortic endothelial cells. Atherosclerosis 161, 365–374.

Lee, Y.W., Hennig, B., Toborek, M., 2003. Redox-regulated mechanisms of IL-4-induced MCP-1 expression in human vascular endothelial cells. Am. J. Physiol., Heart Circ. Physiol. 284, H185–H192.

Li, H., Cybulsky, M.I., Gimbrone Jr., M.A., Libby, P., 1993. An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. Arterioscler. Thromb. 13, 197–204.

Lukacs, N.W., Strieter, R.M., Elner, V., Evanoff, H.L., Burdick, M.D., Kunkel, SL., 1995. Production of chemokines, interleukin-8 and monocyte chemoattractant protein-1, during monocyte: endothelial cell interactions. Blood 86, 2767–2773.

Lusis, A.J., 2000. Atherosclerosis. Nature 407, 233–241.

Marui, N., Offermann, M.K., Swerlick, R., Kunsch, C., Rosen, C.A., Ahmad, M., Alexander, R.W., Medford, R.M., 1993. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. J. Clin. Invest. 92, 1866–1874.

Muto, K., Kuroda, T., Kawato, H., Karasawa, A., Kubo, K., Nakamizo, N., 1998. Synthesis and pharmacological activity of stereoisomers of 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3,5-pyridine-dicarboxylic acid methyl 1-(phenylmethyl)-3-piperidinyl ester. Arzneimittelforschung 38, 1662–1665.

Nakashima, Y., Raines, E.W., Plump, A.S., Breslow, J.L., Ross, R., 1998. Upregulation of VCAM-1 and ICAM-1 at atherosclerosis-prone sites on the endothelium in the ApoE-deficient mouse. Arterioscler. Thromb. Vasc. Biol. 18, 842–851.

Nayler, W.G., 1999. Review of preclinical data of calcium channel blockers and atherosclerosis. J. Cardiovasc. Pharmacol. 33, S7–S11.

Nelken, N.A., Coughlin, S.R., Gordon, D., Wilcox, J.N., 1991. Monocyte chemoattractant protein-1 in human atheromatous plaques. J. Clin. Invest. 88, 1121–1127.

Oka, S., Kamata, H., Kamata, K., Yagisawa, H., Hirata, H., 2000. *N*-acetylcysteine suppresses TNF-induced NF-kappaB activation through inhibition of IkappaB kinases. FEBS Lett. 28, 196–202.

Omori, M., Ogino, T., Than, T.A., Okada, S., 2002. Monochloramine inhibits the expression of E-selectin and intercellular adhesion molecule-1 induced by TNF-alpha through the suppression of NF-kappaB activation in human endothelial cells. Free Radic. Res. 36, 845–852.

Pitt, B., Byington, R.P., Furberg, C.D., Hunninghake, D.B., Mancini, G.B., Miller, M.E., Riley, W., 2000. Effect of amlodipine on the progression of atherosclerosis and the occurrence of clinical events. PREVENT Investigators. Circulation 102, 1503–1510.

Richardson, M., Hadcock, S.J., DeReske, M., Cybulsky, M.I., 1994. Increased expression in vivo of VCAM-1 and E-selectin by the aortic endothelium of normolipemic and hyperlipemic diabetic rabbits. Arterioscler. Thromb. 14, 760–769.

Ross, R., 1989. Atherosclerosis—an inflammatory disease. N. Engl. J. Med. 340, 115–126.

Rus, H.G., Vlaicu, R., Niculescu, F., 1996. Interleukin-6 and interleukin-8 protein and gene expression in human arterial atherosclerotic wall. Atherosclerosis 127, 263–271.

Sanada, S., Node, K., Minamino, T., Takashima, S., Ogai, A., Asanuma, H., Ogita, H., Liao, Y., Asakura, M., Kim, J., Hori, M., Kitakaze, M., 2003. Long-acting Ca2+ blockers prevent myocardial remodeling induced by chronic NO inhibition in rats. Hypertension 41, 963–967.

Sundell, C.L., Somers, P.K., Meng, C.Q., Hoong, L.K., Suen, K.L., Hill, R.R., Landers, L.K., Chapman, A., Butteiger, D., Jones, M., Edwards, D., Daugherty, A., Wasserman, M.A., Alexander, R.W., Medford, R.M., Saxena, U., 2003. AGI-1067: a multifunctional phenolic antioxidant, lipid modulator, anti-inflammatory and antiatherosclerotic agent. J. Pharmacol. Exp. Ther. 305, 1116–1123.

Takeya, M., Yoshimura, T., Leonard, E.J., Takahashi, K., 1993. Detection of monocyte chemoattractant protein-1 in human atherosclerotic lesions by an anti-monocyte chemoattractant protein-1 monoclonal antibody. Human Pathol. 24, 534–539.

Tardif, J.C., Gregoire, J., Schwartz, L., Title, L., Laramee, L., Reeves, F., Lesperance, J., Bourassa, M.G., L'Allier, P.L., Glass, M., Lambert, J., Guertin, M.C., 2003. Canadian Antioxidant Restenosis Trial (CART-1) Investigators. Effects of AGI-1067 and probucol after percutaneous coronary interventions. Circulation 107, 552–558.

Van der Wal, A.C., Das, P.K., Tigges, A.J., Becker, A.E., 1992. Adhesion molecules on the endothelium and mononuclear cells in human atherosclerotic lesions. Am. J. Pathol. 141, 1427–1433.

Weber, C., Erl, W., Pietsch, A., Strobel, M., Ziegler-Heitbrock, H.W., Weber, P.C., 1994. Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-kappa B mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. Arterioscler. Thromb. 14, 1665–1673.

Wu, D., Koga, T., Martin, K.R., Meydani, M., 1999. Effect of vitamin E on human aortic endothelial cell production of chemokines and adhesion to monocytes. Atherosclerosis 147, 297–307.

Yamashita, T., Kawashima, S., Ozaki, M., Rikitake, Y., Hirase, T., Inoue, N., Hirata, K., Yokoyama, M., 2001. A calcium channel blocker, benidipine, inhibits intimal thickening in the carotid artery of mice by increasing nitric oxide production. J. Hypertens. 19, 451–458.

Yao, K., Ina, Y., Nagashima, K., Ohmori, K., Ohno, T., 2000. Antioxidant effects of calcium antagonists in rat brain homogenates. Biol. Pharm. Bull. 23, 766–769.

Yao, K., Ina, Y., Sonoda, R., Nagashima, K., Ohmori, K., Ohno, T., 2003. Protective effects of benidipine on hydrogen peroxide-induced injury in rat isolated hearts. J. Pharm. Pharmacol. 55, 109–114.

Zapolska-Downar, D., Zapolski-Downar, A., Markiewski, M., Ciechanowicz, A., Kaczmarczyk, M., Naruszewicz, M., 2000. Selective inhibition by alpha-tocopherol of vascular cell adhesion molecule-1

expression in human vascular endothelial cells. Biochem. Biophys. Res. Commun. 274, 609–615.

Zapolska-Downar, D., Zapolski-Downar, A., Markiewski, M., Ciechanowicz, A., Kaczmarczyk, M., Naruszewicz, M., 2001. Selective inhibition by probucol of vascular cell adhesion molecule-1 (VCAM-1) expression in human vascular endothelial cells. Atherosclerosis 155, 123–130.

Zhang, X., Wang, L.Y., Jiang, T.Y., Zhang, H.P., Dou, Y., Zhao, J.H., Zhao, H., Qiao, Z.D., Qiao, J.T., 2002. Effects of testosterone and 17-beta-estradiol on TNF-alpha-induced E-selectin and VCAM-1 expression in endothelial cells. Analysis of the underlying receptor pathways. Life Sci. 71, 15–29.