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# Cilostazol Suppresses Superoxide Production and Expression of Adhesion Molecules in Human Endothelial Cells via Mediation of cAMP-dependent Protein Kinase-mediated Maxi-K Channel Activation

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***Running title*:** Cilostazol via cAMP-PK-mediated maxi-K channel opening

***Abbreviation***: BKCa, large conductance $Ca^{2+}$-activated $K^{+}$; PKA, cAMP-dependent protein kinase; PKG, cGMP-dependent protein kinase; NF-κB, nuclear factor-kappaB; VCAM-1, vascular cell adhesion molecule-1: MCP-1, monocyte chemoattractant protein-1.

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**Abstract**

This study shows whether increased intracellular cAMP level by cilostazol is directly coupled to its maxi-K channel activation in human endothelial cells. Cilostazol (1 μM) increased the $K^+$ currents in the human endothelial cells by activating maxi-K channels, which was abolished by iberiotoxin (100 nM), a maxi-K channel blocker. Upon incubation of human coronary artery endothelial cells with TNF-α (50 ng/ml), monocyte adhesion significantly increased with increased superoxide generation and expression of VCAM-1 and MCP-1 accompanied by increased degradation of IκBα in cytoplasm, and activation of NF-κB p65 in nucleus. All these variables were significantly suppressed by cilostazol (10 μM), which were antagonized by iberiotoxin (1 μM) as well as by KT5720 (300 nM, cAMP-dependent protein kinase inhibitor), but not by KT5823 (300 nM, cGMP-dependent protein kinase inhibitor). In the human endothelial cells transfected with siRNA targeting maxi-K channels, cilostazol did not suppress the superoxide generation, VCAM-1 and MCP-1 expressions, and monocyte adhesion as contrasted to the wild type cells. These findings were similarly evident with BMS 204352, a maxi-K channel opener, and forskolin and dibutyryl cAMP. In conclusion, increased cAMP level by cilostazol is directly coupled to its maxi-K channel opening action via protein kinase activation in human endothelial cells, thereby suppressing TNF-α–stimulated superoxide production and expression of adhesion molecules.

## Introduction

It is well established that potassium channel activity is the main determinant of membrane potential, and associated $K^+$ efflux causes hyperpolarization, thereby leading to inhibition of voltage-gated calcium channels and promotion of vascular relaxation. Although multiple classes of potassium channels are expressed at varying densities in different vascular beds, the large conductance $Ca^{2+}$-activated $K^+$ (BKCa) channels is the predominant potassium channel species in most arteries (Nelson and Quayle, 1995). Specifically, increased cyclic nucleotide-linked protein kinase A and protein kinase G levels promote opening of the BKCa channels, which leads to the membrane hyperpolarization and inhibits voltage-gated calcium channels (Torphy, 1994).

Cilostazol has been introduced to increase the intracellular cyclic AMP level by blocking its hydrolysis by type III phosphodiesterase (Kimura et al., 1985). Its actions to inhibit platelet aggregation and thrombosis are ascribed to the increased cyclic AMP level in platelets and vascular smooth muscles (Tanaka et al., 1989). Most recently, cilostazol was demonstrated to increase the outward $K^+$ currents in SK-N-SH cells (human brain neuroblastoma cells, Hong et al., 2003) by opening of the maxi-$K^+$ channels. On the other hand, the presence of large conductance $Ca^{2+}$-activated $K^+$ channels has been shown in many types of endothelial cells (Rusko et al. 1992; Baron et al. 1996; Nilius et al. 1997) as well as in the EA.hy926 cell line (Haburcak et al. 1997). The maxi-K channel mRNA expression of the $\alpha$ subunit, but not $\beta$ subunit, was detected in the endothelium (Papassotiriou et al., 2000).

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Recently, Shin et al. (2004) have reported that cilostazol significantly suppressed remnant lipoprotein particles-stimulated NAD(P)H oxidase–dependent superoxide formation and cytokine production in human umbilical vein endothelial cells. Most recently, Park et al. (2005) have addressed that cilostazol significantly reduced expression of adhesion molecules and chemokines in association with reduced monocyte adhesion to human umbilical vein endothelial cells. Additionally, the anti-atherogenic effect of cilostazol in cholesterol-fed Ldlr-null mice was ascribed to its property to suppress superoxide and TNF-α formation, thereby reducing NF-κB activation, VCAM-1/MCP-1 expressions, and monocyte recruitments (Lee et al., 2005). However, it is not clear whether increase in cAMP level and activation of PKA by cilostazol are directly coupled to its opening action of maxi-K channels in the human endothelial cells. Given the PKA activation is linked to the maxi-K channel opening in the human endothelial cells, it is likely that cilostazol effects to suppress the NAD(P)H oxidase–dependent superoxide production and to inhibit the expression of adhesion molecules in association with reduced monocyte adhesion to the endothelial cells might be significantly inhibited by PKA inhibitor and maxi-K channel blocker.

The present study was designed to (1) determine whether in the human endothelial cells cilostazol increases the outward $K^+$ currents, (2) suppresses the NAD(P)H oxidase–dependent superoxide production, (3) inhibits the expressions of adhesion molecule and chemokine including VCAM-1 and MCP-1, and (4) inhibits activation of transcription factor nuclear factor-kappaB (NF-κB) in the absence and the presence of cAMP-dependent protein kinase (PKA) inhibitors (KT 5720 and Rp-

cAMPS) and cGMP-dependent protein kinase (PKG) inhibitors (KT 5823 and Rp-cGMPS) and maxi-K channel blocker (iberiotoxin). Further, to confirm implication of the maxi-K channels in the action of cilostazol, we identified whether cilostazol suppresses the superoxide production and VCAM-1 expression in the human endothelial cells transfected with oligonucleotide of maxi-K channel siRNA to knockout the channels.

## Materials and Methods

**Cell Cultures.** Two human endothelial cells were used: For the electrophysiological study human umbilical vein endothelial cells [CRL-1730, American Type Culture Collection (Manassas, VA)] and for the biochemical study human coronary artery endothelial cells (cc-2585; Cambrex, Walkersville, MD) were cultured in the endothelial cell basal media-2 (EGM-2) Bullet kit media (Clonetics, BioWhittaker, San Diego, CA). Cells were grown to confluence at 37°C in 5% $CO_2$ and used for experiments at not greater than passage 6. U937 (CRL-1593.2; American Type Culture Collection, Manassas, VA) cells were cultured in RPMI 1640 medium containing 10% fetal bovine serum.

**Recording of the Whole-cell $K^+$ Current.** The membrane currents were recorded in the whole-cell configuration of the human umbilical vein endothelial cells, using Axopatch 1C amplifier (Axon instruments, Union, CA). All experimental parameters, such as pulse generation and data acquisition, were controlled using the PatchPro software. Recording electrodes were pulled from thin-walled borosilicate capillaries (Clark Electromedical Instruments, Pangbourne, UK) using a PP-83 vertical puller (Narishige, Tokyo, Japan). The voltage and current signals were filtered at 0.5 ~ 1.0 kHz and were sampled at a rate of 1 ~ 3 kHz. We used patch pipettes with a resistance of 3 ~ 4 MΩ. The normal bath solution (mM) for the whole-cell recordings was: NaCl, 140; KCl, 5.4; $NaH_2PO_4$, 0.33; $CaCl_2$, 1.8; $MgCl_2$, 0.5; HEPES, 5; glucose, 16.6; adjusted to pH 7.4 with NaOH. Pipettes were filled with (mM): K-aspartate, 100; KCl, 25; NaCl, 5; $MgCl_2$, 1; Mg-ATP, 4; EGTA 10; $Ca_2Cl_2$ 6; HEPES, 10;

with the pH adjusted to 7.25 using KOH (pCa = 6.5).

**Measurement of Superoxide Anion.** Confluent cells were exposed to TNF-α for 24 hours. Human coronary artery endothelial cell homogenates (100 μg protein/well) were placed into the luminometer (Microlumat LB96P, EG & G Berthold, Wildbad, Germany). Immediately before recording chemiluminescence, NADH and NADPH (final concentration, 100 μmol/L each) were added, and dark-adapted lucigenin (*bis*-N-methylacridinium nitrate, 5 μmol/L) was added via an autodispenser. Each experiment was performed in triplicate.

**VCAM-1 assay.** Endothelial cells were plated on the 96-well tissue culture plates at a density of $2\times10^4$ cells/well. Confluent cells received EGM-2 with 0.5% fetal bovine serum plus various concentrations of drugs 15 min before stimulation with TNF-α, and then were exposed to TNF-α for 4 hours. After treatment with 2% paraformaldehyde, the cells were washing twice with Hank's balanced salt solution, and then incubated for 1 h with antibodies specific to human VCAM-1 (R&D Systems, Minneapolis, ML). The cells were incubated for 1 h with second antibody. After washed the wells four times, then the second antibody binding was detected by reaction of tetramethylbenzidine with $H_2O_2$ (TMB Substrate Reagent Set; BD Biosciences, Bedford, MA). The absorbance at 450 nm was measured using ELISA reader (Bio-Tek instruments, Winooski, VT).

**MCP-1 Assay.** For analysis of MCP-1 level in supernatants, endothelial cells were seeded at

$1\times10^5$ cells/well in the 48-well tissue culture plates for 24 h. To quantify the amount of MCP-1 protein, the conditioned media derived from confluent endothelial cells were isolated and centrifuged to remove nonadherent cell debris. MCP-1 levels in the supernatant were analyzed by ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacture's instruction.

**Adhesion Assay.** For the adhesion assay, endothelial cells were plated on six-well dishes at a density of $1\times10^6$ cells/well. On the next day, confluent cells received EGM-2 with 0.5% fetal bovine serum plus drug 15 min before stimulation with TNF-α, and then were exposed to TNF-α for 4 h. Thereafter, human monocytoid U937 cells ($3\times10^5$ cells/well) were added to each monolayer and incubated under rotating conditions (63 rpm) at room temperature. Ten minutes later, nonadhering cells were removed by gentle washing with MCDB131 (Invitrogen, Carsbad, CA), and monolayers were fixed with 1% paraformaldehyde.

**Western Blot Analyses.** After exposure to TNF-α for 4 h, cells were lysed in lysis buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 10% glycerol, 100 μg/mL phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 5 mM sodium flouride. For preparation of nuclear extracts, cells were lysed in buffer A (10 mM HEPES, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.25% Tween 20, 1 mM dithotheritol, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 15 μg/mL aprotinin). After 5 min incubation at 4°C, nuclei were collected by

centrifugation at 4000 rpm, and the pellets were resuspended in buffer B (20 mM HEPES, 25% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.25% Tween 20, 0.2 mM EDTA, 1 mM dithiothreitol, 100 μg/mL phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, and 15 μg/mL aprotinin) and centrifuged at 12000 rpm, 30 μg of total protein of each sample was loaded into 10% SDS-polyacrylamide electrophoresis gel and transferred to nitrocellulose membrane (Amersham Biosciences, Inc., Piscataway, NJ). The blocked membrane was then incubated with antibodies to NF-κB p65, IκB-α and and Maxi-K α-subunit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The immunoreactive bands visualized with chemiluminescent reagent of the Supersignal West Dura Extended Duration Substrate Kit (Pierce Chemical, Rockford, IL). The signals of the bands were quantified using the GS-710 caliberated imaging densitometer (Bio-Rad, Hercules, CA). The results were expressed as a relative density.

**Maxi-K channel knockdown using Stealth™ siRNA oligonucleotide.** The Stealth™ siRNA oligonucleotide was synthesized by Invitrogen with following sequence: complementary to human maxi-K mRNA (Genbank accession nos. nm 002547, beginning at position 1524 target sequence site) : The oligonucleotides of Maxi-K siRNA were sense, 5'-CCUGGAGCUUGAAGCUCUGUUCAAA-3', and antisense, 5'-UUUGAACAGAGCUUCAAGCUCCAGG-3'. The stealth™ siRNA negative control Duplex (Invitrogen) was used as a control oligonucleotide. Transfection efficiency was monitored using a fluorescent oligonucleotide (BLOCK-iT Fluorescent oligo, Invitrogen) and

estimated to be approximately 80 ~ 85%.

At 50-70% confluence, the stealth™ siRNA molecules were transfected into the endothelial cells using Lipofectamine 2000 and Plus reagent following Invitrogen's protocols. The final concentration of 100 nM stealth™ siRNA oligonucleotide was empirically determined to maximally suppress target RNA expression, and the stealth™ siRNA oligonucleotide was transfected to the medium 48 h prior to the drug treatment. The ability of the stealth™ siRNA oligonucleotide to knockdown maxi-K channel expression was analyzed by maxi-K channel protein detection using anti-maxi K channel antibody and by maxi-K channel gene detection using reverse transcription PCR on the whole cell extracts. The expressions of maxi-K channel gene and protein in the stealth™ siRNA oligonucleotide transfected-endothelial cells were inhibited to approximately 20% of control.

After siRNA transfection, expressions of the Maxi-K channel mRNA and protein were determined by reverse transcription-polymerase chain reaction (RT-PCR) and Western blot assays. Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH). PCR was performed according to the manufacturer's specifications with the following primers: Maxi-K sense, 5'-AGATTGAGGAAGACACATG-3'; antisense, 5'-CAATGGCTATTAGGAGC-3'. β-actin cDNA as an internal control was amplified by use of sense, 5'-TCATGAAGTGTGACGTTGACATCCGT-3'; and antisense 5'-CCTAGAAGCATTTGCGGTGCACGATG-3' primers.

**Drugs.** Cilostazol (OPC-13013) [6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)butoxy]-3,4-dihydro-2-

(1*H*)-quinolinone], generously donated from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan), was dissolved in dimethyl sulfoxide as a 10 mM stock solution. TNF-α (Upstate Biotechnology, Lake Placid, NY) was dissolved in the phosphate-buffered saline as a 10 μg/ml stock solution. Iberiotoxin was from Upstate Biotechnology. BMS 204352 was generously donated from the Korea Research Institute of Chemical Technology (Daejon, Korea) and dissolved in dimethyl sulfoxide as a 10 mM stock solution. Others were forskolin (Tocris Cookson Inc., Ballwin, MO) and dibutyryl cAMP (Sigma-Aldrich, St. Louis, MO), and Rp-cGMPs and Rp-cAMPs (Alexis Biochemicals, San Diego, CA).

**Statistical analyses.** Data are expressed as mean ± S.E.M. The comparison of changes in variable parameters between vehicle and cilostazol treatment groups without and with inhibitors was analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests as a post hoc comparison. Student's *t*-test was used for analysis of values between the data of wild type and siRNA-transfected groups. A value of $P < 0.05$ was considered to be statistically significant.

**Results**

**Activation of Maxi-K Channels by Cilostazol.** Action of cilostazol to increase the activity of maxi-K channels was identified in the whole-cell configuration. To record the BKCa currents, the cells were bathed in normal Tyrode's solution. The outward currents were generated by a depolarizing voltage step from -60 to +80 mV (Fig. 1A). These currents were mostly blocked by application of iberiotoxin (100 nM, Fig. 1B). The fraction of the current that was iberiotoxin-sensitive was determined by subtracting the currents in the presence of 100 nM iberiotoxin from that of the control condition (Fig. 1C). Within 2 min of exposing the cells to cilostazol, the amplitude of iberiotoxin-sensitive BKCa currents was greatly increased throughout the entire range of the voltage (Fig. 1D~F). At +70 mV, cilostazol increased the currents from 382.12 ± 53.26 pA to 766.02 ± 99.63 pA at 1 μM, 1442.38 ± 123.15 pA at 10 μM, and 1766 ± 110.23 pA at 30 μM. This stimulatory effect was almost reversed (> 90%, data not shown) when cilostazol was washed out. Fig. 1G summarizes the current-voltage (*I-V*) relationship of the cilostazol-induced increases of the BKCa currents. Taken together, cilostazol (1, 10, and 30 μM at +70 mV) significantly and concentration-dependently increased the steady-state $K^+$ outward currents ($P < 0.05$, $P < 0.01$).

To test whether the increase of iberiotoxin-sensitive BKCa currents by cilostazol was mediated by the PKA and/or PKG pathway, we determined the effect of PKA inhibitor, KT 5720, and PKG inhibitor, KT 5823, on the increase of iberiotoxin-sensitive BKCa currents stimulated by cilostazol. KT 5720 and KT 5823 (300 nM, each) alone had no effect on iberiotoxin-sensitive BKCa currents (Fig. 2). By contrast, KT 5720 (300 nM) significantly reduced cilostazol-induced increase of iberiotoxin-sensitive BKCa currents (control: 352.11 ± 25.32 pA; KT 5720 alone: 364.43 ± 30.25 pA; KT 5720 + cilostazol (1 μM): 395.89 ± 35.54 pA, Fig. 2C), whereas the PKG inhibition by KT 5823 had no effect on the cilostazol-induced increase of iberiotoxin-sensitive BKCa currents (Control: 452.37 ± 35.63

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pA; KT 5823 (300 nM) alone: 431.44 ± 45.85 pA; KT 5823 + cilostazol (1 μM): 802.98 ± 88.96 pA) (Fig 2F). These results suggest that PKA stimulation is responsible for cilostazol-stimulated increased BKCa currents.

**TNF-α-Induced NAD(P)H Oxidase–Dependent Superoxide Production.** When measured by recording of chemiluminescence, superoxide production from the endothelial cells in response to NAD(P)H (control, 7.7 ± 1.0 counts $s^{-1}$ mg $protein^{-1}$) was significantly elevated by incubation of endothelial cells in the medium containing 50 ng/mL TNF-α (18.7 ± 1.6 counts $s^{-1}$ mg $protein^{-1}$, $P < 0.001$) for 24 h (Fig. 3). TNF-α-stimulated superoxide formation was markedly reduced by application of cilostazol (10 μM ) to 11.7± 1.9 counts $s^{-1}$ mg $protein^{-1}$ ($P < 0.05$). Cilostazol (10 μM)-induced decreased superoxide level was significantly reversed by PKA inhibitors, KT 5720 (300 nM, $P < 0.05$) and Rp-cAMPS (30 nM, $P < 0.05$), respectively, but little affected by PKG inhibitors, KT 5823 (300 nM) and Rp-cGMPS (30 nM). Cilostazol-induced suppression of superoxide production was significantly antagonized by 1 μM of iberiotoxin, a maxi-K channel blocker ($P < 0.05$) (Fig. 3).

**TNF-α-stimulated VCAM-1 and MCP-1 Expression.** When endothelial cells were incubated with increasing concentration of TNF-α (1 ~ 100 ng/mL) for 4 h to determine VCAM-1, expression of VCAM-1 concentration-dependently increased and reached a maximum at 10 to 50 ng/mL of TNF-α (Fig. 4A Insets). TNF-α (50 ng/mL)-stimulated increased VCAM-1 expression was concentration-dependently suppressed by cilostazol (10 and 30 μM, $P < 0.01$). Cilostazol (10 μM)-suppressed

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VCAM-1 level was reversed by KT 5720 (300 nM, $P < 0.05$) and 1 μM of iberiotoxin, ($P < 0.05$), but it was little affected by KT 5823 (300 nM) (Fig. 4A).

MCP-1 level was measured in the supernatant after incubation for 24 h. Similarly, TNF-α (50 ng/mL)-stimulated increased MCP-1 expression was significantly suppressed by cilostazol (10 μM, $P < 0.001$), which was reversed by KT 5720 (300 nM, $P < 0.05$) and Rp-cAMPS (30 nM, $P < 0.05$), respectively, and 1 μM of iberiotoxin, ($P < 0.05$), but it was not affected by either KT 5823 (300 nM) or Rp-cGMPS (Fig. 4B).

**TNF-α-stimulated Adhesion of Monocytes.** Incubation of endothelial cells with TNF-α (50 ng/mL) for 4 h (basal levels, 7.01 ± 2.11 cells/mm$^2$) caused increase in monocyte adhesion to 57.01 ± 3.84 cells/mm$^2$ (8-fold). The increased adhesion of monocytes to human endothelial cells was significantly suppressed by cilostazol (10 μM, $P < 0.001$), which was markedly antagonized by iberiotoxin (1 μM, $P < 0.05$). Cilostazol (10 μM)-induced suppressed monocyte adhesion was significantly reversed by KT 5720 (300 nM, $P < 0.05$) and 1 μM of iberiotoxin, ($P < 0.05$), but not by KT 5823 (300 nM) (Fig. 5).

**TNF-α-stimulated Degradation of IκBα and Activation of NF-κB.** To examine whether cilostazol action to suppress the transcription factor NF-κB activation is inhibited by the PKA/PKG inhibitors, we assessed degradation of IκBα in the cytoplasm and activation of NF-κB in the nuclear

extracts under treatment with each protein kinase inhibitor by Western blotting. Treatment of human endothelial cells with TNF-α (50 ng/mL) significantly increased degradation of IκBα ($P < 0.001$) and markedly activated NF-κB p65 ($P < 0.01$). TNF-α-induced IκBα degradation and NF-κB p65 activation were significantly reversed by cilostazol (10 μM), all of which were antagonized by iberiotoxin (1 μM, $P < 0.05$) and KT 5720 (300 nM, $P < 0.05$), but not by KT 5823 (300 nM) (Fig. 6).

**Analysis of Maxi-K Channel Knockdown Cells.** The ability of the stealth™ siRNA oligonucleotide to knockdown the maxi-K channel expression was analyzed by RT-PCR and Western blot assay in the whole cell extracts. When two types of human maxi-K mRNA beginning at position of 1524 and 1726 target sequences were tried, the transfection efficiency of the former one was more prominent than the latter (74.6% and 50% measured by the quantity of mRNA, respectively). Transfection with siRNA oligonucleotide to the endothelial cells resulted in approximately 82% ($P < 0.001$) and 77% ($P < 0.001$) reduction in maxi-K channel mRNA and protein expression, respectively (Fig. 7A, B).

To further confirm implication of the maxi-K channel in the biochemical effects of cilostazol, we tested whether cilostazol increases the BKCa currents on the human endothelial cells transfected with maxi-K channel siRNA oligonucleotide. In the cells transfected with the stealth™ siRNA negative control used as a control oligonucleotide, cilostazol (1 μM) showed increases in BKCa currents as shown in the wild type cells (Fig. 7C). However, recordings of $K^+$ outward currents were

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significantly reduced by the knockdown of BKCa channels by transfection with siRNA oligonucleotide. Application of 1 μM cilostazol did not increase the outward $K^+$ current (control: 283.13 ± 18.99 pA, cilostazol: 298.58 ± 22.58 pA, at +70 mV, Fig. 7Da,b). Application of iberiotoxin (100 nM) with cilostazol was also without effect on the outward current (control: 283.13 ± 18.99 pA, cilostazol plus iberiotoxin: 259.43 ± 20.63 pA, at +70 mV, Fig. 7Dc). Knockdown of BKCa channels by transfection with siRNA oligonucleotide abrogated the BKCa currents by approximately 92% based on the responsiveness of iberiotoxin, indicating that cilostazol increases the outward $K^+$ currents in the human endothelial cells by opening of the maxi-K channels.

**Effects of Maxi-K Channel Knockdown on the Cilostazol Actions. T**ransfection of stealth™ siRNA oligonucleotide (maxi-K mRNA beginning at position of 1524 target sequence) to the endothelial cells resulted in the loss of cilostazol effect to suppress the TNF-α-stimulated superoxide production (Fig. 8A), VCAM-1 expression (Fig. 8B) and monocyte adhesion to endothelial cells (Fig. 8C) as contrasted to the wild type endothelial cells. Similar findings were evident with BMS-204352 (10 μM), a maxi-K channel opener. Likewise, forskolin, adenylate cyclase activator and dibutyryl cAMP did not elicit the suppression of the TNF-α-stimulated superoxide production (Fig. 8A), VCAM-1 expression (Fig. 8B) and monocyte adhesion to endothelial cells (Fig. 8C) in the endothelial cells transfected with siRNA. These results provide conclusive evidence that maxi-K channel activation is required for the actions of cilostazol and cAMP in the human endothelial cells.

**Discussion**

The present study shows that 1) cilostazol increased the outward $K^+$ current in the endothelial cells by opening of the maxi-K channels, and 2) cilostazol significantly suppressed TNF-α-induced increased NAD(P)H oxidase–dependent superoxide production, expression of VCAM-1, MCP-1 and monocyte adhesion in association with IκBα degradation and NF-κB p65 activation, all of which were significantly reversed by PKA inhibitors, KT 5720 and Rp-cAMPS and iberiotoxin, a maxi-K channel blocker, but not by PKG inhibitors, KT 5823 and Rp-cGMPS. Upon transfection with siRNA oligonucleotide targeting the maxi-K channel α subunit of the endothelial cells, cilostazol effects to suppress the TNF-α-stimulated superoxide production and VCAM-1 expression were almost abolished as contrasted to those shown in the wild type endothelial cells. The similar findings were evident with BMS-204352 (a maxi-K channel opener), forskolin (an adenylate cyclase activator) and dibutyryl cAMP (a membrane-permeable derivative of cAMP).

Cilostazol is known to increase intracellular cyclic AMP level by inhibiting phosphodiesterase type III (Kimura et al., 1985). Lee et al. (2005) observed a significant elevation of cAMP expression in the proximal ascending aorta including aortic sinus of mice fed 0.2% w/w cilostazol-supplemented high-fat diet. Recently, Hong et al. (2003) found that cilostazol increased the outward $K^+$ current in the SK-N-SH cells (human brain neuroblastoma cell line) by activating maxi-K channels (large conductance $Ca^{2+}$-activated $K^+$ channels) that is antagonized by iberiotoxin, a maxi-K channel blocker (Galvez et al., 1990). In agreement with these results, the present electrophysiological study showed

an increase in outward $K^+$ currents in the endothelial cells which was antagonizable by iberiotoxin. Our results further revealed that cilostazol significantly suppressed TNF-α-stimulated increase in monocyte adhesion with suppression of VCAM-1 and MCP-1 expression and reduction of NAD(P)H oxidase-dependent superoxide generation in consistent with the findings of Park et al. (2005). All these variables were significantly antagonized by iberiotoxin, a maxi-K channel blocker, and more intriguingly reversed by PKA inhibitors, KT 5720 and Rp-cAMPS, and but not by PKG inhibitors, KT 5823 and Rp-cGMPS.

NF-κB (a redox-sensitive transcription factor) is present in the cytosol as a heterodimer composed of NF-κB1(p50) and Rel (p65) subunits bound to the inhibitor protein IκBα/β. Upon activation, NF-κB translocates from the cytosol to the nucleus of the cell, binds to specific DNA sequences, and initiates transcription (Baeuerle, 1998). As shown in the results of Park et al. (2005), our present results also showed that cilostazol significantly suppressed not only TNF-α-induced IκBα degradation but also TNF-α-induced activation and translocation of the NF-κB p65 subunit into the nucleus. Further, cilostazol effects were consistently antagonized by iberiotoxin ($P < 0.05$) and KT 5720 ($P < 0.05$), but not by KT 5823. These results are supported by the reports of Ollivier et al. (1996) and Aizawa et al. (2003), in that PKA activation by cAMP-elevating agents, such as forskolin and dibutyryl cAMP, inhibited TNF-α-induced NF-κB-dependent reporter gene expression and reduced NF-κB–dependent adhesion molecule and chemokine expressions. Thus, it is likely indicated that suppression by cilostazol of NF-κB nuclear transcription is mediated via PKA activation and maxi-K

channel opening. However, PKG activation is not implicated in the cilostazol effects.

To define the direct relationships between cyclic AMP and maxi-K channel opening induced by cilostazol in the endothelial cells, we identified whether cilostazol suppresses TNF-α-stimulated superoxide production and VCAM-1 expression in the endothelial cells transfected with siRNA targeting the maxi-K channel α-subunit in comparison with those in the wild type endothelial cells. In the endothelial cells transfected with siRNAs, cilostazol failed to activate the outward $K^+$ currents in line with loss of suppression of the superoxide production and VCAM-1 expression and monocyte adhesion as contrasted to those shown in the wild type endothelial cells. Similarly, BMS-204352 (Cheney et al., 2001, maxi-$K^+$ channel opener), forskolin (adenylate cyclase activator) and dibutyryl cAMP (a membrane-permeable derivative of cAMP) also showed lack of their effects to suppress superoxide production, VCAM-1 expression and monocyte adhesion. These results indicate that maxi-K channel activation is critically implicated in the actions of cilostazol and cAMP in the human endothelial cells.

In the smooth muscle cells, cross-activation of the cGMP-dependent protein kinase has been demonstrated, in that the catalytic subunit of protein kinase A opens the large conductance $Ca^{2+}$-activated $K^+$ channels in patches from rat tail artery myocytes (Schubert et al., 1999), and cAMP-stimulating agents enhance the activity of the large conductance $Ca^{2+}$-activated $K^+$ channels in myocytes from coronary arteries (White et al., 2000). Moreover, Barman et al. (2003) have documented that cAMP-elevating agents (forskolin and 8–4-chlorophenylthio-cAMP) open BKCa

channels in cAMP-induced pulmonary vasodilation. They addressed that treatment with KT5823 (PKG inhibitor), but not KT 5720 (PKA inhibitor), inhibited the effect of cAMP-stimulated BKCa channel activity indicating PKG-dependent and PKA-independent signaling pathways, indicative of the striking contrast to the endothelial cells. On the other hand, Watanabe et al. (2003) have reported that cilostazol increases coronary flow reserve and flow-dependent coronary dilation attributing to nitric oxide in patients with vasospastic angina. However, Haburcak et al. (1997) early emphasized that nitric oxide alone neither activated BKCa channels directly nor modulated preactivated BKCa channels in cultured EA.hy926 cells, indicating lack of a direct modulatory effect of PKG activity on large conductance BKCa channels in cultured endothelial cells. Thus it is speculated that even though cilostazol releases nitric oxide from endothelial cells (Nakamura et al., 2001), nitric oxide-stimulated cGMP-dependent protein kinase activity is not implicated in the regulation of maxi-K channel activation in the human endothelial cells.

In conclusion, cilostazol inhibits TNF-α–stimulated superoxide production and expression of adhesion molecules and chemokines, and consequently suppresses monocyte adhesion to the endothelial cells via mediation of cAMP-dependent protein kinase-coupled maxi-K channel opening.

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**Footnotes**

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**Figure Legends**

Fig. 1. Effect of cilostazol on the Maxi-K channels in human umbilical vein endothelial cells in the whole-cell configuration. Current traces are shown for 600-ms depolarizing pulses from –80 mV to voltages between –60 mV and +60 mV in steps of 10 mV in control conditions (A) and in the presence of 100 nM iberiotoxin (Ibtx) (B). Ibtx -sensitive currents are shown in panel (C). D~F: The effect of cilostazol (1, 10 and 30 μM) on Ibtx-sensitive BKCa currents. G: Current-voltage (I-V) relationships of Ibtx-sensitive BKCa currents in the absence (○) and presence of 1 μM (●), 10 μM (□), and 30 μM (▲) cilostazol, respectively. The current-voltage relationships from six cells. Cilostazol (1, 10, and 30 μM at +70 mV) significantly and concentration-dependently increased the steady-state $K^+$ outward currents ($P < 0.05$, $P < 0.01$).

Fig. 2. Upper: Effect of 300 nM KT 5720 (A) on the cilostazol-stimulated Ibtx-sensitive BKCa currents (B). C: Summary of Ibtx-sensitive BKCa currents obtained from control, KT 5720 (300 nM), and KT 5720 (300 nM) + 1 μM cilostazol at +70 mV. Lower: Effect of 300 nM KT 5823 (D) on the cilostazol-stimulated Ibtx-sensitive BKCa currents (E).F: Summary of Ibtx-sensitive BKCa currents obtained from control, KT 5823 (300 nM), and KT 5823 (300 nM) + 1 μM cilostazol at +70 mV. Results are expressed as mean ± S.E.M. from 6 experiments. $^{\dagger\dagger}P < 0.01$ vs. KT 5823 alone.

Fig. 3. Inhibitory effect of cilostazol on the TNF-α-stimulated NAD(P)H oxidase-dependent superoxide production in the endothelial cells in the presence of KT 5720 and Rp-cAMPS (inhibitors

of cAMP-dependent protein kinase activity) and KT 5823 and Rp-cGMPS (inhibitors of cGMP-dependent protein kinase activity) in comparison with effect of iberiotoxin (a maxi-$K^+$ channel blocker). Results are expressed as mean ± S.E.M. of 6 experiments. $^{###}$ $P < 0.001$ vs. None; *** $P < 0.001$ vs. TNF-α alone. $^{††}$ $P < 0.01$; $^{†††}$ $P < 0.001$ vs. Cilostazol alone.

Fig. 4. (A) Inhibitory effect of cilostazol on the TNF-α-stimulated VCAM-1 expression in the endothelial cells in the presence of KT 5720 and KT 5823 in comparison with iberiotoxin. Inset: Concentration-dependent increase in VCAM-1 expression by TNF-α. (B) Inhibitory effect of cilostazol on the TNF-α-stimulated MCP-1 expression in the presence of KT 5720 and Rp-cAMPS (inhibitors of cAMP-dependent protein kinase activity) and KT 5823 and Rp-cGMPS (inhibitors of cGMP-dependent protein kinase activity) in comparison with iberiotoxin. Inset: Concentration-dependent increase in MCP-1 expression by TNF-α. Results are expressed as mean ± S.E.M. of 4 experiments. . $^{#}$ $P < 0.05$; $^{##}$ $P < 0.01$; $^{###}$ $P < 0.001$ vs. None; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ vs. TNF-α alone; $^{†}$ $P < 0.05$; $^{††}$ $P < 0.01$; $^{†††}$ $P < 0.001$ vs. 10 μM Cilostazol alone.

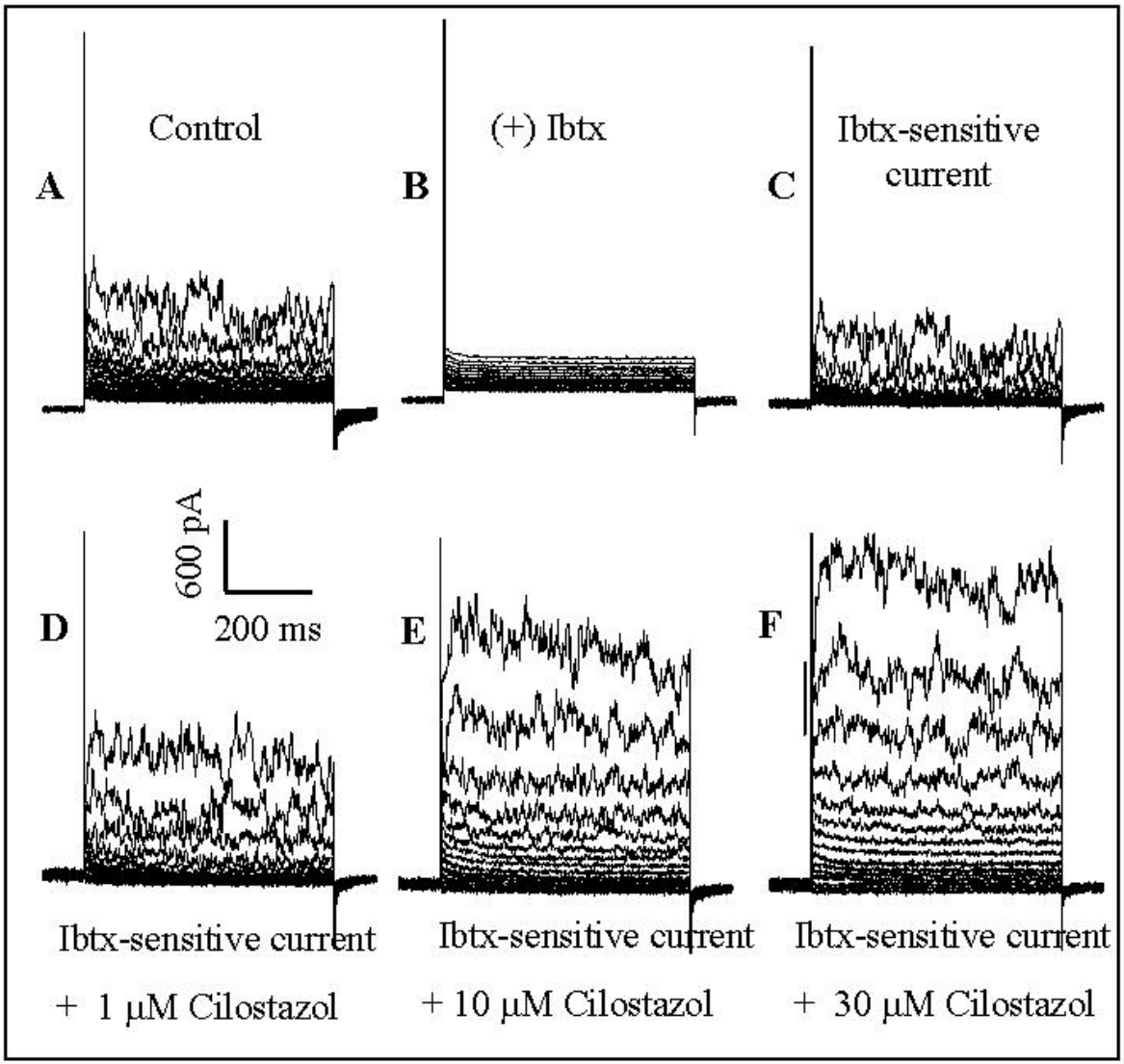
Fig. 5. Inhibitory effect of cilostazol on the TNF-α-stimulated monocyte adhesion to the endothelial cells in the presence of KT 5720 and KT 5823 in comparison with effect of iberiotoxin. Results are expressed as mean ± S.E.M. of 4 experiments. $^{###}$ $P < 0.001$ vs. None; *** $P < 0.001$ vs. TNF-α alone; $^{††}$ $P < 0.01$; $^{†††}$ $P < 0.001$ vs. 10 μM Cilostazol alone.

Fig. 6. Western blot assays (upper) and densitometric analysis (lower). Inhibition by cilostazol on the TNF-α-stimulated NF-κB activation in the nucleus and IκBα degradation in the cytoplasm in the presence of KT 5720 and KT 5823 in comparison with effect of iberiotoxin. Results are expressed as mean ± S.E.M. of 4 experiments. $^{\#\#}$ $P < 0.01$; $^{\#\#\#}$ $P < 0.001$ vs. None; * $P < 0.05$; ** $P < 0.01$ vs. TNF-α alone; $^{\dagger}$ $P < 0.05$; $^{\dagger\dagger}$ $P < 0.01$ vs. 10 μM Cilostazol alone.

Fig. 7. Analysis of maxi-K channel-knockdown cells. The ability of the stealth™ siRNA oligonucleotide to abrogate maxi-K channel expression was analyzed by RT-PCR (A) and Western blot assay (B) in the whole cell extracts. Transfection with stealth™ siRNA oligonucleotide in the human endothelial cells resulted in approximately 18% and 23% reduction in maxi-K channel gene and protein expression, respectively. The experiment was repeated three times each with duplicates. Results are expressed as means ± S.E.M. *** $P< 0.001$ vs. Negative control. (C) The stealth™ siRNA negative control Duplex (Invitrogen) was used as a control oligonucleotide, and effect of cilostazol was examined on the negative control cells. (D) Effect of cilostazol on the maxi-K channel-knockdown cells. Superimposed current traces were elicited by 600 ms pulses between -60 mV and +60 mV from a holding potential of -80 mV under the presence of vehicle (a), 1 μM cilostazol (b), and (c) 1 μM cilostazol + iberiotoxin (Ibtx, 100 nM). The current traces are representative from 3 experiments.

Fig. 8. TNF-α-induced NAD(P)H oxidase-dependent superoxide (A) and VCAM-1 expression (B) and

adhesion of monocytes (C) in the human coronary artery endothelial cells of the wild type (WT), and cells transfected with maxi-K channel siRNA oligonucleotide. In the cells transfected with siRNA oligonucleotide, TNF-α-stimulated variables were not affected by cilostazol (10 μM), BMS-204352 (10 μM) and forskolin (10 μM) and dibutyryl cAMP (Db-cAMP, 100 μM) as contrasted to the wild type cells. Results are expressed as means ± S.E.M. from 4 experiments. $^{\#\#}$ $P < 0.01$; $^{\#\#\#}$ $P < 0.001$ vs. Control; * $P < 0.05$; ** $P < 0.01$ vs. Vehicle; $^{\dagger}$ $P < 0.05$; $^{\dagger\dagger}$ $P < 0.01$ vs. Wild type cells.



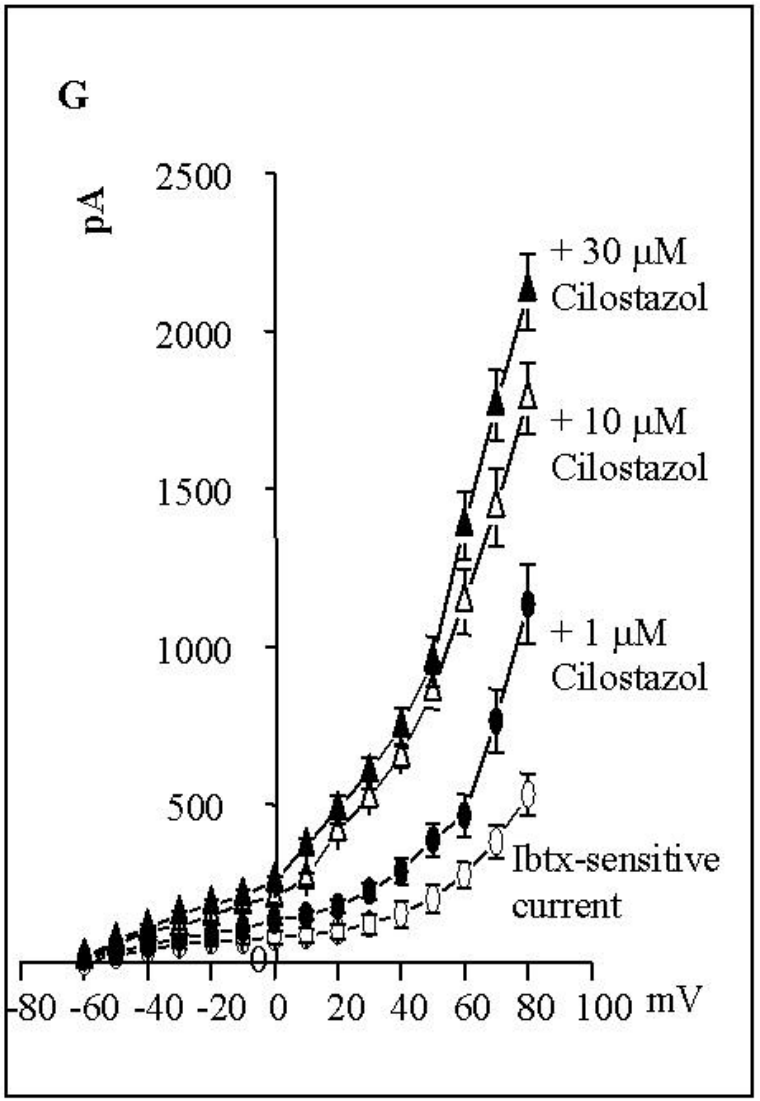
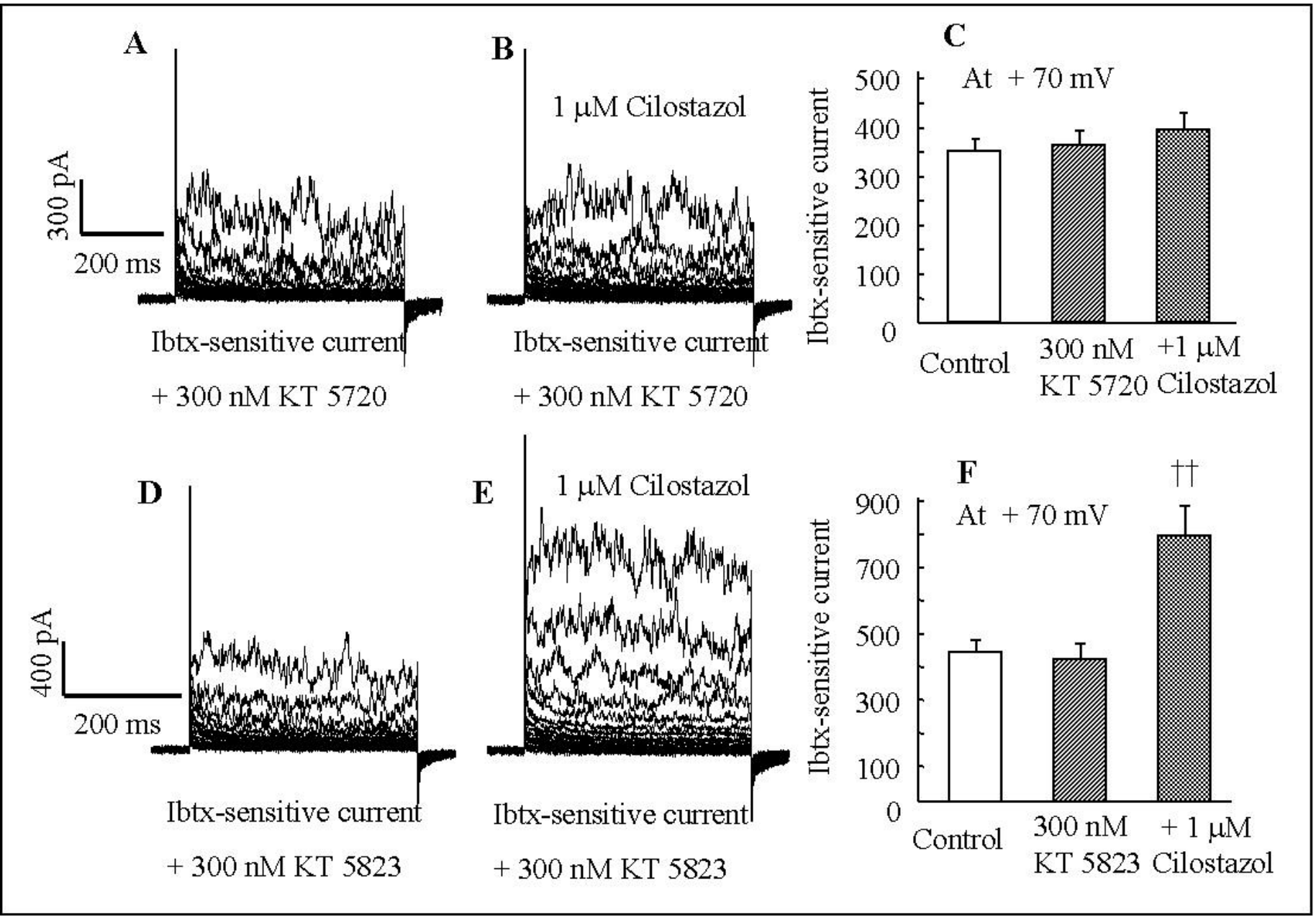




Fig. 1

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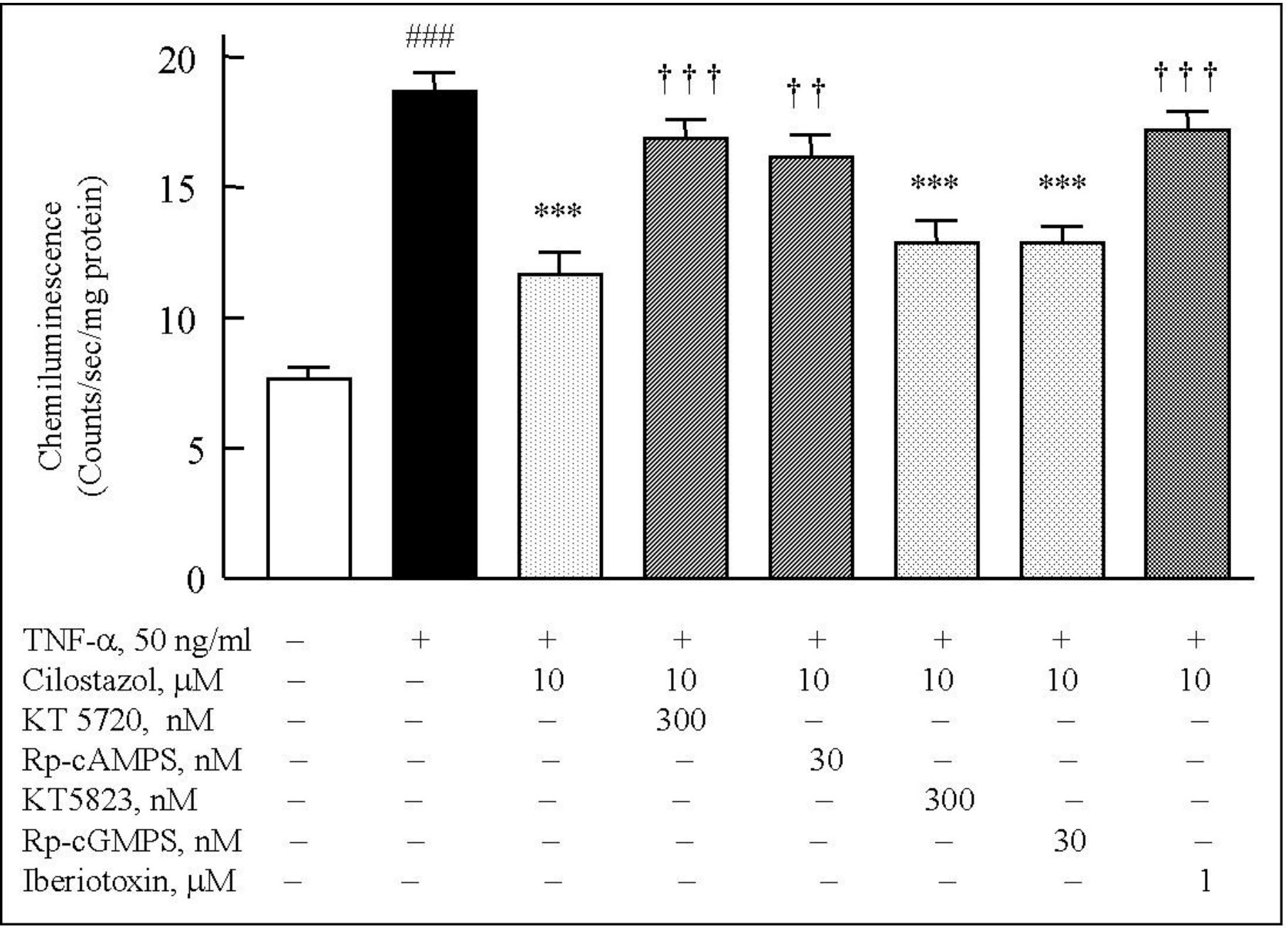


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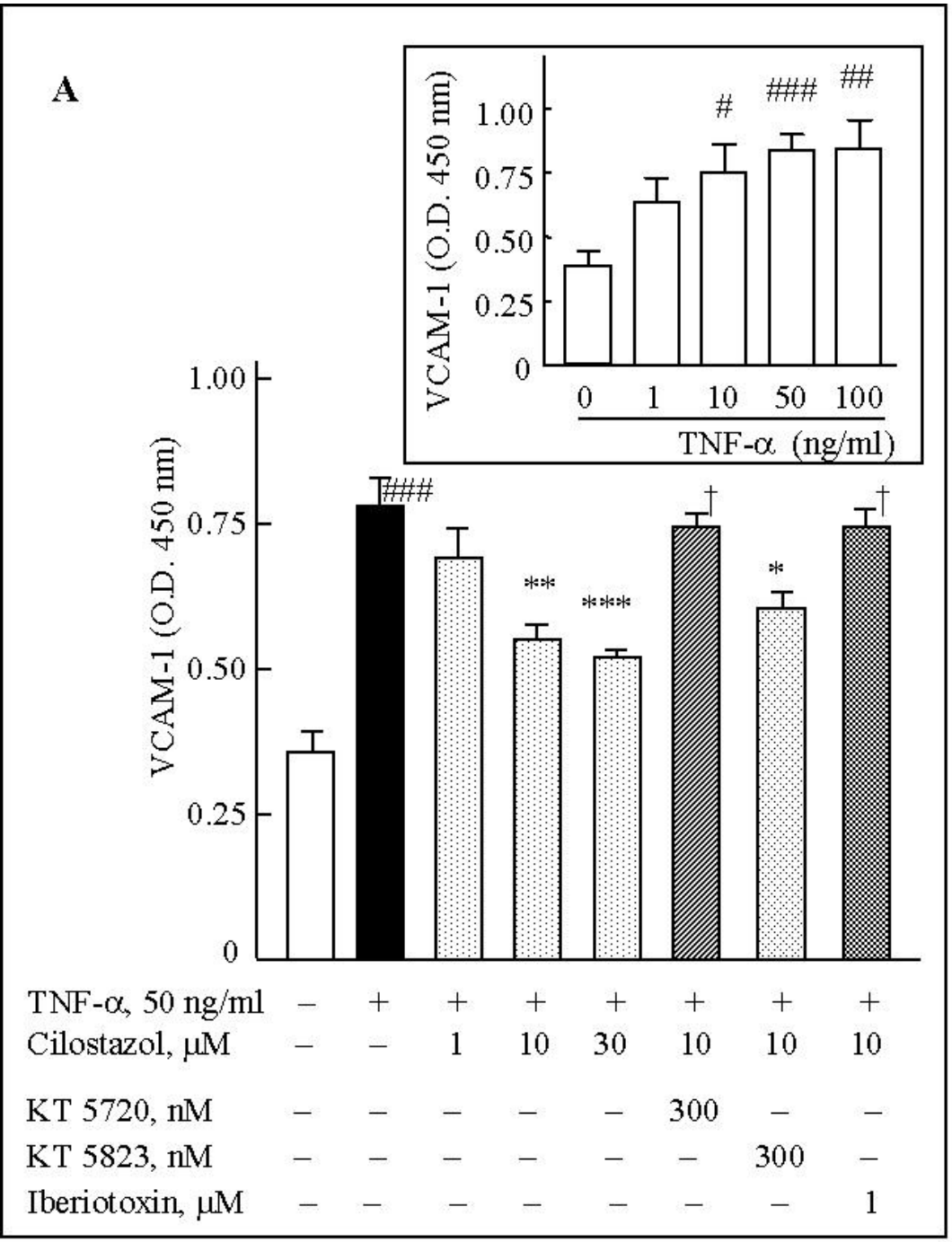


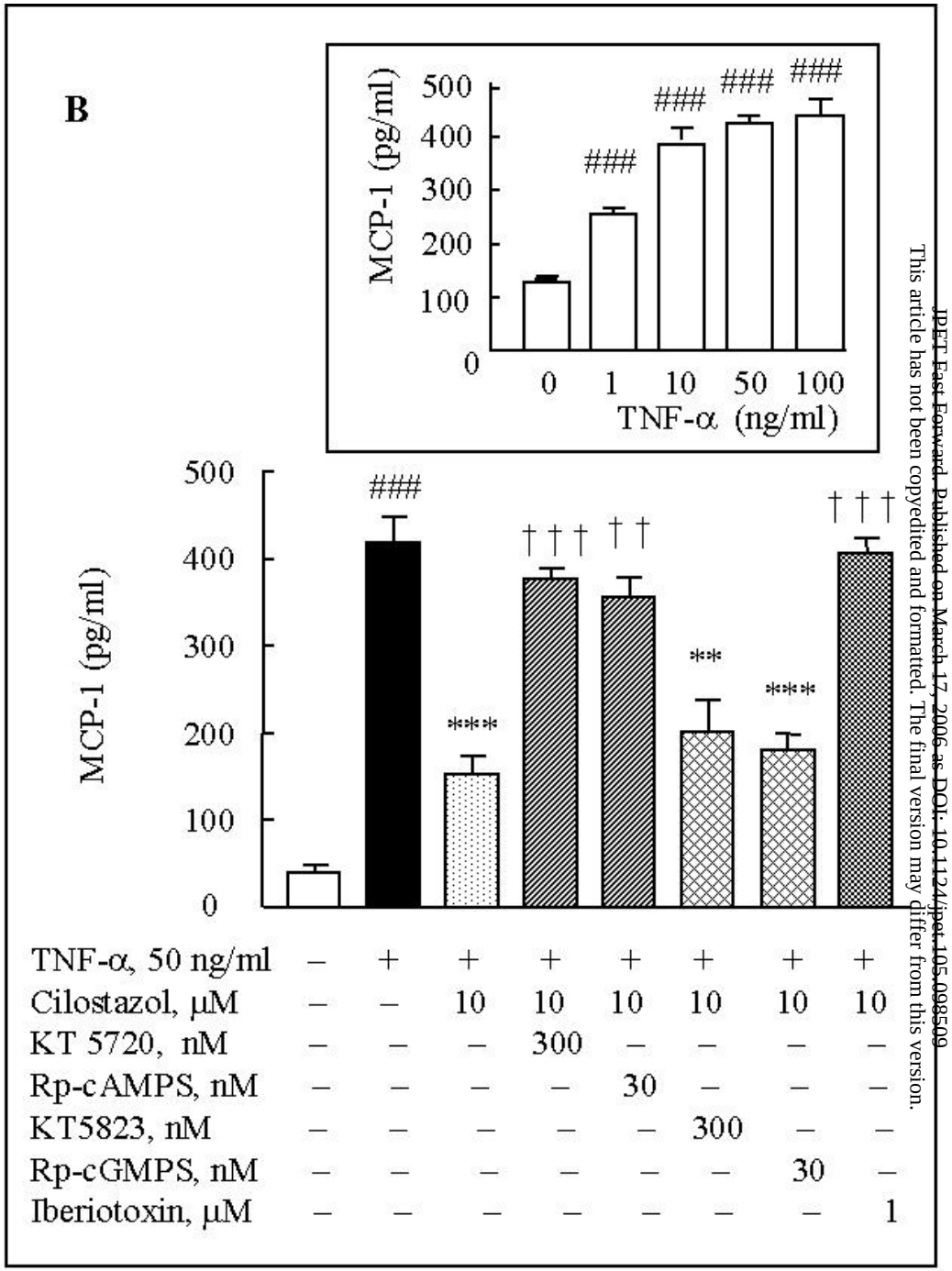


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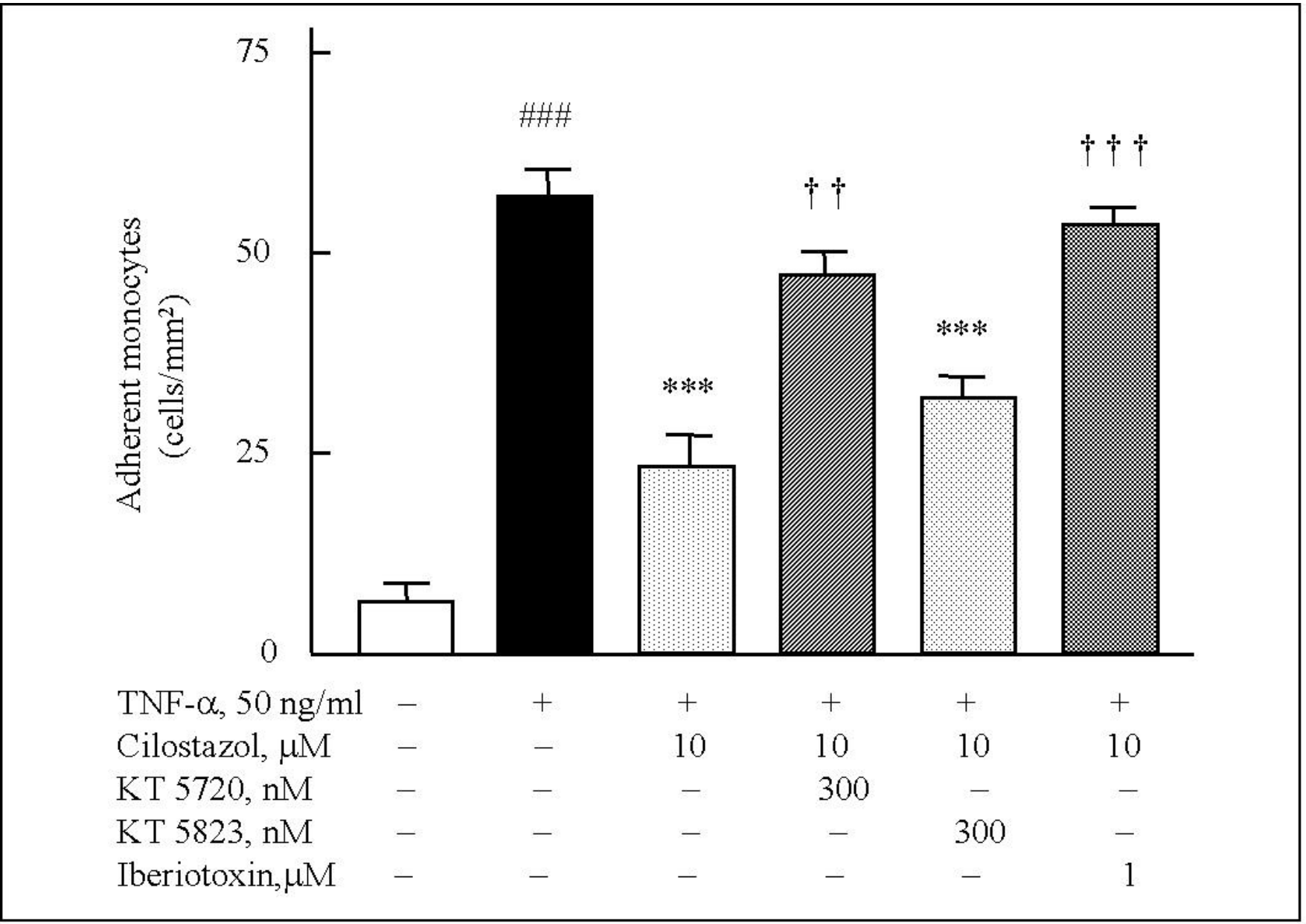



Fig. 5

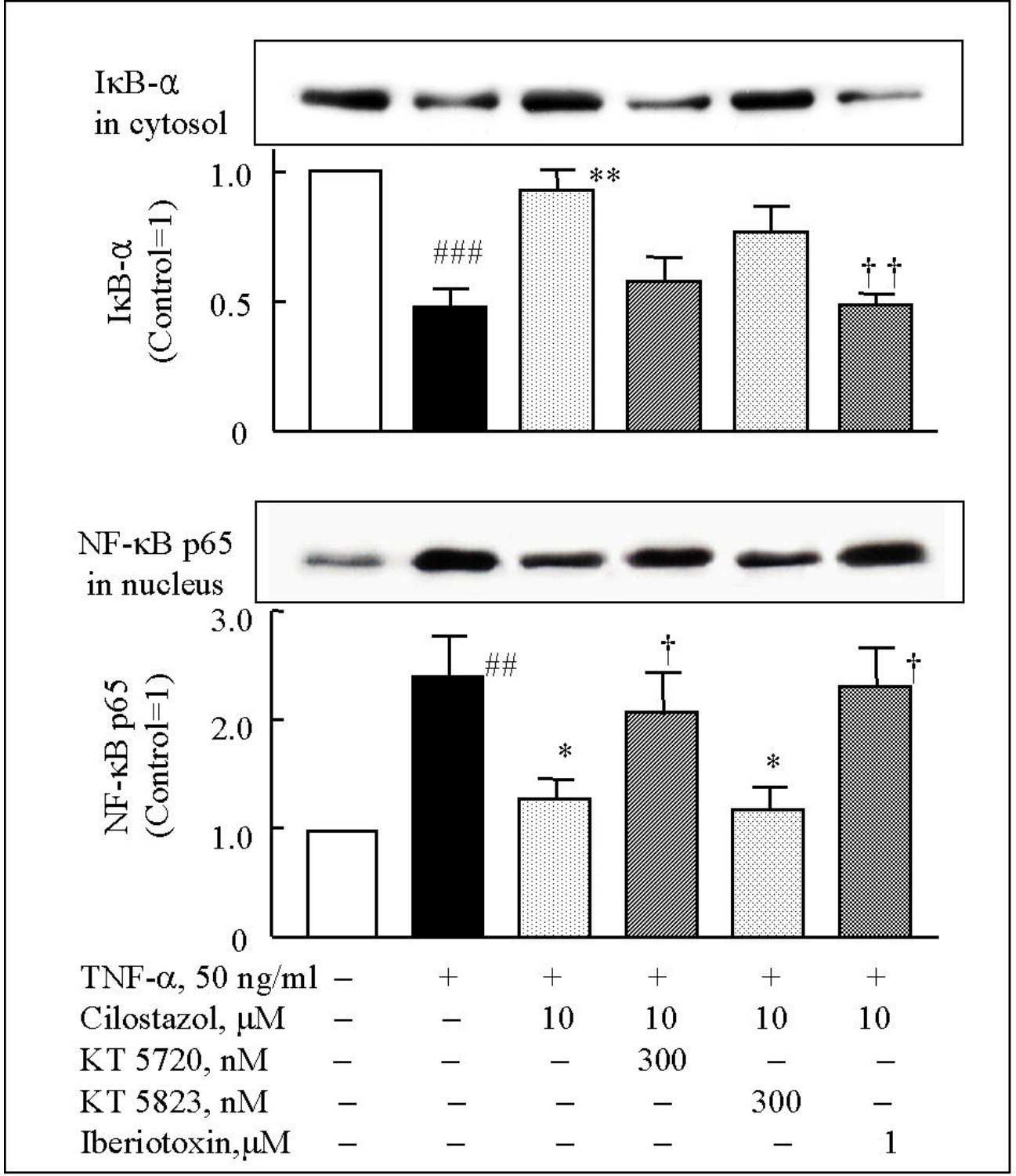
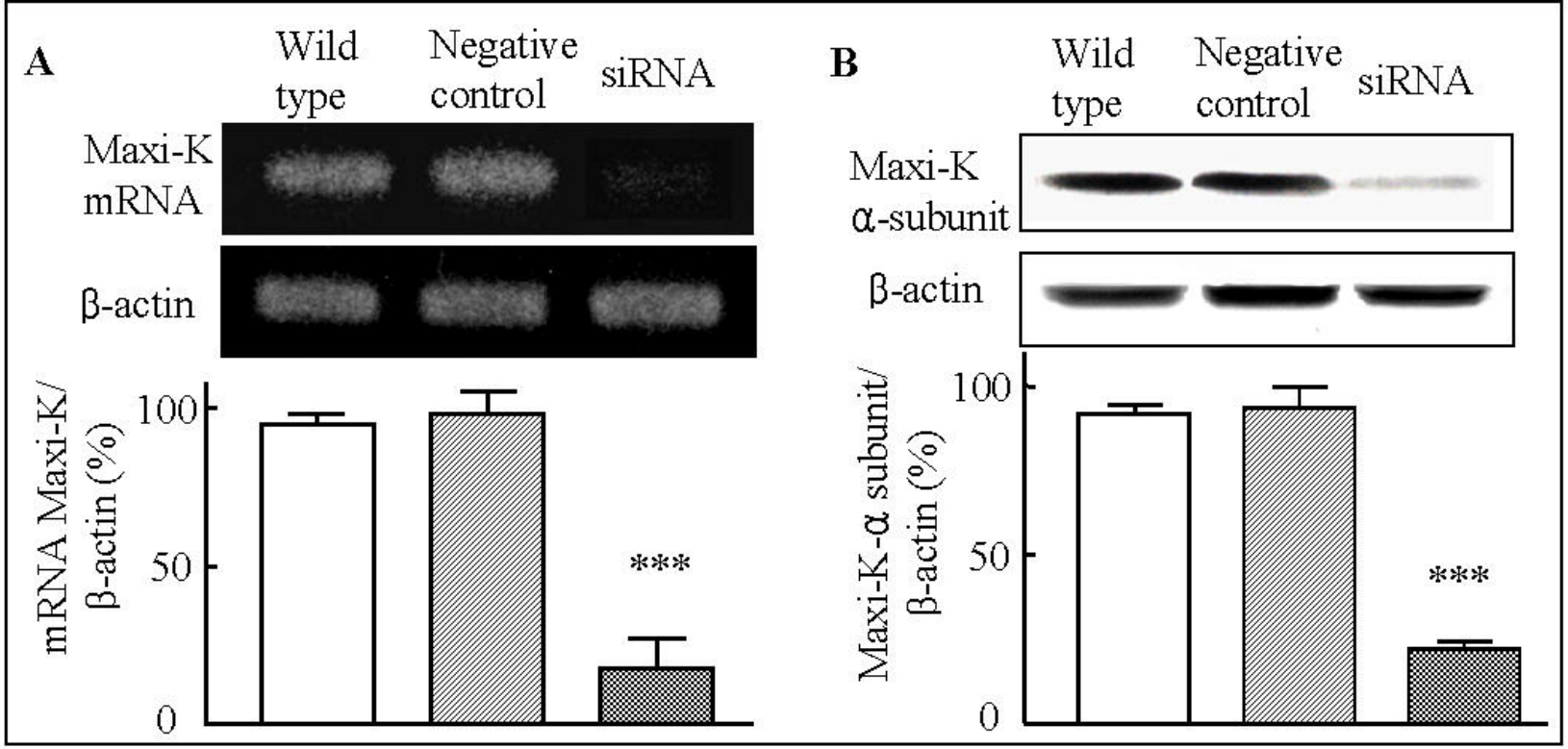



Fig. 6



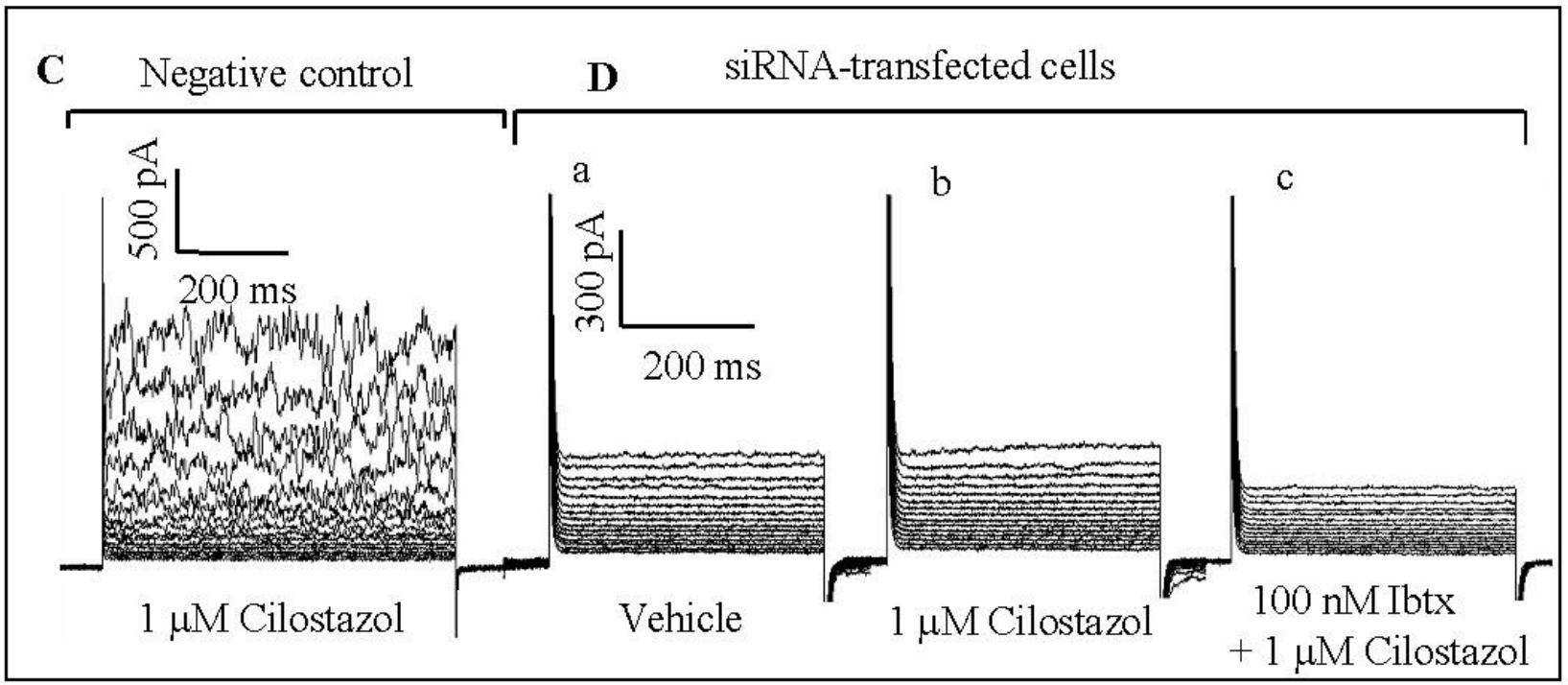








Fig. 7

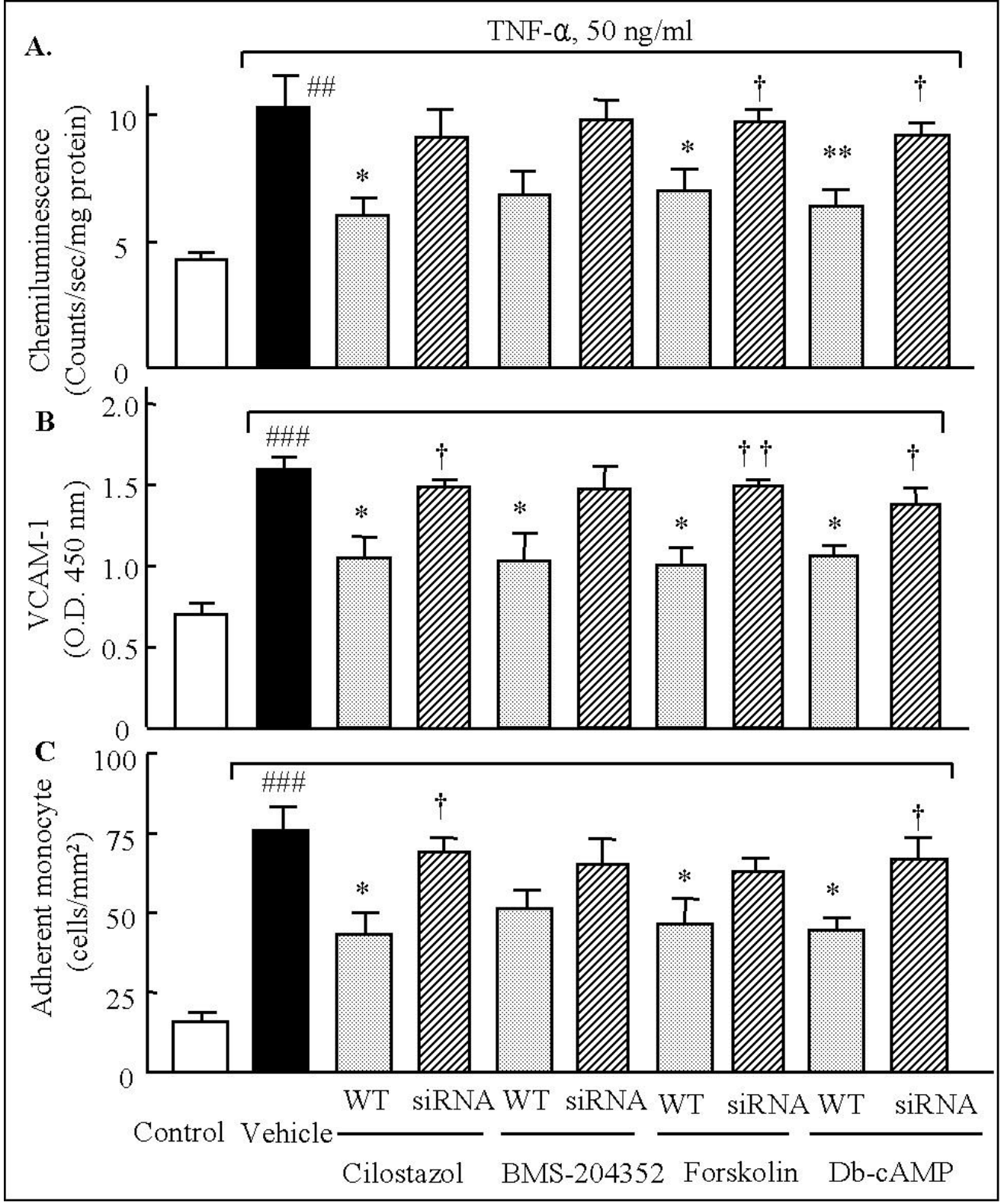



Fig. 8