

# Cilostazol inhibits cytokine-induced nuclear factor- $\kappa$ B activation via AMP-activated protein kinase activation in vascular endothelial cells

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## KEYWORDS

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Endothelial cells;  
Cyclic AMP

**Aims** Cilostazol is a selective inhibitor of phosphodiesterase 3 that increases intracellular cyclic AMP (cAMP) levels and activates protein kinase A, thereby inhibiting platelet aggregation and inducing peripheral vasodilation. We hypothesized that cilostazol may prevent inflammatory cytokine induced-nuclear factor (NF)- $\kappa$ B activation by activating AMP-activated protein kinase (AMPK) in vascular endothelial cells.

**Methods and results** Cilostazol was observed to activate AMPK and its downstream target, acetyl-CoA carboxylase, in human umbilical vein endothelial cells (HUVEC). Phosphorylation of AMPK with cilostazol was not affected by co-treatment with an adenylate cyclase inhibitor, SQ 22536, and a cell-permeable cAMP analogue, pCTP-cAMP, did not induce AMPK phosphorylation and had no effect on cilostazol-induced AMPK phosphorylation, suggesting that cilostazol-induced AMPK activation occurs through a signalling pathway independent of cyclic AMP. Cilostazol also dose-dependently inhibited tumour necrosis factor alpha (TNF $\alpha$ )-induced NF- $\kappa$ B activation and TNF $\alpha$ -induced I $\kappa$ B kinase activity. Furthermore, cilostazol attenuated the TNF $\alpha$ -induced gene expression of various pro-inflammatory and cell adhesion molecules, such as vascular cell adhesion molecule-1, E-selectin, intercellular adhesion molecule-1, monocyte chemoattractant protein-1 (MCP-1), and PECAM-1 in HUVEC. RNA interference of AMPK $\alpha$ 1 or the AMPK inhibitor compound C attenuated cilostazol-induced inhibition of NF- $\kappa$ B activation by TNF $\alpha$ . **Conclusion** In the light of these findings, we suggest that cilostazol might attenuate the cytokine-induced expression of adhesion molecule genes by inhibiting NF- $\kappa$ B following AMPK activation.

## 1. Introduction

Treatment of thrombotic disease requires a delicate balance between the prevention of new thrombotic events and management of bleeding complications. Many antiplatelet and anticoagulant agents have been used to this end, with varying degrees of success. Among the many antiplatelet agents tested to date, cilostazol, which selectively targets phosphodiesterase 3 (PDE3), has several unique features. Cilostazol is classified as an antiplatelet agent because it inhibits the platelet aggregation induced by collagen, 5'-adenosine diphosphate (ADP), epinephrine, and arachidonic acid.<sup>1</sup> Unlike other antiplatelet agents, cilostazol not only inhibits platelet function, but also appears to have beneficial effects on endothelial cell function.<sup>1</sup> Since receiving approval in the USA for the treatment of intermittent claudication in 1999, cilostazol continues to demonstrate promise in the treatment of cardiovascular disorders.

New data regarding the role of cilostazol in the prevention of recurrent cerebral infarction, the prevention of re-stenosis, and the treatment of peripheral artery diseases, have increased recent interest in the drug.<sup>2–5</sup> However, because of the action mechanism of a PDE inhibitor, there have been concerns about the cardiovascular safety of cilostazol in patients with left ventricular dysfunction.<sup>6,7</sup> Furthermore, little is known about the clinical experience with cilostazol in acute myocardial infarction patients.<sup>8</sup>

Vascular endothelial cells play an important role in maintaining the antithrombotic properties of blood vessels, and functional damage to endothelial cells results in thrombus formation along the vessel wall.<sup>9</sup> The interaction that occurs between platelets and endothelium within the micro- and macro-vascular circulation has thrombotic effects by altering the status of platelet activation. Platelets, which are not activated by normal endothelium, are activated when they encounter activated endothelial cells following exposure to oxidative stress, for example, in patients with hypertension, diabetes mellitus, or hyperlipidaemia.<sup>10</sup>

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The activated nuclear factor (NF)- $\kappa$ B has been identified in human atherosclerotic plaques, but is absent, or present only in very small amounts in vessels devoid of atherosclerosis.<sup>11</sup> A number of genes whose products have been implicated in the development of atherosclerosis are regulated by NF- $\kappa$ B. Various leukocyte adhesion molecules, such as the vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin, as well as various chemokines (chemoattractant cytokines), monocyte chemoattractant protein-1 (MCP-1), and IL-8, recruit circulating mononuclear leukocytes to the arterial intima.<sup>12–14</sup> The induction of other NF- $\kappa$ B-dependent genes, including tissue factors, might tip the pro/anti-coagulant balance of the endothelium towards coagulation. Still other products of target genes, including cyclin D1, may induce cell proliferation or stimulate cell survival at atherosclerotic deposits. Therefore, the coordinated induction of NF- $\kappa$ B-dependent genes might promote atherosclerosis.<sup>15</sup>

Cilostazol is unique in that it targets the endothelium, thereby inhibiting thrombosis and improving endothelial cell function, and reducing the number of partially activated platelets by interacting with activated endothelial cells. However, the exact mechanism by which cilostazol preserves endothelial function remains to be elucidated. In the present study, we hypothesized that cilostazol may prevent NF- $\kappa$ B activation in endothelial cells exposed to inflammatory cytokines. We examined the effects of cilostazol on NF- $\kappa$ B activation, as well as the expression of NF- $\kappa$ B-mediated genes, such as VCAM-1, ICAM-1, E-selectin, and MCP-1, in vascular endothelial cells. We found that cilostazol inhibits the cytokine-induced expression of pro-inflammatory and adhesion molecule genes by suppressing NF- $\kappa$ B activity via AMP-activated protein kinase (AMPK) activation and not via the cyclic AMP (cAMP)/protein kinase A (PKA) pathway.

## 2. Methods

### 2.1 Cell culture

Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA, USA) and cultured in EGM2 medium supplemented with 2% FCS in the standard fashion. The cells in this experiment were used within 3–4 passages and were examined to ensure that they demonstrated the specific characteristics of endothelial cells. SVEC4 cells (murine endothelial cell line; ATCC, Rockville, MD, USA) were also cultured in DMEM containing 10% FCS and observed to demonstrate the typical cobblestone morphological appearance of endothelial cells.<sup>16</sup> THP-1 cells, a human monocytic cell line (ATCC), were grown in RPMI-1640 medium containing 10% FCS.

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health and with the Declaration of Helsinki.

### 2.2 Western blot analysis

HUVEC treated with tumour necrosis factor alpha (TNF $\alpha$ ) in the presence or absence of metformin for various intervals were lysed using cell lysis buffer (Cell Signalling, Beverly, MA, USA) with 1 mM PMSF. The protein concentration of each sample was measured using a Bio-Rad detergent-compatible protein assay. Subsequently,  $\beta$ -mercaptoethanol was added to a final concentration of 1%, after which each sample was denatured by boiling for 3 min. Samples containing 10  $\mu$ g of protein were resolved by electrophoresis on 12% sodium dodecyl sulphate (SDS)–polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride

(PVDF) membrane (Bio-Rad, Tokyo, Japan), after which they were incubated with anti-phospho-Thr-172 AMPK polyclonal antibody and anti-phospho-Ser-79 acetyl-CoA carboxylase (ACC) polyclonal antibody (1:1000, Cell Signalling). For the I $\kappa$ B experiments, the membranes were incubated with I $\kappa$ B $\alpha$  antibody or phospho-I $\kappa$ B $\alpha$  antibody (1:1000, Cell Signalling). The binding of each of these antibodies was detected using sheep anti-rabbit IgG horse-radish peroxidase (1:20 000) and an ECL Plus system (Amersham, Buckinghamshire, UK).

### 2.3 Nuclear factor- $\kappa$ B activation

To study NF- $\kappa$ B activation, SVEC4 cells were stably transfected with a *cis*-reporter plasmid containing the luciferase reporter gene linked to five repeats of NF- $\kappa$ B binding sites (pNF- $\kappa$ B-Luc; Stratagene, La Jolla, CA, USA), as previously described.<sup>17</sup> For this, the pNF- $\kappa$ B-Luc plasmid was transfected, together with a pSV2neo helper plasmid, (Clontech, Palo Alto, CA, USA) into SVEC4 cells using a FuGEN 6 transfection reagent (Boehringer Mannheim, Mannheim, Germany). The cells were then cultured in the presence of G418 (Clontech) at a concentration of 500  $\mu$ g/mL, and the medium was replaced every 2–3 days. Approximately 3 weeks after transfection, G418-resistant clones were isolated using a cloning cylinder and analysed individually for the expression of luciferase activity. Several clones were also selected for the analysis of NF- $\kappa$ B activation. Luciferase activity was measured using a luciferase assay kit (Stratagene).

We also measured changes in the levels of NF- $\kappa$ B p50 and p65 in nuclear extracts from HUVEC using a transcription factor assay kit (Active Motif Japan, Tokyo, Japan). Nuclear extracts were prepared with a NE-PER nuclear extraction reagent (Pierce, Rockford, IL, USA), after which p50 and p65 were quantified using recombinant NF- $\kappa$ B p50 and p65 protein (Active Motif) as the standard.

### 2.4 I $\kappa$ B kinase assay

I $\kappa$ B kinase (IKK) activity was examined using an immune complex kinase assay with GST-I $\kappa$ B $\alpha$  (1–55) as the substrate, as previously described.<sup>18</sup> Briefly, the cells were solubilized in ice-cold buffer, and then centrifuged at 15 000  $\times$  g for 20 min. IKK $\alpha$  and IKK $\beta$  were recovered from the cell lysate by immunoprecipitation, after which the immune complexes were incubated with 20  $\mu$ L of reaction buffer containing 20 mM HEPES/NaOH (pH 7.4), 10 mM MgCl<sub>2</sub>, 50 mM NaCl, 100 mM Na3VO<sub>4</sub>, 20 mM  $\beta$ -glycerophosphate, 1 mM DTT, 100  $\mu$ M ATP, 0.1  $\mu$ Ci [ $\gamma$ -<sup>32</sup>P]ATP, and 10  $\mu$ g GST-I $\kappa$ B $\alpha$  (1–55), at 30°C for 20 min. Following SDS–PAGE, GST-I $\kappa$ B $\alpha$  phosphorylation was estimated using an Imaging plate (Fuji Film, Tokyo, Japan).

### 2.5 siRNA transfection

The day before transfection, the plates were inoculated with an appropriate number of HUVEC in serum-containing medium to ensure 50–70% confluence the following day. Control siRNA, and LKB1 siRNA (siL1 or siL2) or Ca<sup>2+</sup>/calmodulin-dependent protein kinase  $\beta$  (CaMKK $\beta$ ) siRNA (siC1 or siC2) (Santa Cruz Biotechnology, Santa Cruz, CA, USA and Dharmacon, Lafayette, CO, USA) mixed with siLentFect (Bio-Rad) were added to each plate of the cells at a concentration of 10 nM. Forty-eight hours after transfection, AMPK activation induced by cilostazol was assessed.

AMPK siRNA (Santa Cruz Biotechnology) mixed with siLentFect was added to SVEC4 cells at a concentration of 10 nM. Forty-eight hours after transfection, TNF $\alpha$ -induced NF- $\kappa$ B activity was compared with that of control SVEC4 cells.

### 2.6 Real-time PCR of human umbilical vein endothelial cells mRNA

For quantitative measurement of mRNA, 2  $\mu$ g of total RNA was treated with DNase I for 15 min and subsequently used for cDNA synthesis. Reverse transcription was performed using a SuperScript

Pre-amplification System (Gibco-BRL, Gaithersburg, MD, USA) with random oligonucleotide primers. The following primers were used: ICAM-1 forward 5'-CCGGAAGGTGTATGAACTGA-3', reverse 5'-GGCAGCGTAGGGTAAGGTT-3'; VCAM-1 forward 5'-GGCAGAGTACGCAAACTT-3', reverse 5'-GGCTGTAGTCCCCGTTAG-3'; E-selectin forward 5'-GCCTTGAATCAGACGGAAGC-3', reverse 5'-TGATGGGTGTTGCGGTTTC-3'; MCP-1 forward 5'-CAAAGTGAAGCTCGCACTCTC-3', reverse 5'-GCTGCAGATTCTTGGGTTGTG-3'; PECAM forward 5'-CAAAGACAACCCTACTGAAGAC-3', reverse 5'-CGCAATGATCAAGAGAGCAATG-3'; P-selectin forward 5'-AGACAGGCCACCGAATATGAG-3', reverse 5'-GGCCGTCAGTCGAGTTGT-3'; and GAPDH forward 5'-GGAGAAGGCTGGGCTCAT-3', reverse 5'-TGATGGCATGGACTGTGGTC-3'. A typical reaction (50  $\mu$ L) contained 1 of 50 of reverse transcription (RT)-generated cDNA and 200 nM of primer in 1  $\times$  SYBR Green RealTime Master Mix (Toyobo, Tokyo, Japan) buffer. The PCRs were carried out in a LineGene system (BioFlux, Tokyo, Japan) under the following conditions: 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 30 s.

## 2.7 Adhesion assay under static conditions

THP-1 cells were labelled with BCECF-AM (Calbiochem, San Diego, CA, USA), placed on a confluent HUVEC monolayer ( $1 \times 10^4$  per well) in a 96-well plate ( $1 \times 10^5$  THP-1 cells per well), and allowed to adhere for 10 min. After non-adherent cells were removed, the fluorescent intensity of adhered and total cells applied to the well was measured with a fluorescence plate reader (Fluoroskan Ascent FL, GMI, Inc., Ramsey, MN, USA). The ratio of adherent to total cells was expressed as adhesion (%).

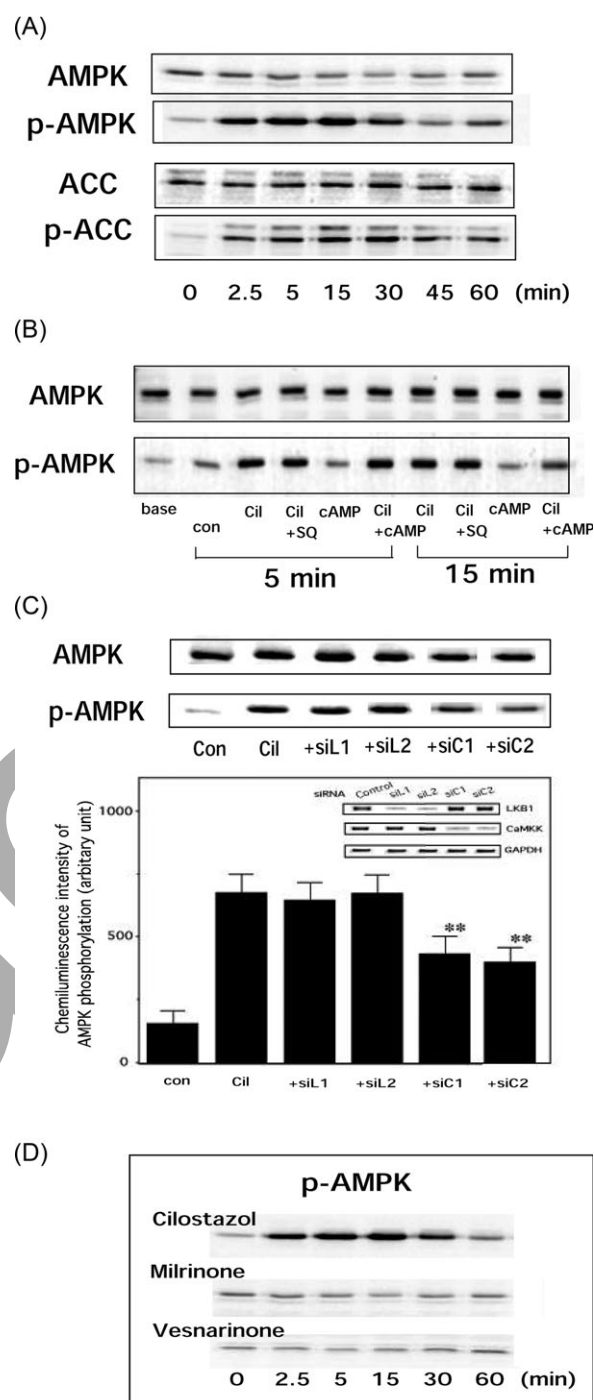
## 2.8 Statistical analysis

Data are presented as the mean  $\pm$  SEM. Multiple comparisons were evaluated by ANOVA followed by Fisher's protected least significant difference test. A value of  $P < 0.05$  was considered statistically significant.

## 3. Results

### 3.1 Cilostazol activates AMP-activated protein kinase in human umbilical vein endothelial cells

Treatment of HUVEC with cilostazol resulted in time-dependent activation of AMPK, as monitored by phosphorylation of AMPK and its down-stream target, ACC (Figure 1A). Phosphorylation of AMPK with cilostazol was not affected by co-treatment with an adenylate cyclase inhibitor SQ 22536 (Figure 1B). A cell-permeable cAMP analogue pCTP-cAMP (100  $\mu$ M) did not induce AMPK phosphorylation, and had no effect on cilostazol-induced AMPK phosphorylation (Figure 1B). Thus, cilostazol activates AMPK independent of cAMP in vascular endothelial cells. AMPK is controlled by upstream kinases, which have been identified as LKB1 or CaMKK $\beta$ .<sup>19</sup> Both LKB1 and CaMKK $\beta$  are expressed in HUVEC (data not shown). In order to assess whether CaMKK $\beta$  or LKB1 might act as an AMPK kinase (AMPKK) in cilostazol-treated cells, we used a siRNA approach to knock down the expression of LKB1 or CaMKK $\beta$ . Compared with the results following transfection using control siRNA, cilostazol-induced AMPK activation was significantly reduced in cells treated with CaMKK siRNA (siC1 or siC2), but not in cells treated with LKB1 siRNA (siL1 or siL2) (Figure 1C). We also examined whether other PDE3 inhibitors activate AMPK. Neither milrinone nor vesnarinone could induce AMPK activation (Figure 1D).



**Figure 1** (A) Cilostazol activates AMP-activated protein kinase (AMPK) in vascular endothelial cells. Human umbilical vein endothelial cells (HUVEC) were treated with cilostazol (100  $\mu$ M) for the indicated time periods before lysis, after which each cell lysate sample was probed with antibodies specific for phosphorylated forms of AMPK and acetyl-CoA carboxylase (ACC). (B) HUVEC were treated with cilostazol (100  $\mu$ M) alone or in the presence of an adenylate cyclase inhibitor SQ 22536 (10  $\mu$ M) or a cell-permeable cyclic AMP (cAMP) analogue pCTP-cAMP (100  $\mu$ M). After 5 and 15 min of incubation, the cells were lysed and p-AMPK was analysed. Three independent studies showed similar results. (C) Cilostazol activates AMPK, which was significantly attenuated in HUVEC transfected with CaMKK $\beta$  siRNA (siC1 or siC2: 10 nM) but not with LKB1 siRNA (siL1 or siL2: 10 nM). Inset (lower figure): 48 h after cells were transfected with control siRNA, siL1, siL2, siC1, or siC2, the mRNA levels of LKB1, CaMKK $\beta$ , and GAPDH were determined. (D) Cilostazol, but not milrinone or vesnarinone, activates AMPK in vascular endothelial cells. HUVEC were treated with cilostazol (100  $\mu$ M), milrinone (100  $\mu$ M), or vesnarinone (100  $\mu$ M) for the indicated time periods before lysis, after which each cell lysate sample was probed with antibodies specific for phosphorylated AMPK.

### 3.2 Cilostazol inhibits NF- $\kappa$ B activation and inhibits vascular cell adhesion molecule-1, E-selectin, intercellular adhesion molecule-1, monocyte chemoattractant protein-1, and PECAM-1 mRNA induction

We initially examined the effect of incubation of cilostazol with TNF $\alpha$  for 2 h on NF- $\kappa$ B activation in SVEC4 cells. TNF $\alpha$  induced a 7-fold increase in NF- $\kappa$ B-mediated reporter gene expression. Cilostazol dose-dependently suppressed TNF $\alpha$ -elicited activation of NF- $\kappa$ B (Figure 2A). We then examined the effect of siRNA specific for AMPK $\alpha$ 1 on cilostazol-induced NF- $\kappa$ B inhibition, which was partially but significantly attenuated in AMPK $\alpha$ 1 siRNA-transfected cells, compared with cells transfected with control siRNA (Figure 2A).

We also measured p50 and p65 in nuclear extracts from untreated HUVEC and from those treated with TNF $\alpha$  in the presence (30 or 100  $\mu$ M) and absence of cilostazol. Both p50 and p65 markedly increased 30 min after stimulation with TNF $\alpha$ , from very low levels. This increase was dose-dependently inhibited by cilostazol (Figure 2B).

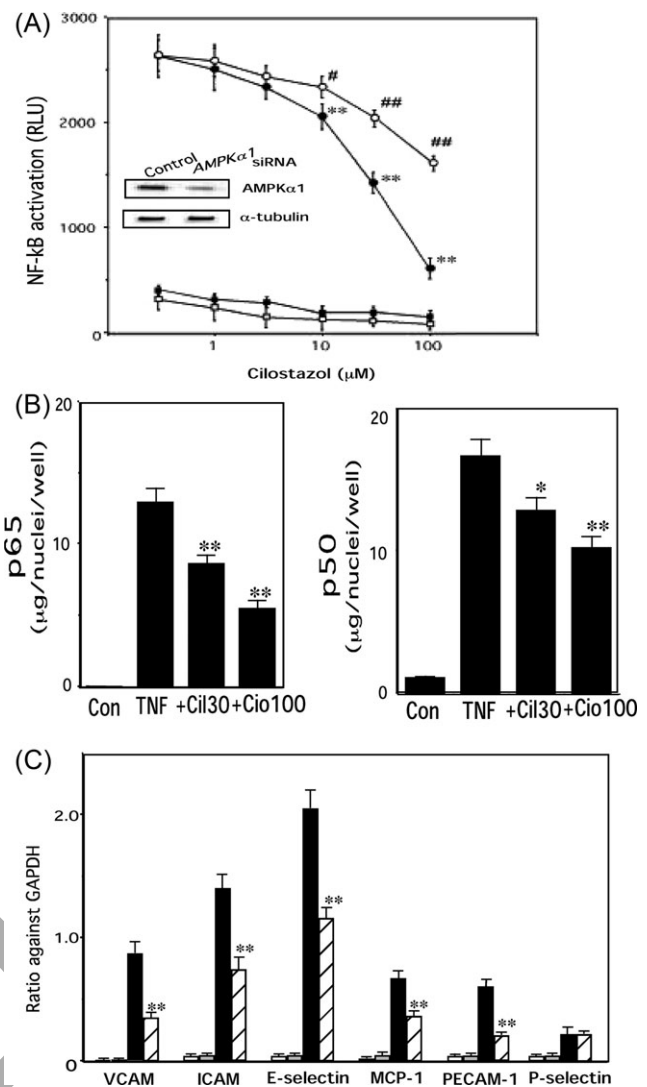
Incubation for 24 h with TNF $\alpha$  substantially induced the gene expression of VCAM-1, E-selectin, ICAM-1, and MCP-1. Induction of TNF $\alpha$ -induced gene expression was markedly suppressed by co-treatment with an NF- $\kappa$ B inhibitor, BAY11-7082, which is known selectively and irreversibly to inhibit cytokine-induced I $\kappa$ B phosphorylation,<sup>20</sup> suggesting that induction of these genes may be NF- $\kappa$ B-dependent (data not shown). Cilostazol significantly inhibited TNF $\alpha$ -induced gene expression (Figure 2C). We also examined the effect of cilostazol on TNF $\alpha$ -induced gene expression of PECAM-1 and P-selectin, adhesion molecules relevant in platelet-endothelium interaction. PECAM-1 mRNA was substantially induced by TNF $\alpha$ , which was clearly inhibited by cilostazol. Although P-selectin mRNA was modestly induced by TNF $\alpha$ , cilostazol did not affect the induction of P-selectin gene expression (Figure 2C).

### 3.3 Cilostazol inhibits adhesion of monocytic cells via suppressing NF- $\kappa$ B activation

Treating HUVEC with TNF $\alpha$  for 4 h significantly increased THP-1 cell adhesion. Pretreatment with cilostazol inhibited the TNF $\alpha$ -induced adhesion of THP-1 cell to HUVEC in a dose-dependent manner (Figure 3A). An NF- $\kappa$ B inhibitor BAY11-7082 markedly inhibited TNF $\alpha$ -induced THP-1 cell adhesion to HUVEC (Figure 3A). We then examined the effect of siRNA specific for AMPK $\alpha$ 1 on cilostazol-induced inhibition of THP-1 cell adhesion, which was significantly attenuated in AMPK $\alpha$ 1 siRNA-transfected cells, compared with cells transfected with control siRNA (Figure 3B).

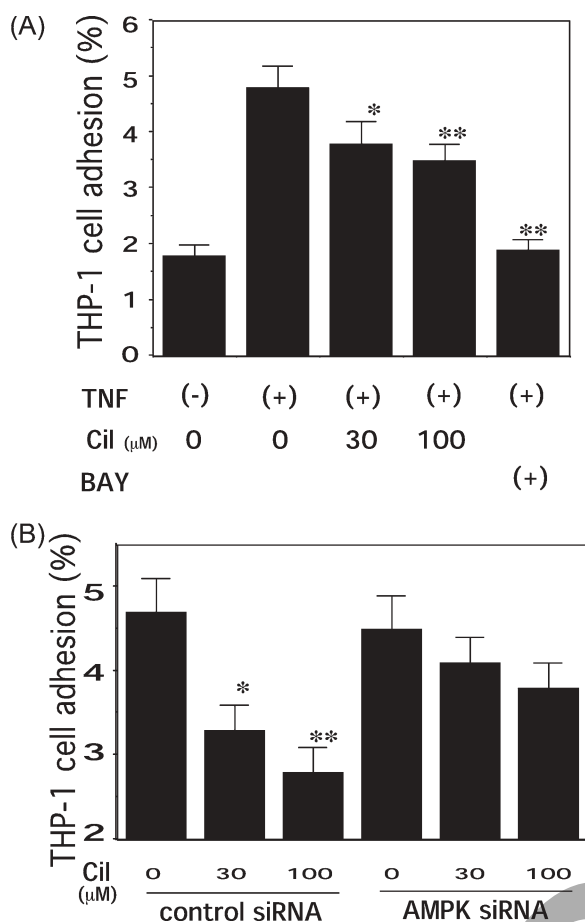
### 3.3 TNF $\alpha$ stimulates I $\kappa$ B phosphorylation by inducing I $\kappa$ B kinase activity, while cilostazol inhibits TNF $\alpha$ -induced I $\kappa$ B kinase activity and I $\kappa$ B phosphorylation

We first determined whether TNF $\alpha$ -induced NF- $\kappa$ B activation might occur through phosphorylation and subsequent degradation of I $\kappa$ B. To determine whether TNF $\alpha$  might induce I $\kappa$ B $\alpha$  phosphorylation in HUVEC, western blot analysis using the antiphospho-Ser32 of I $\kappa$ B $\alpha$  antibody was performed. TNF $\alpha$  was observed to induce I $\kappa$ B phosphorylation within 15 min,



**Figure 2** (A) Cilostazol inhibits tumour necrosis factor alpha (TNF $\alpha$ )-induced nuclear factor (NF)- $\kappa$ B-activation in SVEC4 cells. Cilostazol dose-dependently suppressed TNF $\alpha$ -activated NF- $\kappa$ B-dependent transcriptional activity, which was significantly attenuated in cells transfected with AMP-activated protein kinase (AMPK) siRNA (10 nM). Closed (control scrambled siRNA) and open squares (AMPK siRNA) represent the results in the absence of TNF $\alpha$ , whereas closed (control scrambled siRNA) and open circles (AMPK siRNA) represent the results in the presence of TNF $\alpha$  ( $n = 6$ ). Inset: Cells were transfected with AMPK $\alpha$ 1 or control siRNA for 48 h, after which the protein levels of AMPK $\alpha$ 1 were determined by western blot analysis. Results represent the means  $\pm$  SEM ( $n = 4$ ). \*\* $P < 0.05$ , \*\*\* $P < 0.01$  vs. NF- $\kappa$ B activity in the absence of cilostazol, # $P < 0.05$ , ## $P < 0.01$  vs. NF- $\kappa$ B activity in the presence of cilostazol. (B) Human umbilical vein endothelial cells (HUVEC) were stimulated with TNF $\alpha$  in the presence or absence of cilostazol (Cil30: 30  $\mu$ M, Cil100: 100  $\mu$ M) for 30 min. NF- $\kappa$ B p65 or p50 subunits were quantified within nuclear extracts using a transcription factor assay kit. Results represent the means  $\pm$  SEM ( $n = 4$ ). \* $P < 0.05$ , \*\*\* $P < 0.01$ . (C) Effects of cilostazol on TNF $\alpha$ -induced vascular cell adhesion molecule-1 (VCAM-1), E-selectin, intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractant protein-1 (MCP-1), PECAM-1, and P-selectin mRNA expression in HUVEC. Cilostazol (30  $\mu$ M) significantly inhibited VCAM-1, E-selectin, ICAM-1, MCP-1, and PECAM-1 mRNA levels. White bars: control, grey bars: control treated with cilostazol, black bars: TNF $\alpha$ , hatched bars: TNF $\alpha$  treated with cilostazol. Data represent the means  $\pm$  SEM ( $n = 4$ ) and are expressed as a ratio of GAPDH. \*\* $P < 0.01$  compared with the value of TNF $\alpha$ .

and decreased levels of phospho-I $\kappa$ B $\alpha$  were observed at 60 min (Figure 4A). The blot was then re-probed with anti-I $\kappa$ B antibody, producing evidence of significant degradation within 15–30 min. After this, I $\kappa$ B synthesis was

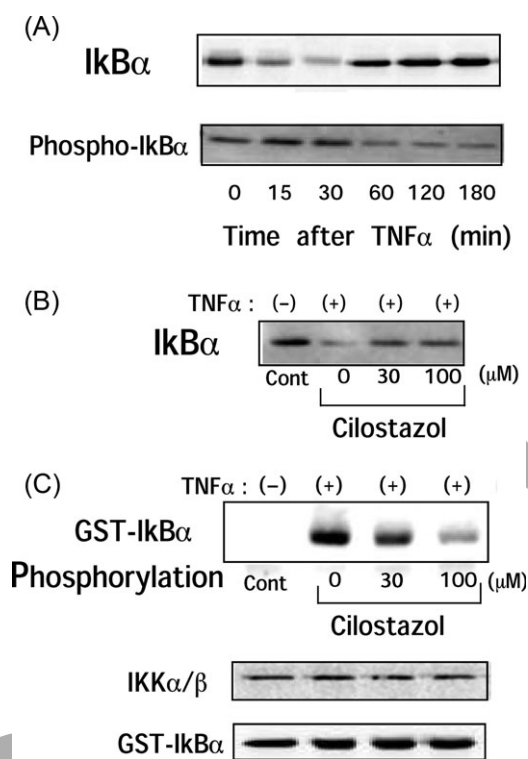


**Figure 3** (A) Human umbilical vein endothelial cells (HUVEC) were pre-treated with cilostazol (30 or 100  $\mu$ M) for 30 min and then incubated in the presence of tumour necrosis factor alpha (TNF $\alpha$ ), and static adhesion assays were performed. The effect of BAY11-7082 (10  $\mu$ M) on TNF $\alpha$ -induced cell adhesion was also examined. (B) Cilostazol dose-dependently suppressed TNF $\alpha$ -activated THP-1 cell adhesion that was significantly attenuated in HUVEC transfected with AMPK siRNA (10 nM).  $n = 6$ , \* $P < 0.05$ , \*\* $P < 0.01$ .

re-activated, possibly by NF- $\kappa$ B, at 60 min (Figure 4A). Next, the effect of cilostazol on TNF $\alpha$ -induced I $\kappa$ B $\alpha$  degradation was determined 30 min after exposure to TNF $\alpha$ . Cilostazol partially inhibited TNF $\alpha$ -induced I $\kappa$ B $\alpha$  degradation (Figure 4B). A radiolabelled phosphorylated GST-I $\kappa$ B $\alpha$ -specific band was detected in TNF $\alpha$ -treated cells, while it was undetectable in untreated cells, thus demonstrating induction of IKK activity by TNF $\alpha$  (Figure 4C). IKK activity was dose-dependently inhibited by the treatment of the cells with cilostazol (Figure 4C). The remaining half of the immunoprecipitated samples were analysed by western blot analysis using anti-IKK $\alpha$ / $\beta$  antibody, which showed identical expression levels of IKK, confirming expression of IKK in these cells. Identical amounts of GST-I $\kappa$ B were also detected when an equal volume of kinase reaction mixture was loaded into the SDS-PAGE column, followed by western blot analysis using anti-I $\kappa$ B antibody (Figure 4C).

### 3.4 AMP-activated protein kinase inhibition restored and cyclic AMP enhanced cilostazol-induced inhibition of nuclear factor- $\kappa$ B activation by tumour necrosis factor alpha

An AMPK inhibitor compound C restored cilostazol-induced inhibition of NF- $\kappa$ B activation by TNF $\alpha$ . This NF- $\kappa$ B activation

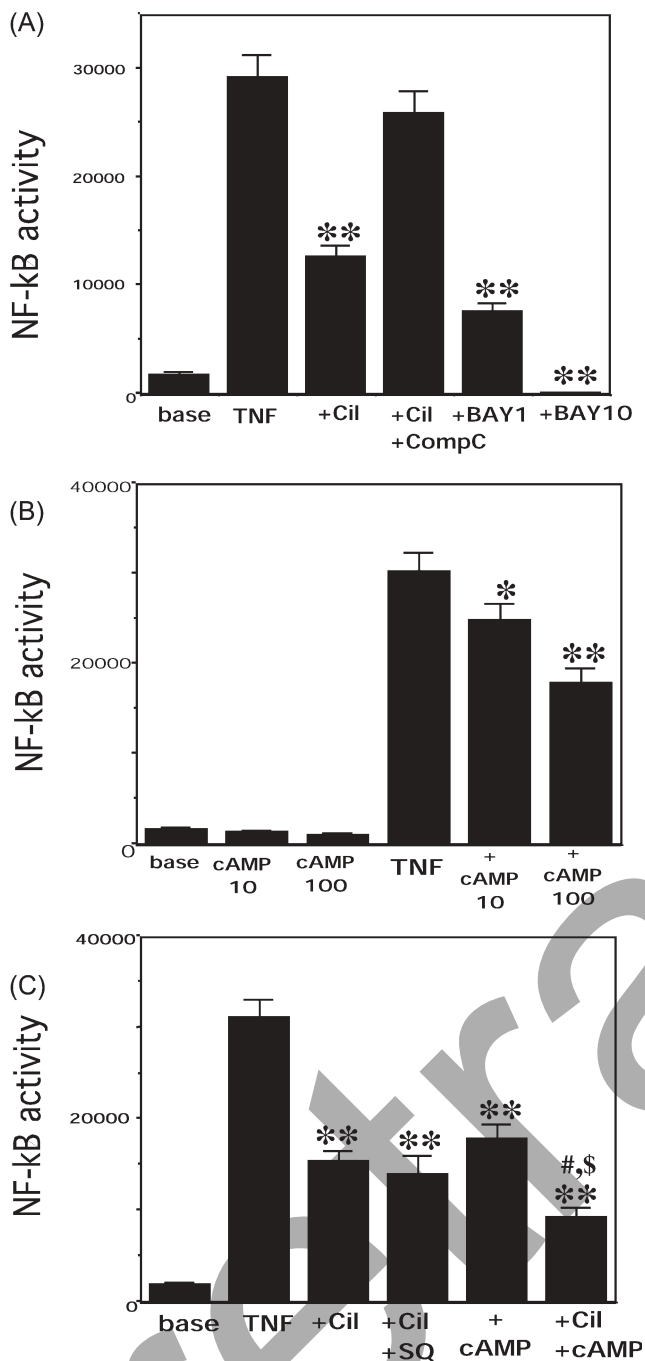


**Figure 4** (A) Human umbilical vein endothelial cells (HUVEC) were incubated with tumour necrosis factor alpha (TNF $\alpha$ ) for 0–180 min. The cells were lysed and subjected to western blot analysis using anti-I $\kappa$ B $\alpha$  and anti-phospho-I $\kappa$ B $\alpha$  antibodies. (B) The effect of cilostazol on I $\kappa$ B $\alpha$  degradation in HUVEC. Cells were incubated for 30 min with cilostazol (30 and 100  $\mu$ M), followed by TNF $\alpha$  for 30 min. Cells were then lysed and subjected to western blot analysis using anti-I $\kappa$ B $\alpha$  antibody. (C) The effect of cilostazol on IKK activity in HUVEC. Cells were incubated for 30 min with cilostazol (30 and 100  $\mu$ M), followed by TNF $\alpha$  for 15 min. Cells were then lysed and immunoprecipitated with anti-IKK $\alpha$ / $\beta$  antibody and used for kinase assay using recombinant I $\kappa$ B $\alpha$  as a substrate. Note that equal band densities for IKK $\alpha$ / $\beta$  and GST-I $\kappa$ B $\alpha$  were observed. Three independent studies showed similar results.

by TNF $\alpha$  was completely inhibited by the NF- $\kappa$ B inhibitor BAY11-7082 (Figure 5A). A cell-permeable cAMP analogue pCTP-cAMP dose-dependently suppressed NF- $\kappa$ B activation by TNF $\alpha$  (Figure 5B). Although an adenylate cyclase inhibitor SQ 22536 had no effect on cilostazol-induced inhibition of NF- $\kappa$ B activation by TNF $\alpha$ , pCTP-cAMP enhanced cilostazol-induced inhibition of NF- $\kappa$ B activation by TNF $\alpha$  (Figure 5C).

## 4. Discussion

In the present study, we demonstrated that cilostazol inhibits TNF $\alpha$ -induced NF- $\kappa$ B activation in vascular endothelial cells. Cilostazol inhibited the NF- $\kappa$ B-dependent gene expression of various inflammatory and cell adhesion molecules, including VCAM-1, E-selectin, ICAM-1, and MCP-1. We demonstrated AMPK activation by cilostazol in HUVEC and examined whether this might be associated with the inhibition of cytokine-induced NF- $\kappa$ B activation. Transfection of AMPK $\alpha$ 1 siRNA, which caused marked inhibition of AMPK $\alpha$ 1 expression, significantly attenuated cilostazol-induced inhibition of NF- $\kappa$ B activation by TNF $\alpha$  in endothelial cells. An AMPK inhibitor compound C also restored cilostazol-induced inhibition of NF- $\kappa$ B activation by TNF $\alpha$ . An AMPK activator AICAR was observed to suppress



**Figure 5** (A) An AMP-activated protein kinase (AMPK) inhibitor compound C (Comp C; 1  $\mu$ M) restored cilostazol-induced inhibition of NF- $\kappa$ B activation by tumour necrosis factor alpha (TNF $\alpha$ ) in SVEC4 cells. This NF- $\kappa$ B activation by TNF $\alpha$  was completely inhibited by the NF- $\kappa$ B inhibitor BAY11-7082 (BAY1 or BAY10; 1 or 10  $\mu$ M). (B) Cyclic AMP inhibited TNF $\alpha$ -elicited NF- $\kappa$ B activation. A cell-permeable cyclic AMP analogue pCTP-cAMP dose-dependently suppressed NF- $\kappa$ B activation by TNF $\alpha$  in SVEC4 cells (cAMP 10 or cAMP 100: pCTP-cAMP 10 or 100  $\mu$ M). (C) Cilostazol and cAMP additively inhibited TNF $\alpha$ -elicited NF- $\kappa$ B activation. Although an adenylate cyclase inhibitor SQ 22536 (10  $\mu$ M) had no effect on cilostazol-induced inhibition of NF- $\kappa$ B activation by TNF $\alpha$ , a cell-permeable cAMP analogue pCTP-cAMP (100  $\mu$ M) enhanced cilostazol-induced inhibition of NF- $\kappa$ B activation by TNF $\alpha$  in SVEC4 cells. Results represent the means  $\pm$  SEM ( $n = 4$ ). \* $P < 0.05$ , \*\* $P < 0.01$  vs. NF- $\kappa$ B activity by TNF $\alpha$ . # $P < 0.05$  vs. Cil, \$ $P < 0.05$  vs. cAMP.

cytokine-induced NF- $\kappa$ B activation in vascular endothelial cells.<sup>21</sup> These data suggest that AMPK activation by cilostazol may be responsible for the inhibition of NF- $\kappa$ B activation.

Although we did not perform a strict kinase assay for AMPK, it is likely that cilostazol activates AMPK since the extent of AMPK phosphorylation at Thr-172 strongly reflects its activity,<sup>22</sup> and since phosphorylation of the AMPK consensus substrate, ACC, at Ser-79 was observed. It remains elucidated how cilostazol activates AMPK, but our data that the down-regulation of CaMKK $\beta$  using RNA interference modestly but significantly inhibited cilostazol-induced AMPK activation, suggest that CaMKK $\beta$  might at least partly mediate the effect of cilostazol on AMPK activation. This ability of AMPK activation appears to be specific for cilostazol among PDE3 inhibitors since milrinone or vesnarinone could not activate AMPK in HUVEC.

We demonstrated that cilostazol inhibits the expression of pro-inflammatory and adhesion molecule genes by blocking phosphorylation and subsequent degradation of I $\kappa$ B- $\alpha$ . These data suggest that cilostazol might suppress TNF $\alpha$ -induced NF- $\kappa$ B activation prior to I $\kappa$ B phosphorylation. We further demonstrated the stimulation of I $\kappa$ B- $\alpha$  phosphorylation by TNF $\alpha$  through the induction of IKK activity, and inhibition of IKK activity and TNF $\alpha$ -induced I $\kappa$ B- $\alpha$  phosphorylation by cilostazol. Thus, cilostazol-activated AMPK may suppress NF- $\kappa$ B activation by inhibiting IKK activity in vascular endothelial cells. It has been reported that AICAR attenuates LPS-induced activation of NF- $\kappa$ B via down-regulation of I $\kappa$ B kinase  $\alpha/\beta$  activity in glial cells.<sup>23</sup> This could be the same mechanism as we showed in this study in vascular endothelial cells, suggesting that AMPK activation may inhibit cytokine-induced NF- $\kappa$ B activation by suppressing IKK activity.

We investigated adenosine uptake using [ $^3$ H]adenosin in HUVEC. As shown in Supplementary material online, Figure S1, the adenosine uptake was dose-dependently inhibited by cilostazol in HUVEC. The increased plasma levels of adenosine due to cilostazol-induced inhibition may play a vasculo-protective role *in vivo*. We also examined the effect of cilostazol treatment on cAMP and cGMP levels in HUVEC (see Supplementary material online, Table S1). cGMP levels were low at the basal level, and the increase by cilostazol was also modest. However, this observation could be more evident *in vivo*, because cAMP elevating substances exist *in vivo*, and also NO increases cGMP levels *in vivo*. Thus, this observation could be considered as another underlying mechanism for cilostazol to play a protective role on vascular endothelial cells.

Cilostazol is a selective inhibitor of PDE3 by which it may increase intracellular cAMP and activate protein kinase A (PKA), thereby inhibiting platelet aggregation and inducing peripheral vasodilation *in vivo*.<sup>2,24</sup> Thus, we examined whether cAMP might be associated with cilostazol-induced activation of AMPK. We found that phosphorylation of AMPK with cilostazol was not affected by co-treatment with an adenylate cyclase inhibitor SQ 22536 and that a cell-permeable cAMP analogue pCTP-cAMP did not cause AMPK phosphorylation, and had no effect on cilostazol-induced phosphorylation of AMPK. Thus, AMPK activation by cilostazol independent of cAMP appears to protect against endothelial inflammation.

Although cAMP did not affect cilostazol-induced AMPK activation, we confirmed that elevated levels of cAMP inhibit cytokine-induced NF- $\kappa$ B activation in cultured endothelial cells. However, cilostazol alone, unless adenylate cyclase activators are also present, induces a modest

increase in cAMP levels in cultured cells.<sup>25</sup> Thus, suppression of NF- $\kappa$ B by cilostazol might be due to AMPK activation, rather than the cAMP pathway in cultured cells, however, in the context of increasing cAMP levels *in vivo*, cilostazol might inhibit platelet aggregation and induce peripheral vasodilation to protect endothelial cells against inflammation via cAMP elevation *in vivo*. AMPK activation by cilostazol in the setting of elevated cAMP levels might serve a protective role for endothelial cells by the additive inhibitory effects on cytokine-induced NF- $\kappa$ B activation. Recently, cross-talk between a cAMP/cAMP-dependent protein kinase and the AMPK signalling pathway has been demonstrated in an insulin-secreting cell line.<sup>26</sup> However, the mechanism of cross-talk between cAMP and AMPK remains to be elucidated in vascular endothelial cells.

In conclusion, we demonstrated that cilostazol inhibits the expression of various pro-inflammatory and adhesion molecule genes by blocking NF- $\kappa$ B activation in vascular endothelial cells. Through AMPK activation, cilostazol attenuates the phosphorylation and subsequent degradation of I $\kappa$ B $\alpha$  by inhibiting IKK activity, resulting in suppression of cytokine-induced NF- $\kappa$ B activation. Although experiments on cultured cells do not necessarily represent the events that occur *in vivo*, particularly with regard to the possible cAMP elevating effect, our findings suggest that cilostazol-induced AMPK activation might have beneficial effects on endothelial functions in addition to its selective inhibitory action of PDE3, thereby inhibiting platelet aggregation and inducing peripheral vasodilation.<sup>2,24</sup>

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## Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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