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# Synergistic Efficacy of Concurrent Treatment with Cilostazol and Probucol on the Suppression of Reactive Oxygen Species and Inflammatory Markers in Cultured Human Coronary Artery Endothelial Cells

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**In the present study, we aimed to identify the synergistic effects of concurrent treatment of low concentrations of cilostazol and probucol to inhibit the oxidative stress with suppression of inflammatory markers in the cultured human coronary artery endothelial cells (HCAECs). Combination of cilostazol (0.3 ~ 3 $\mu$M) with probucol (0.03 ~ 0.3 $\mu$M) significantly suppressed TNF-$\alpha$-stimulated NAD(P)H-dependent superoxide, lipopolysaccharide (LPS)-induced intracellular reactive oxygen species (ROS) production and TNF-$\alpha$ release in comparison with probucol or cilostazol alone. The combination of cilostazol (0.3 ~ 3 $\mu$M) with probucol (0.1 ~ 0.3 $\mu$M) inhibited the expression of vascular cell adhesion molecule-1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) more significantly than did the monotherapy with either probucol or cilostazol. In line with these results, combination therapy significantly suppressed monocyte adhesion to endothelial cells. Taken together, it is suggested that the synergistic effectiveness of the combination therapy with cilostazol and probucol may provide a beneficial therapeutic window in preventing atherosclerosis and protecting from cerebral ischemic injury.**

**Key Words: Cilostazol, Probucol, VCAM-1, MCP-1, Monocyte adhesion, Synergism, Endothelial cells**

## INTRODUCTION

Atherosclerosis is a chronic inflammatory process with increased oxidative stress by which the adhesion of monocytes to the vascular endothelium and their subsequent migration into the vessel wall are increased (Libby, 1995; Ross, 1993). In the inflammatory and proliferative responses of the endothelium, adhesion molecules (VCAM-1), chemokines (MCP-1) and cytokines (interleukin-1$\beta$ and tumor necrosis factor-$\alpha$) are secreted by the activated endothelial cells in the atherosclerotic lesions (Bevilacqua et al, 1994; Reape et al, 1999). VCAM-1 has been reported to exert a dominant role in the initiation of atherosclerosis (Cybulsky et al, 2001; Dansky, 2001). An increased MCP-1 expression was also demonstrated to be implicated in the monocyte adhesion to the endothelium (Gu et al, 1998).

Cilostazol was first introduced to inhibit phosphodiesterase type 3 and to suppress cyclic AMP degradation (Kimura et al, 1985). Recently, cilostazol has been demonstrated to scavenge the hydroxyl and peroxyl radicals (Kim et al, 2002; Choi et al, 2002), and to increase the $K^+$ currents in SK-N-SH (human brain neuroblastoma) cells by opening the maxi-K channels (Hong et al, 2003). Most recently, cilostazol was reported to exert a cell-protective effect by suppressing remnant lipoprotein particle (RLP)-stimulated NAD(P)H oxidase-dependent superoxide formation and cytokine (TNF-$\alpha$ and IL-1$\beta$) production in the human umbilical vein endothelial cells (Shin et al, 2004). Park et al. (2005) identified the anti-inflammatory action of cilostazol, in that cilostazol inhibits RLP-stimulated increased monocyte adhesion to HUVECs concurrently with suppression of expressions of VCAM-1 and MCP-1.

Probucol, a potent lipid-soluble antioxidant, has been reported to prevent atherogenesis by acting as an antioxidant and suppressing the oxidative modification of low density lipoprotein (LDL) (Carew et al, 1987; Kuzuya et al 1993). In line with these facts, probucol was demonstrated to decrease mononuclear cell adhesion to vascular endothelium in the cholesterol-fed rabbit both *in vitro* and *in vivo* studies (Ferns et al, 1993; Faruqi et al, 1994). Interest in probucol has been raised by Tardif et al. (1997) who demonstrated that probucol significantly reduces the rate of coronary artery restenosis after coronary angioplasty in humans. Zapolska-Downar et al. (2001) have further demonstrated the anti-atherogenic effect of probucol, in that probucol downregulates adhesion of monocytes in association with

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**ABBREVIATIONS:** HCAEC, human coronary artery endothelial cell; ROS, reactive oxygen species; LPS, lipopolysaccharide; VCAM, vascular cell adhesion molecule-1; MCP, monocyte chemoattractant protein-1; TNF-a, tumor necrosis factor-$\alpha$; IL-1$\beta$, interleukin-1$\beta$.

reduced VCAM-1 expression.

Both cilostazol and probucol have been approved to be safe and efficient in their respective therapeutic categories with some different and similar action mechanisms. Nevertheless, some hitherto uncharacterized untoward effects may limit their long-term uses in clinics. It has been reported that probucol may lower high density lipoprotein level in plasma (Johansson et al, 1995) and rarely prolong the Q-T interval with proarrhythmic risk (Reinoehl et al, 1996). Cilostazol was reported to show side effects such as headache in some patients (Mallikaarjun et al, 1999). To provide a beneficial therapeutic window with fewer side effects, we examined the combination therapy with low concentrations of cilostazol and probucol in the *in vitro* experiment. Thus, in the present study, we determined effects of low concentration of cilostazol and probucol in combination on the TNF-$\alpha$-stimulated NAD(P)H-dependent superoxide, lipopolysaccharide (LPS)-induced intracellular ROS production, TNF-$\alpha$ release, expression of VCAM-1 and MCP-1 and monocyte adhesion to the endothelial cells, and their effects were compared with the effect of the monotherapy. It is highly likely that the combination therapy may provide new therapeutic window in coronary artery restenosis and ischemic brain injury with few side effects.

## METHODS

### *Cell cultures*

HCAEC (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 (FBS).

### *Measurement of superoxide anion*

Endothelial homogenates (100 $\mu$g protein/well) were placed into the luminometer (Microlumat LB96P, EG & G Berthold). Immediately before recording chemiluminescence, NADH and NADPH (final concentration, 100 $\mu$M each) were added, and dark-adapted lucigenin (*bis-N*-methylacridinium nitrate, 5 $\mu$M) was added via an autodispenser. Each experiment was performed in triplicate.

### *Assay of intracellular ROS*

Measurement of intracellular ROS was based on ROS-mediated conversion of non-fluorescent DCFH-DA into DCFH. DCFH is able to react with ROS, and to be rapidly oxidized to the highly fluorescent 2,7-dichlorofluorescein. The intensity of fluorescence reflects the level of oxidative stress. Cells were incubated in the dark for 2 hours at 37°C in 50 mM phosphate buffer, pH 7.4, containing 5 $\mu$M DCFH-DA. The quantity of DCFH fluorescence was measured at an emission wavelength of 530 nm and an excitation wavelength of 485 nm by using FLUOstar OPTIMA (BMG LABTECH GmbH, Germany).

### *VCAM-1 assay*

Cells were seeded $2\times10^4$ cells/well in 96-well tissue culture plates containing TNF-$\alpha$ for 4 hours. After treatment with 2% paraformaldehyde, cells were incubated for 1 hour with antibodies specific to human VCAM-1 (R&D Systems, Minneapolis, MN) and then further incubated for 1 hour with secondary antibody. The second antibody binding was detected by reaction of tetramethylbenzidine with $H_2O_2$ (TMB Substrate Reagent Set; BD Biosciences). Absorbance at 450 nm was measured using ELISA reader.

### *MCP-1 and TNF-$\alpha$ assay*

The amounts of MCP-1 and TNF-$\alpha$ in HCAECs supernatants were measured with ELISA kits (R&D Systems, Minneapolis, ML) according to the manufacturer's protocol.

### *Adhesion assay*

For the adhesion assay, HCAECs were plated on six-well dishes at a density of $1.2\times10^5$ cells/well. On the next day, cells received EGM-2 with 0.5% FBS plus drug 15 min before stimulation with TNF-$\alpha$ and then were exposed to TNF-$\alpha$ for 4 hours. 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. After removing non-adhering cells, monolayers were fixed with 1% paraformaldehyde. The number of ad-

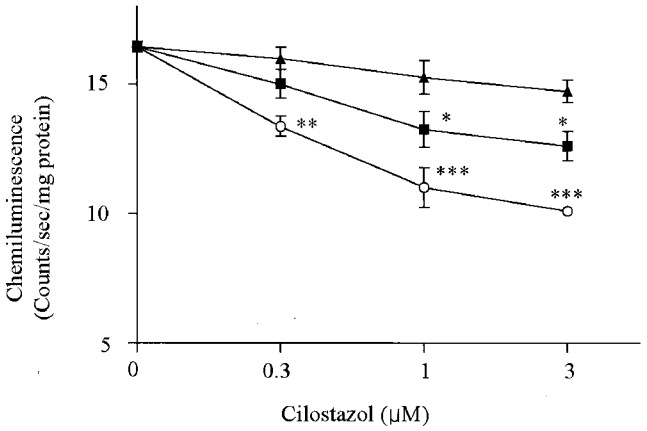


**Fig. 1.** Effects of cilostazol alone and in combination of cilostazol with probucol on the NAD(P)H-dependent superoxide production in the HCAECs stimulated by TNF-$\alpha$ (50 ng/ml). The significant effect was evident by cilostazol in combination with probucol. Results are expressed as mean±S.E.M. of four experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. cilostazol alone. Significant differences were shown between cilostazol alone and cilostazol plus probucol 0.03 $\mu$M or cilostazol plus 0.1 $\mu$M probucol groups by two-way repeated measures ANOVA.

herent cells was counted in six different fields using an ocular grid (0.01 $mm^2$ per field).

### *Drugs*

Cilostazol {6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl) butoxy]-3, 4-dihydro-2(1*H*)-quinolinone} and probucol [4,4'-(isopropylidenedithio)bis(2,6-di-tert-butylphenol)] were donated by Otsuka Pharmaceutical Co. Ltd., and both were dissolved in dimethyl sulfoxide as a 10 mM stock solution.

### *Statistical analysis*

The results are expressed as mean±S.E.M. The comparison of changes in superoxide, intracellular ROS and VCAM-1 between groups was analyzed by repeated measures ANOVA, followed by Turkey's multiple comparison tests as a post hoc comparison. Differences were analyzed by one-way analysis of variance combined with Turkey's test multiple comparison tests, and $p<0.05$ was considered statistically significant.

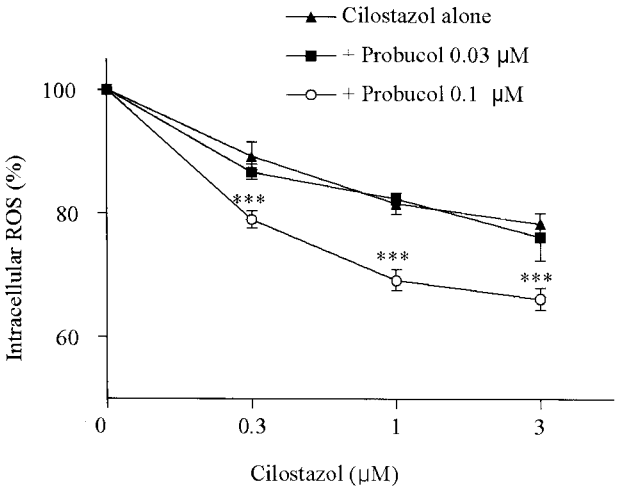


**Fig. 2.** Inhibitory effect of cilostazol in combination with probucol on the intracellular ROS levels stimulated by in the HCAECs LPS (1 $\mu$g/ml). LPS-stimulated intracellular ROS was significantly decreased by cilostazol plus 0.1 $\mu$M probucol in combination. ***$p<0.001$ vs. cilostazol alone. Results are expressed as mean±S.E.M. of four experiments. Significant differences were shown between cilostazol alone and cilostazol plus 0.1 $\mu$M probucol groups by two-way repeated measures ANOVA.

## RESULTS

### *Inhibitory effects on superoxide formation and intracellular ROS production*

The synergistic effects of coadministration of probucol and cilostazol were determined in the cultured HCAECs. When measured by recording of chemiluminescence, superoxide production in response to NAD(P)H (control, 9.2±0.8 counts/s/mg protein) was significantly elevated by incubation in the medium containing 50 ng/ml TNF-$\alpha$ (16.4±0.2 counts/s/mg protein, $p<0.001$) for 4 hours. Cilostazol (0.3, 1 and 3 $\mu$M) showed a marginal reduction in superoxide formation. Coadministration of cilostazol (0.3, 1 and 3 $\mu$M) with 0.03 $\mu$M or 0.1 $\mu$M probucol, showed that superoxide production was significantly decreased ($p<0.01$ and $p<0.001$, respectively) in comparison with cilostazol alone (Fig. 1).

Following incubation of confluent cells with LPS (1 $\mu$g/ml) for 1 hours, intracellular ROS was significantly increased to 123.3±1.9% (control=100%), which was marginally decreased by 0.03 $\mu$M probucol (112.3±3.2%) and 0.1 $\mu$M probucol (110.9±2.7%). The addition of 0.03 $\mu$M probucol to cilostazol (0.3, 1 or 3 $\mu$M) showed little changes in LPS-induced intracellular ROS. When of 0.1 $\mu$M probucol was combined with cilostazol (0.3, 1 and 3 $\mu$M), intracellular ROS was markedly decreased ($p<0.001$), the degree of which was significantly larger than that of cilostazol monotherapy (Fig. 2).

### *Inhibitory effects on TNF-a formation*

Incubation of HCAECs with LPS (1 $\mu$g/ml) for 18 hours largely increased, TNF-$\alpha$ level (baseline, 20.7±3.8 pg/ml) to 104.7±6.4 pg/ml. Cilostazol (0.3, 1 $\mu$M) and probucol (0.3 $\mu$M) singly had no effect on TNF-$\alpha$ production, however, combination of cilostazol with probucol significantly decreased the TNF-$\alpha$ level to 78.3 ±5.7 pg/ml ($p<0.01$) and

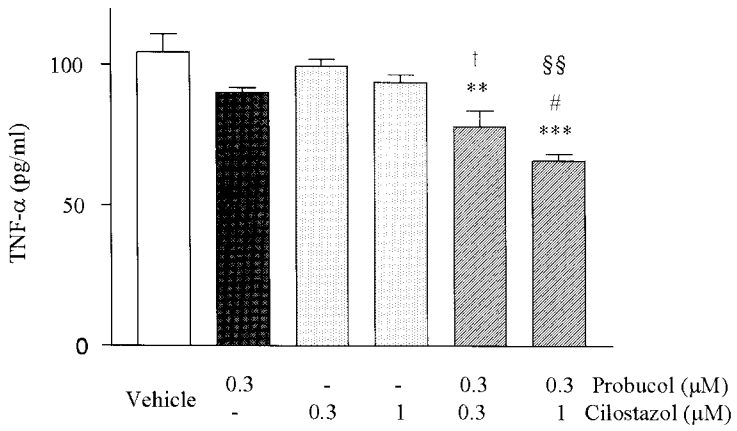


**Fig. 3.** Effects of cilostazol and probucol alone and their combination on the TNF-$\alpha$ formation stimulated by in the HCAECs LPS (1 $\mu$g/ml). Increased TNF-$\alpha$ by LPS was significantly decreased by cilostazol and probucol in combination. Results are expressed as mean±S.E.M. of three experiments. **$p<0.01$, ***$p<0.001$ vs. vehicle, $^{\#}p<0.05$ vs. 0.3 $\mu$M probucol alone, $^{\dagger}p<0.05$ vs. 0.3 $\mu$M cilostazol alone, $^{\S\S}p<0.01$ vs. 1 $\mu$M cilostazol alone.

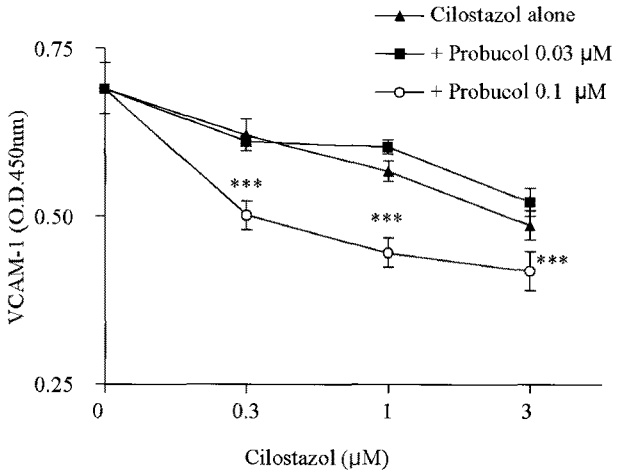


**Fig. 4.** Inhibitory effect of cilostazol in combination with probucol on the VCAM-1expression in the HCAECs. TNF-$\alpha$ (50 ng/ml)-induced increased VCAM-1expression was significantly decreased by treatment with cilostazol and 0.1 $\mu$M probucol in combination. Results are expressed as mean±S.E.M. of four experiments. ***p< 0.001 vs. cilostazol alone. Significant differences were shown between cilostazol plus 0.03 $\mu$M probucol and cilostazol plus 0.1 $\mu$M probucol groups by two-way repeated measures ANOVA.

66.6±2.5 pg/ml ($p<0.001$) (Fig. 3).

### *Effects on VCAM-1 and MCP-1*

TNF-$\alpha$ (50 ng/ml) (baseline, 0.35±0.03 $OD_{450}$) caused a large increase of VCAM-1 expression (0.69±0.03 $OD_{450}$), to approximately 97%. Probucol in the ranges of 0.03~0.3 $\mu$M showed little reduction in VCAM-1 expression (data not shown). Inhibition of VCAM-1 expression by combination of cilostazol (0.3, 1 and 3 $\mu$M) with 0.1 $\mu$M probucol was clearly observed with a significance ($p<0.001$) when compared with treatment with cilostazol alone (Fig. 4).

Under treatment with TNF-$\alpha$ (50 ng/ml), MCP-1 level (baseline, 43.0±2.8 pg/ml) was markedly increased to 293.2± 12.4 pg/ml, which was significantly suppressed by the combination of 0.3 $\mu$M cilostazol with 0.3 $\mu$M probucol (243.9± 7.6 pg/ml, $p<0.05$) and 1 $\mu$M cilostazol with 0.3 $\mu$M probucol (226.1±8.0 pg/ml, $p<0.05$). Inhibition of MCP-1 expression by the combination therapy was significantly greater than that of probucol or cilostazol alone (Fig. 5).

### *Effects on monocyte adherence to HCAECs*

Adhesion of human monocytoid U937 cells to HCAECs was assayed under stimulation with TNF-$\alpha$ for 4 hours. TNF-$\alpha$ (50 ng/ml) caused a large increase in monocyte adhesion to HCAECs from 14.4±2.0 cells/$mm^2$ (baseline) to 58.1±3.3 cells/$mm^2$ (4-fold). TNF-$\alpha$-stimulated monocyte

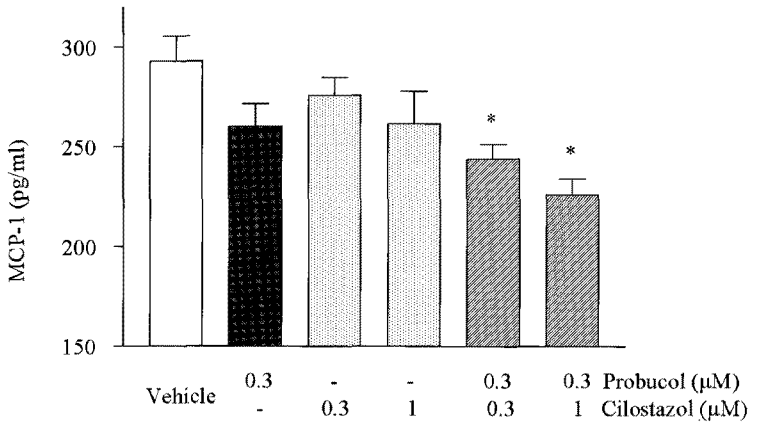


**Fig. 5.** Effects of cilostazol and probucol in combination on the MCP-1 expression stimulated in the HCAECs by TNF-$\alpha$ (50 ng/ml). Concurrent treatment with 0.3 $\mu$M probucol plus 0.3 or 1 $\mu$M cilostazol significantly decreased MCP-1 expression in comparison to effect of probucol and cilostazol alone. Results are expressed as mean±S.E.M. of three experiments. *p<0.05 vs. vehicle.

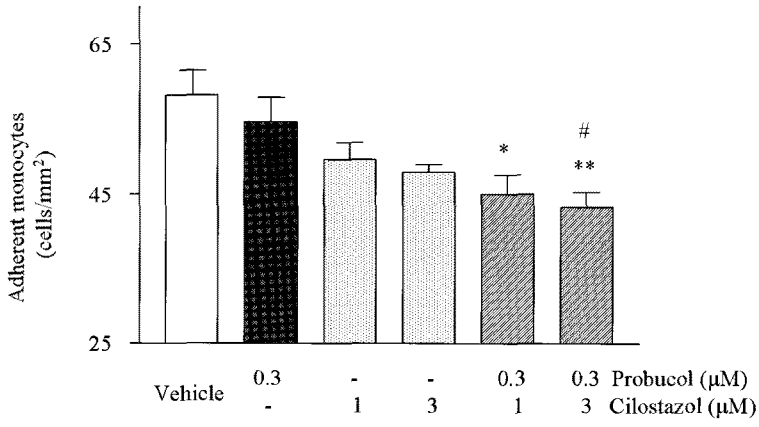


**Fig. 6.** Effects of cilostazol and probucol alone and their combination on the monocyte adhesion to HCAECs. TNF-$\alpha$ (50 ng/ml)-stimulated adherent monocytes were significantly decreased by treatment with cilostazol and probucol in combination. Results are expressed as mean±S.E.M. of four experiments. *p<0.05, **p<0.01 vs. vehicle, #p<0.05 vs. probucol alone.

adhesion was little affected by cilostazol or probucol alone. Combination of cilostazol (1, 3 $\mu$M) with 0.3 $\mu$M probucol significantly inhibited adhesion of monocyte to 45.0±2.5 cells/mm$^2$ ($p<0.05$) and 43.2±2.1 cells/mm$^2$ ($p<0.01$) (Fig. 6).

## DISCUSSION

In the present study, we observed that the concurrent treatment with low concentrations of cilostazol and probucol in the cultured HCAECs synergistically and significantly reduced oxidative stress and inflammatory markers when compared with the effects of the monotherapy with either cilostazol or probucol.

A mounting evidence has emphasized that ROS, including $H_2O_2$ and hydroxyl radical, induce apoptosis (Li et al, 1997), and that the apoptotic processes play an important role in the cell death in the atherosclerotic plaque (Kockx et al, 1998). Therefore, prevention of oxidative stress-mediated cell injury is an area of active investigation. In the present study, combination of cilostazol with 0.03 and 0.1 $\mu$M probucol synergistically reduced NAD(P)H-dependent superoxide formation and intracellular ROS production in comparison with the monotherapy. Similarly, LPS-stimulated TNF-$\alpha$ production was also significantly decreased by the concurrent administration of probucol (0.3 $\mu$M) with 0.3 and 1 $\mu$M cilostazol. Considering the reported results that cilostazol has a property to scavenge the hydroxyl and peroxyl radicals (Kim et al, 2002; Choi et al, 2002), and protects cell injury by suppressing NAD(P)H oxidase-dependent superoxide formation and cytokine (TNF-$\alpha$ and IL-1$\beta$) production in the HUVECs (Shin et al, 2004), it is highly possible that the combination therapy with cilostazol and probucol may exert more beneficial effects in reducing the cell death with reduced DNA fragmentation.

VCAM-1 plays an important role in the early atherogenesis. Fruebis et al. (1997; 1999) demonstrated that probucol prevents atherosclerotic lesion formation in the aortic wall of LDL receptor-deficient rabbits accompanied with reduction of VCAM-1 expression and downregulation of adhesion of monocytes (Zapolska-Downar et al, 2001). In the present study, the combination therapy with cilostazol and either 0.1 or 0.3 $\mu$M probucol markedly inhibited VCAM-1 and MCP-1, while low concentration of individual probucol or cilostazol alone showed a marginal reduction in VCAM-1 expression. Inhibition of the adherence of human monocytoid U937 cells to HCAECs by the combination therapy was much stronger then the effect of individual administration of probucol or cilostazol, suggesting that the combined therapy elicits additional substantial and significant inhibition of these variables.

Regarding the action mechanism by which cilostazol reduces expression of adhesion molecules (VCAM-1, ICAM-1 and E-selectin) and chemokine (MCP-1), Park et al. (2005; 2006) have recently suggested that cilostazol inhibits remnant lipoprotein particle-stimulated increased monocyte adhesion to human umbilical vein endothelial cells by suppression of NF-$\kappa$B via cyclic AMP-dependent protein kinase-activated maxi-K channel opening that is inhibited by iberiotoxin, a maxi-K channel blocker. In light of these results, the maxi-K channel opening-coupled suppression of NF-$\kappa$B by cilostazol appears to largely contribute to suppression of expression of adhesion molecules (VCAM-1) and chemokine (MCP-1). Furthermore, the antioxidant action of cilostazol and probucol may synergistically contribute to the inhibition of expression of adhesion molecules and chemokine (Kim et al, 2002; Bilenko et al, 2003), in agreement with the report of Sekiya et al. (1998) in that the combined treatment with probucol and cilostazol proved to be safe and effective in preventing acute poststenting complications and suppressing chronic restenosis in patients subjected to coronary angioplasty.

Most recently, Park et al. (2007) confirmed the beneficial synergistic effects of concurrent treatment with low doses of cilostazol and probucol against focal cerebral ischemic infarct in rats. They showed that the ischemic infarct area and volume induced by 2-hour occlusion of middle cerebral artery (MCA) and 22-hour reperfusion in rat brain were significantly reduced, when received cilostazol (20 mg/kg, twice) and probucol (30 mg/kg, twice), accompanied with prominent improvement of neurological function via reducing the inflammatory and oxidative processes, and apoptotic cell death in the ischemic brain in comparison with the effect of cilostazol or probucol monotherapy.

Taken together, the combination therapy with low concentrations of both drugs may clinically provide more effective therapeutic potential in reducing restenosis, preventing atherosclerosis and protecting from cerebral ischemic injury with few side effects.

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