

Blockade of Phosphodiesterase-III Protects Against Oxygen-Glucose Deprivation in Endothelial Cells by Upregulation of VE-Cadherin

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Abstract: We recently reported that a phosphodiesterase-III inhibitor, cilostazol, prevented the hemorrhagic transformation induced by focal cerebral ischemia in mice treated with tissue plasminogen activator (tPA) and that it reversed tPA-induced cell damage by protecting the neurovascular unit, particularly endothelial cells. However, the mechanisms of cilostazol action are still not clearly defined. The adheren junction (AJ) protein, VE-cadherin, is a known mediator of endothelial barrier sealing and maintenance. Therefore, we tested whether cilostazol might promote expression of adhesion molecules in endothelial cells, thereby preventing deterioration of endothelial barrier functions. Human brain microvascular endothelial cells were exposed to 6-h oxygen-glucose deprivation (OGD). We compared cilostazol with aspirin treatments and examined 2 representative AJ proteins: VE-cadherin and platelet endothelial cell adhesion molecule-1 (PECAM-1). A protein kinase A (PKA) inhibitor, LY294002 (a PI3-K inhibitor), db-cAMP, and Rp-cAMPS were used to assess the roles of cAMP, PKA, and PI3-K signaling, respectively, in cilostazol-induced responses. Cilostazol and db-cAMP prevented OGD-stress injury in endothelial cells by promoting VE-cadherin expression, but not PECAM-1. Aspirin did not prevent cell damage. PI3-K inhibition by LY294002 had no influence on the effects of cilostazol, but inhibition of cAMP/PKA with PKA inhibitor and Rp-cAMPS suppressed cilostazol-induced inhibition of cell damage and promotion of VE-cadherin expression. In contrast, OGD stress had no detectable effects on VEGF, VEGF receptor, or angiopoietin-1 levels. Cilostazol promotes VE-cadherin expression through cAMP/PKA-dependent pathways in brain endothelial cells; thus, cilostazol effects on adhesion molecule signaling may provide protection against OGD stress in endothelial cells.

Keywords: Adheren junction, cAMP, endothelial protection, oxygen–glucose deprivation, PDE-III inhibitor, VE-cadherin.

INTRODUCTION

Cilostazol, a selective inhibitor of phosphodiesterase III, is an antiplatelet drug and a vasodilator that causes increased cyclic AMP (cAMP) and cyclic GMP levels [1]. Cilostazol has been approved for use as a vasodilating antiplatelet drug for the treatment of ischemic symptoms in chronic peripheral arterial obstruction or intermittent claudication and for secondary prevention of cerebral infarction (CSPS I) [2]. Cilostazol has also been used after aneurismal subarachnoidal hemorrhage (SAH) to prevent development of delayed cerebral vasospasm [3]. Recently, cilostazol has been shown to be a more effective and safer alternative to aspirin for long-term prevention of the recurrence of ischemic stroke in patients with chronic ischemic stroke [4].

In rats, cilostazol has also been reported to have a neuroprotective effect against ischemic brain injury and to prevent attenuated acute brain infarction induced by middle cerebral artery occlusion (MCAO) and reperfusion [5]. We have also reported that when combined with treatment with normobaric hyperoxia, cilostazol protects mice against focal cere-

bral ischemia [6], and provides neuroprotection against filamental MCAO-mediated increases in metallothionein-1 and -2 [7]. Increasing evidence also indicates that cilostazol may offer endothelial protection via both an inhibition of lipopolysaccharide-induced apoptosis and an inhibition of neutrophil adhesion to endothelial cells [8].

Endothelial cells act as gatekeepers, controlling the infiltration of blood proteins and cells into the vessel wall. This unique characteristic is largely exerted by the coordinated opening and closure of cell-cell junctions. The formation of adhesive structures between cell-cell junctions, including adherens and tight junctions, contributes to the establishment of cell polarity, differentiation, and survival, and is ultimately required for the maintenance of tissue integrity. Adhesion molecules, such as VE-cadherin and claudin-5, can serve as markers for endothelial junctions as their pattern of expression is highly restricted to the endothelial lineages and their organization is quite distinct from more common junctions, such as those found in epithelial cells [9]. VE-cadherin knockout is embryonic lethal and knockout mice show severe defects during developmental angiogenesis [10], suggesting that the function of VE-cadherin is not restricted to promoting cell-cell adhesion.

Since endothelial cells are one of the main constituents of the blood-brain barrier (BBB), the integrity of the BBB is compromised in many disorders of the human central nervous system. Therefore, endothelial breakdown under

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conditions of neuroinflammation and cerebral ischemia, as well as in response to traumas and brain tumors, leads to loss of the protective function of the barrier [11].

Although the protective mechanisms of cilostazol exerted on endothelial cells are still not clearly defined, one of known molecular targets for cilostazol is cAMP. Cilostazol has been reported to be more effective than aspirin in the secondary prevention of all types of stroke in patients and, in particular, in preventing secondary attacks of hemorrhagic stroke in patients (CSPS II) [12]. We also showed that cilostazol protects against hemorrhagic transformation in mouse transient focal cerebral ischemia [13] and that it prevents hemorrhagic transformation induced by focal cerebral ischemia in mice treated with tPA. Cilostazol protected against tPA-induced cerebral injury in mice that had undergone a hemorrhagic transformation, it prevented the loss of claudin-5, a component of the tight junction complex, and it inhibited tPA-induced cell damage in human brain microvascular endothelial cells. These effects may be due, at least in part, to protection of endothelial cells, the neurovascular unit associated with cAMP activity [14]. Accordingly, in the present study, we now examined whether cilostazol protects against endothelial damage by using OGD as an *in vitro* ischemia model. We also aimed to clarify the mechanism whereby cilostazol provides endothelial protection against OGD-induced cell damage.

MATERIALS AND METHODS

Drugs and Materials

For this study, cilostazol was kindly gifted by Otsuka Pharmaceutical Co. Ltd. (Plemaal; Tokushima, Japan). Aspirin was purchased from Wako Pure Chemical Industries (Osaka, Japan). dibutyryl adenosine 3'5' -cyclic monophosphate sodium salt (db-cAMP ; a cAMP-agonist), Rp-cyclic adenosine monophosphorothioate (Rp-cAMPS ; a cAMP-antagonist), Protein kinase A (PKA) inhibitor, and LY294002 (Phosphatidylinositol 3-kinase (PI3-K) inhibitor) were from Sigma-Aldrich (Madison, WI, USA). Human brain microvascular endothelial cells (HBMVECs) were obtained from Cell Systems (Kirkland, WA, USA). EBM-2 medium with endothelial growth supplements were from Lonza (Walkersville, MD, USA). The LDH kit was from Promega (Madison, Wis, USA).

Cell Culture

HBMVECs were maintained in HuMedia EG2 medium supplemented with 10% heat-inactivated horse serum at 37°C under a humidified 5% CO₂ atmosphere. To examine the effect of cilostazol, aspirin, db-cAMP, or Rp-cAMPS on oxygen-glucose deprivation (OGD)-induced cell death, cells were seeded into 12-well plates. After incubating for 2 days, the culture medium was removed, the cells were washed twice with glucose-free Dulbecco's modified Eagle's medium, and then incubated in the same glucose-free medium for 6 h in an oxygen-free incubator (94% N₂, 5% CO₂, and 1% O₂). After OGD, cells were incubated under normal growth conditions for an additional 30 min (reoxygenation). Cilostazol (3, 10, or 30 µM), aspirin (30 or 100 µM), cilostazol (30 µM) plus PKA inhibitor (1 µM),

cilostazol (30 µM) plus LY294002 (10 µM), db-cAMP (100 or 300 µM), or Rp-cAMPS (200 µM) was added to the glucose-free medium before OGD treatment. After the cells were treated, the conditioned medium was harvested and used for determination of LDH, a marker of nonspecific cell damage. Cell morphological changes were observed using an inverted epifluorescence microscope (Olympus, Tokyo, Japan).

Western Blot Analysis

Cells were homogenized in 120 µl ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 50 mM EDTA, 1% Triton X-100, and protease/phosphatase inhibitor mixture). A 3 µg protein sample was subjected to electrophoresis on a 5%-20% gradient SDS-polyacrylamide gel (SuperSep Ace; Wako Pure Chemicals). Separated proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Corporation, Billerica, MA, USA). For immunoblotting, the following primary antibodies were used: polyclonal antibody to VE-cadherin (1:2000 dilution; Abcam, Cambridge, UK), PECAM-1 (1:2000 dilution; Abcam), VEGF (1:2000 dilution; Cell Signaling, Technology Inc., Danvers, MA, USA), VEGF Receptor (1:2000 dilution; Cell Signaling), Phospho-VEGF Receptor (Tyr1175) (1:2000 dilution; Cell Signaling), Angiopoietin-1 (1:2000 dilution; Abcam), and monoclonal antibody to GAPDH (1:2000 dilution; Cell Signaling). The secondary antibody was anti-rabbit HRP-conjugated IgG (1:2000 dilution). The SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Waltham, MA, USA) was used to visualize the immunoreactive bands. A Lumino Imaging Analyzer (FAS-1000; Toyobo Engineering, Osaka, Japan) and a Gel Pro Analyzer (Media Cybernetics, Inc., Bethesda, MD, USA) were used to measure the band intensity.

Statistical Analysis

Data are presented as means ± SEM. Statistical comparisons were made using a one- or two-way analysis of variance followed by Student's *t* test, Turkey's test, or Dunnett's test. *P* < 0.05 was considered to indicate statistical significance.

RESULTS

The Effects of Cilostazol and Aspirin on Cell Damage Induced by Oxygen-Glucose Deprivation (OGD) Stress in Human Brain Microvascular Endothelial Cells

We analyzed changes in cellular morphology and effects on the LDH level under OGD stress in cilostazol-treated endothelial cells. In addition, for comparison purposes, we also assessed the effects of aspirin on OGD stress. The effects of cilostazol and aspirin were evaluated with an LDH kit to determine the extent of cell damage under OGD stress in the human brain microvascular endothelial cells. In endothelial cells, the LDH level was increased by 6-h OGD stress, and this increase was significantly reduced by cilostazol at 3, 10, and 30 µM in a concentration-dependent manner, but was not reduced by aspirin at either 30 or 100 µM (Fig. 1B). Interestingly, after 6 h of cilostazol supplementation under OGD stress, microscopy revealed a

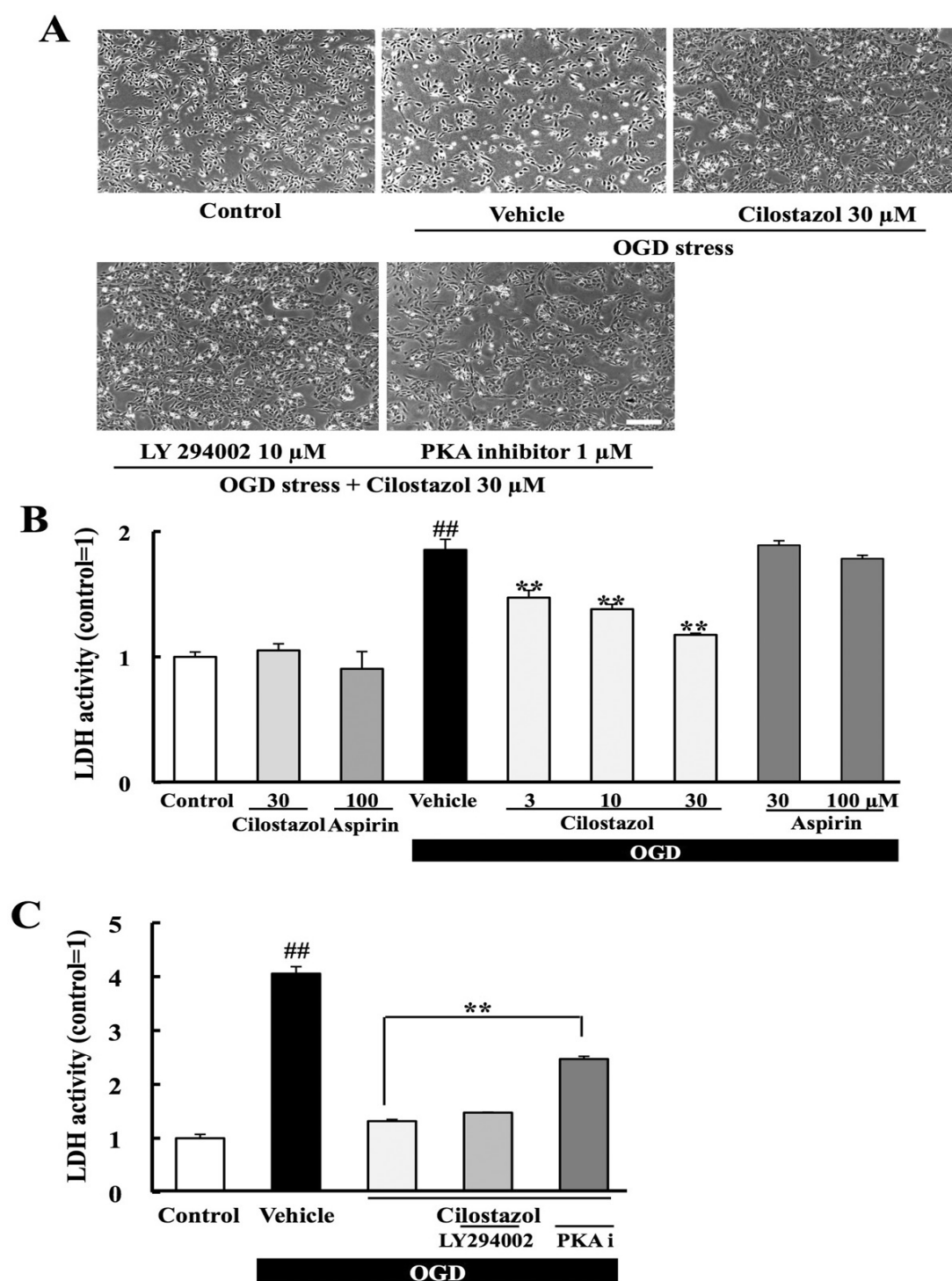


Fig. (1). Effects of cilostazol and aspirin on the cell damage induced by oxygen-glucose deprivation (OGD) stress in human brain microvascular endothelial cells. **(A)** Representative phase microscopy image of endothelial cells at 6 h after control or OGD conditions. Control cells (upper left), cells in OGD conditions (upper center), cells treated with 30 μ M cilostazol in OGD conditions (upper right), cells treated with 30 μ M cilostazol plus LY294002 in OGD conditions (lower left), and cells treated with 30 μ M cilostazol plus PKA inhibitor (PKAi) in OGD conditions (lower right). Scale bar = 50 μ m. **(B)** Cell damage after OGD was assessed by measuring LDH release into the culture medium in endothelial cells. The LDH level was increased by OGD treatment, and the increase was reduced by cilostazol at 3, 10, and 30 μ M in endothelial cells. In contrast, aspirin at 30 or 100 μ M did not reduce endothelial cell damage induced by OGD. $^{##}P < 0.01$ vs. control, $^{**}P < 0.01$ vs. OGD-treated vehicle group (Dunnett's test, $n = 3$ or 4). **(C)** In addition, endothelial cells were treated with the vehicle or 30 μ M cilostazol with or without LY294002 (10 μ M) or PKAi (1 μ M) in 6-h OGD conditions. The prevention of cell damage by cilostazol was inhibited by PKA inhibitor, but the inhibition by LY294002 was not significant. $^{##}P < 0.01$ vs. control and 30 μ M cilostazol alone, $^{**}P < 0.01$ vs. 30 μ M cilostazol alone (Dunnett's test, $n = 3$). Data are expressed as means $\pm$ SEM.

morphological binding of one cell to another, which was not observed in untreated cells (Fig. 1A).

The Effects of Cilostazol on the PI3-K and PKA Pathway in Human Brain Microvascular Endothelial Cells

The pathway whereby cilostazol prevented cell damage induced by OGD stress was evaluated by examining the effect of a PI3-K inhibitor (LY 294002) and a PKA inhibitor on cell damage in cilostazol-treated endothelial cells. The LDH level was decreased by 30 μ M cilostazol, and this decrease was significantly enhanced by 30 μ M cilostazol plus PKA inhibitor, but not by 30 μ M cilostazol plus the PI3-K inhibitor (Fig. 1C). In microscope views, cilostazol plus PKA inhibitor prevented the binding of one cell to another, compared to cilostazol-treated endothelial cells (Fig. 1A).

On the other hand, a PI3-K inhibitor did not inhibit these adhesions induced by OGD stress plus cilostazol. These findings suggest that cilostazol suppressed OGD-induced cell damage via the PKA pathway, not the PI3-K pathway. Given the average plasma concentration of cilostazol orally administered to humans (100 mg/body/day) is about 2 to 10 μ M and may be partially higher in the body, the concentration of cilostazol we used is consistent with a clinical concentration [15].

The Effects of db-cAMP and Rp-cAMPS on Cell Damage Induced by OGD Stress in Human Brain Microvascular Endothelial Cells

Cilostazol increases intracellular cAMP content and accordingly activates PKA signaling [16]. We next examined

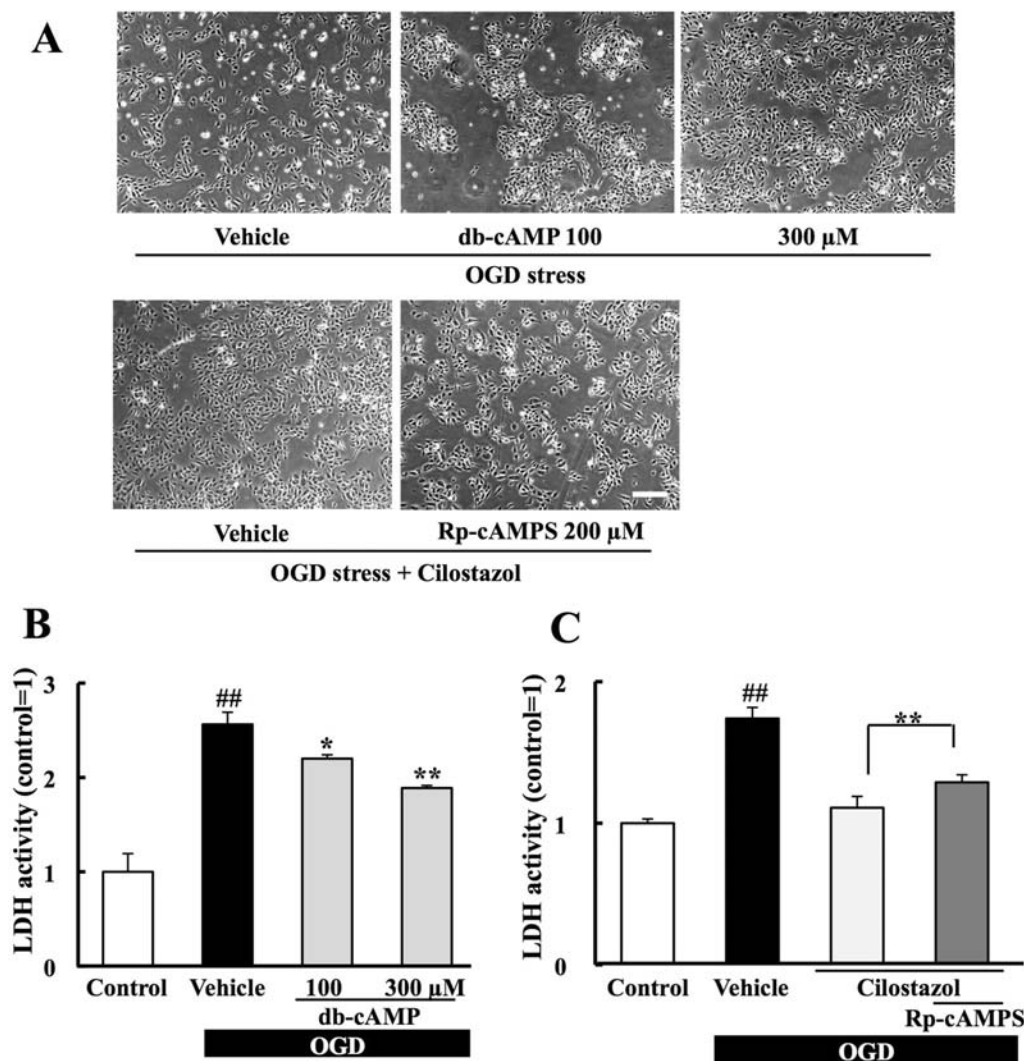


Fig. (2). Effects of db-cAMP and Rp-cAMPS on the cell damage in OGD conditions. **(A)** Representative phase microscopy image of endothelial cells at 6 h after OGD conditions. Cells in OGD conditions (upper left), cells treated with 100 μ M db-cAMP (a cAMP-agonists) in OGD conditions (upper center), with 300 μ M db-cAMP in OGD conditions (upper right), cells treated with 30 μ M cilostazol in OGD conditions (lower left), and cells treated with 30 μ M cilostazol plus Rp-cAMPS (a cAMP-antagonists) at 200 μ M in OGD conditions (lower right). The LDH level was increased by OGD treatment, and the increase was reduced by db-cAMP at 100 and 300 μ M in endothelial cells. $^{###}P < 0.01$ vs. control, $^{*}P < 0.05$, $^{**}P < 0.01$ vs. OGD-treated vehicle group (Dunnett's test, $n = 3$). **(B)** Next, endothelial cells were treated with 30 μ M cilostazol with or without Rp-cAMPS in 6-h OGD conditions. The prevention of cell damage by cilostazol was significantly inhibited by Rp-cAMPS. $^{###}P < 0.01$ vs. control and 30 μ M cilostazol, $^{**}P < 0.01$ vs. 30 μ M cilostazol alone (Turkey's test, $n = 3$ to 5). Data are expressed as means $\pm$ SEM.

the effects of db-cAMP and Rp-cAMPS on cell damage induced by OGD stress in endothelial cells. In microscope views, cAMP caused binding of one cell to another, as was seen with cilostazol. On the other hand, Rp-cAMPS inhibited these cell adhesions induced by OGD stress plus cilostazol (Fig. 2A). In addition, like cilostazol, the increase in LDH under OGD stress was significantly reduced by db-cAMP at 100 and 300 μ M in a concentration-dependent manner (Fig. 2B). In contrast, Rp-cAMPS at 200 μ M significantly prevented the decrease in LDH level and VE-cadherin expression induced by 30 μ M cilostazol (Fig. 2C). These analyses indicate that cilostazol prevented the OGD-induced cell damage via the cAMP/PKA pathway.

The Effects of Cilostazol and db-cAMP on Adheren Junction (AJ) Proteins Induced by OGD Stress in Human Brain Microvascular Endothelial Cells

We next used immunoblotting to examine the alterations in expression of the cell-cell adhesion proteins, VE-cadherin and PECAM-1, in the presence or absence of 6-h OGD stress. VE-cadherin and PECAM-1 are known to be the main AJ proteins and to be a key mediator of endothelial barrier sealing and maintenance [11]. After 6-h OGD stress, VE-cadherin levels were markedly reduced in endothelial cells. In contrast, 30 μ M cilostazol significantly prevented this decrease in VE-cadherin expression, while this effect of 30 μ M cilostazol was significantly attenuated by a PKA

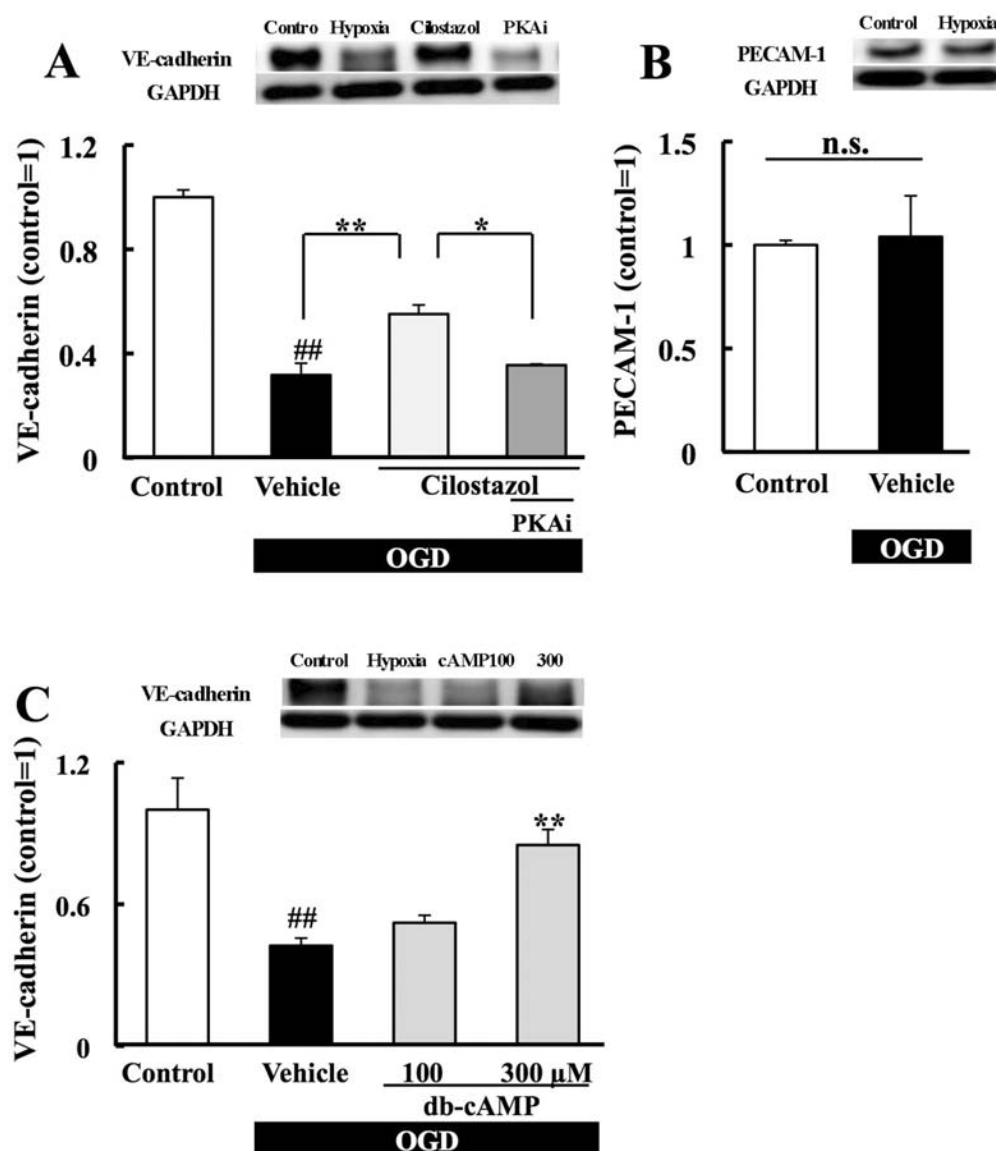


Fig. (3). Effects of cilostazol and db-cAMP on adheren junction's proteins, VE-cadherin, and PECAM-1 in OGD conditions. (A and B) Endothelial cells were treated with the vehicle or 30 μ M cilostazol with or without PKAi (1 μ M) in 6-h OGD conditions. Western blot analysis was performed using antibodies against VE-cadherin and PECAM-1. OGD stress decreased VE-cadherin levels, but not PECAM-1. Cilostazol prevented this OGD-associated decrease in VE-cadherin. The increase in VE-cadherin induced by cilostazol was inhibited by a PKA inhibitor. ^{##}P < 0.01 vs. control, ^{**}P < 0.01 vs. OGD-treated vehicle, and ^{*}P < 0.05 vs. 30 μ M cilostazol plus PKA inhibitor (Turkey's test, n = 3-4). (C) In addition, db-cAMP at 100 and 300 μ M prevented the OGD-associated decrease in VE-cadherin in a concentration-dependent manner. ^{##}P < 0.01 vs. control, ^{**}P < 0.01 vs. OGD-treated vehicle group (Dunnett's test, n = 6). Data are expressed as means $\pm$ SEM.

inhibitor (Fig. 3A). Interestingly, these changes were essentially identical to those observed in the LDH assay and microscope views of the endothelial cells treated with 30 μ M cilostazol and 30 μ M cilostazol plus PKA inhibitor. On the other hand, 6-h OGD stress did not affect PECAM-1 levels (Fig. 3B). Furthermore, db-cAMP at 300 μ M significantly prevented a decrease in VE-cadherin expression, similar to the LDH level (Fig. 3C). These analyses may indicate that cilostazol prevented OGD-induced dissolution of VE-cadherin via the cAMP/PKA pathway.

The Effects of db-cAMP on VEGF and Angiopoietin-1 Induced by OGD Stress in Human Brain Microvascular Endothelial Cells

Finally, to evaluate pathways other than AJ proteins, we used immunoblotting to examine alterations in the exp-

ression of VEGF, VEGF receptor, and angiopoietin-1 (Ang1) in the presence or absence of 6-h OGD stress. No effect was seen for 6-h OGD stress on VEGF (Fig. 4A), phospho-VEGF receptor /VEGF receptor (Fig. 4B), or ang1 levels (Fig. 4C). VEGF or Ang1 levels were not affected by OGD stress in this model.

DISCUSSION

Recently, we showed that cilostazol protects endothelial cells and prevent the hemorrhagic transformation induced by tPA in mice subjected to MCA occlusion and reperfusion [14]. In the present study, we examined whether cilostazol prevents cell damage induced by OGD stress and we found that cilostazol provides endothelial protection via the cAMP/PKA pathway in human brain endothelial cells. In

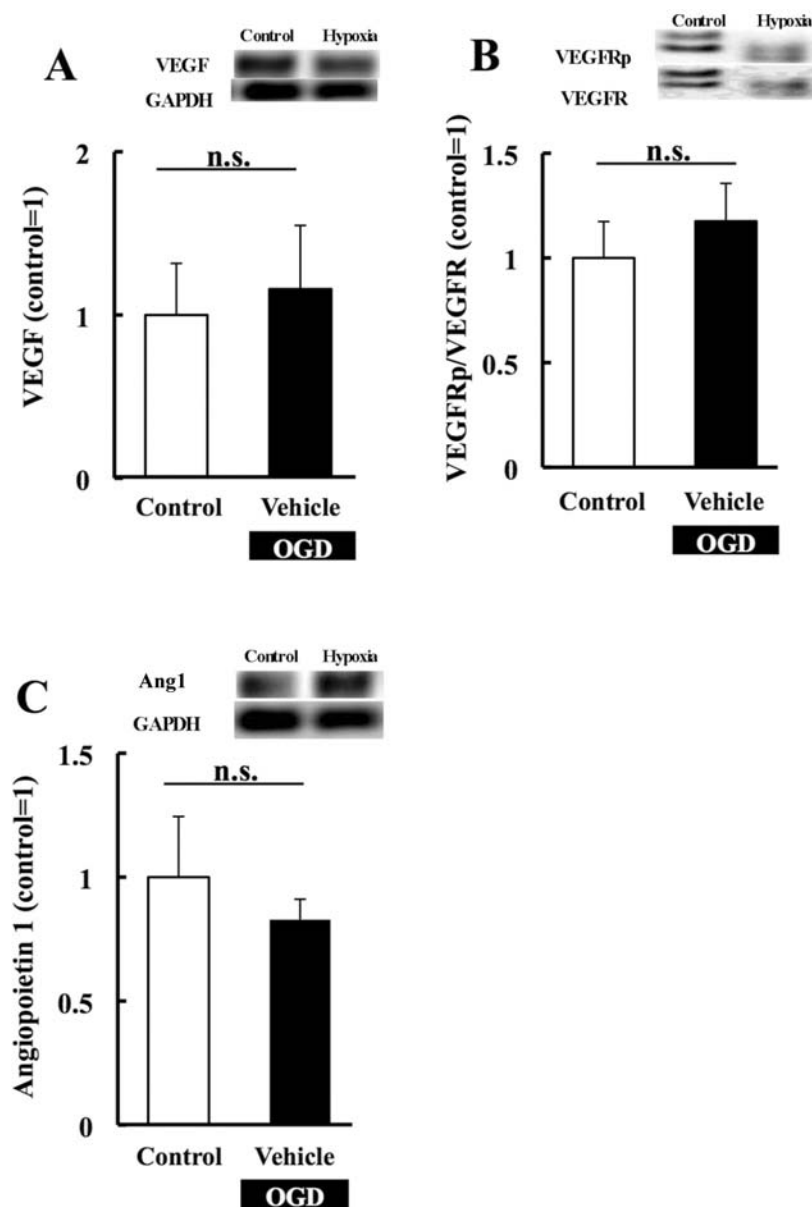


Fig. (4). Effects of db-cAMP on VEGF, VEGF receptor, and angiopoietin-1 in OGD conditions. (A, B, and C) Endothelial cells were treated in control or 6-h OGD conditions. Western blot analysis was performed using antibodies against VEGF, VEGF receptor, and angiopoietin-1 (Ang1). OGD stress did not change all protein levels. (Student's *t* test, *n* = 3 to 6). Data are expressed as means $\pm$ SEM.

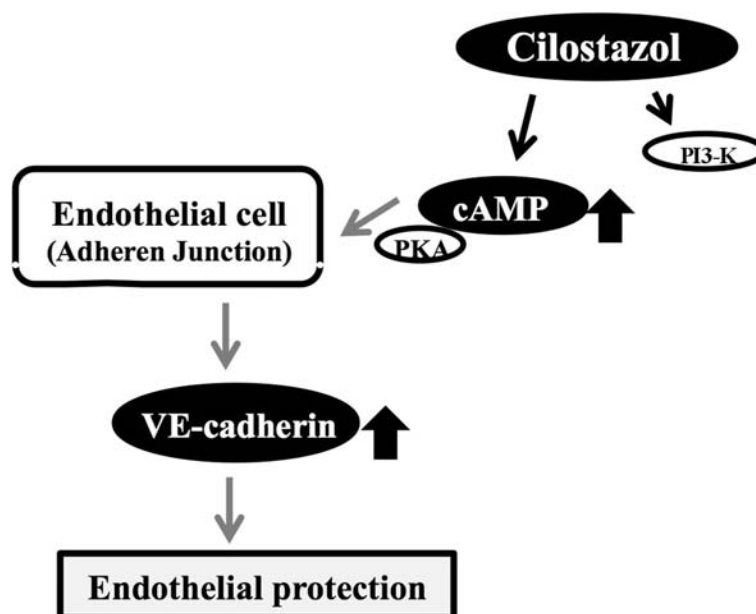


Fig. (5). Schematic diagram depicting the mechanism by which cilostazol inhibits OGD stress in human brain microvasucular cells. The endothelial protective potentials of cilostazol are ascribed to promote VE-cadherin expression through cAMP/PKA-dependent pathways in brain endothelial cells against OGD stress.

addition, the present study suggests that the effects of cilostazol might involve its ability to upregulate VE-cadherin in brain endothelial cells.

The breakdown of the barrier permeability of BBB often accelerates the progression of diseases such as cerebral ischemia and enables entry of exogenous materials into the brain, thus resulting in tissue damage and vasogenic edema. Hence, the regulation of the BBB is one of the crucial control points for the treatment of brain damage. However, the molecular mechanisms involved in barrier disruption by ischemia have not been completely determined. Identification of the molecules responsible for the ischemia-induced disruption of the endothelial barrier may yield new therapeutic targets for intractable diseases.

For these reasons, we focused on adhesion proteins such as VE-cadherin. VE-cadherin is characteristically expressed in the endothelium [17] and is one of the first markers detected in endothelial progenitor cells [18], indicating that it has an important role in development. VE-cadherin is strongly expressed in peripheral and neural endothelial cells, where it appears to be the major cell-cell adhesion molecule. Consequently, it is generally thought that formation of a paracellular barrier requires the initial engagement of cell-cell contacts at the AJ [17].

Our microscopy analysis revealed that cilostazol-treated endothelial cells were morphologically binding, one cell to another, compared to untreated cells. In this *in vitro* model, the degree of VE-cadherin was decreased in the endothelial cells under the OGD stress. Cilostazol prevented a decrease in the LDH level. These changes were essentially identical to those observed in microscope views of the endothelial cells treated in the presence or absence of cilostazol. Our results showed that the expression of VE-cadherin was altered by OGD stress, which resulted in cell damage of endothelial cells, and that this damage was prevented by cilostazol.

Previous study suggested that the cadherin superfamily includes calcium-dependent single-pass adhesion molecules controlling cell architecture and survival [17]. The extracellular domain of VE-cadherin was shown to be released by an unidentified metalloprotease activity during apoptosis of endothelial cells [19]. The cell-cell junctions described above can also act as signaling structures that limit apoptosis. Taken together, in the present study, cilostazol appeared to upregulate VE-cadherin in the endothelial cells, thereby preventing cell damage induced by OGD stress. In addition, increasing evidence has shown that cilostazol also possesses antiapoptotic effects [5] while also having an ability to reduce inflammatory cytokines, such as TNF- α and MCP-1 [20]. Furthermore, VE-cadherin is essential for cell-cell contacts and its intracellular domain connects to catenin complex, which acts as an anchor for the actin cytoskeleton. Cilostazol potentially displayed a protective effect against acute ischemia by preventing an increase in the endothelial permeability through the preservation of the actin cytoskeleton [21]. Among these pleiotropic properties of cilostazol, the protective effects on endothelial cells are thus considered to prevent OGD stress through the upregulation of VE-cadherin.

Unlike VE-cadherin, PECAM1, an immunoglobulin-family cell adhesion molecule expressed in endothelial cells and localized at cell-cell junctions [22], is not confined to the AJs. In the present study, 6-h OGD stress did not decrease PECAM1 levels.

Cilostazol increases intracellular cAMP content and accordingly activates protein kinase A (PKA) and PI3-K/Akt signaling [16]. An increase in intracellular cAMP level in endothelial cells is widely recognized to strengthen the barrier function and to attenuate endothelial permeability both *in vitro* and *in vivo* [23]. A cAMP pathway promotes endothelial barrier function by potentiating VE-cadherin-mediated cell-cell adhesion [24]. In addition, PKA activation

by N6-benzoyl-cAMP (6-Bnz), a specific cAMP analogue for PKA, also promotes endothelial barrier function *in vitro* [25]. Based on these findings, we next evaluated whether cilostazol protects against cell damage by cAMP activation. We found that db-cAMP and PKA inhibitor both prevented cell damage and VE-cadherin reduction after OGD stress in endothelial cells. This result also supports the idea that cAMP/PKA activity may be part of the mechanism underlying cilostazol therapy.

Nevertheless, several questions remain. Interendothelial AJs are dynamic structures, and their adhesive property is finely controlled by various signaling molecules [26]. VEGF is also known to weaken interendothelial cell junctions [27], whereas angiopoietin-1 stabilizes endothelial barrier integrity [28]. VE-cadherin, in particular, can modulate VEGF receptor signaling, which transduces the proangiogenic and permeability effects of VEGF [10]. Indeed, many of the biological responses attributed to VE-cadherin appear to be intimately linked to VEGF signaling [29]. Thus, acute VEGF stimulation could induce an increase in vascular permeability by a reversible disruption of VE-cadherin adhesion [27]. Therefore, we also evaluated the roles of the VEGF on the mechanisms of OGD stress. However, we found no effects on VEGF, at least in the present study. Factors other than VEGF might be altered by cAMP signaling and cAMP/PKA signals might not be the only molecular pathways that modulate VE-cadherin in endothelial cells. Further studies will be needed to clarify the cell signaling mechanisms underlying cAMP/VE-cadherin interactions.

CONCLUSION

A PDE III inhibitor (cilostazol), or other cAMP-modulating drugs, may provide a therapeutic approach for augmenting VE-cadherin and may serve perhaps to protect endothelial cells during ischemic stress. Although cilostazol can trigger a wide range of biological actions in cells, the data presented here suggest that cAMP/PKA signaling pathways may play a pivotal role. In addition, although the initial cell data presented require further confirmation and analysis with *in vivo* models, the present findings indicate that a PDE III inhibitor (cilostazol) may be a promising potential drug that, unlike aspirin, can protect endothelial cells. These results may indicate, in part, the mechanism by which PDE III inhibitor (cilostazol) therapy prevents hemorrhagic complications, as shown in our previous basic and clinical (CSPSII) studies.

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