Cilostazol Prevents Focal Cerebral Ischemic Injury by Enhancing Casein Kinase 2 Phosphorylation and Suppression of Phosphatase and Tensin Homolog Deleted from Chromosome 10 Phosphorylation in Rats

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ABSTRACT

This study shows the in vivo neuroprotective effect of cilostazol against cerebral ischemic injury evoked by subjecting rats to 2-h occlusion of middle cerebral artery (MCAO) followed by 24-h reperfusion. We observed the signaling pathway by which cilostazol suppressed MCAO-induced increased phosphorylation of phosphatase and tensin homolog deleted from chromosome 10 (PTEN) and apoptosis via increased phosphorylation of casein kinase 2 (CK2). When rats received 30 mg/kg cilostazol orally two times at 5 min and 4 h after the completion of ischemia, the infarct area was significantly reduced in the cortex and striatum with improvement of neurological deterioration. Increased DNA fragmentation in the penumbral zone was significantly reduced by cilostazol. Cilostazol significantly elevated phosphorylation levels of CK2, Akt, and cyclic AMP response element-binding protein (CREB) in association with increased Bcl-2 in the ischemic area, whereas the elevated PTEN phosphorylation was significantly reduced, all of which were antagonized by iberiotoxin, a maxi-K channel blocker, administered intracisternally 30 min before ischemia. In conclusion, cilostazol ameliorates the neuronal damage by suppression of apoptotic cell death via the maxi-K channel opening-coupled up-regulation of CK2 phosphorylation and down-regulation of PTEN phosphorylation with resultant increase in the Akt and CREB phosphorylation and increased Bcl-2 protein.

Cilostazol, 6-[4-(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1H)-quinolinone, has been demonstrated to increase the intracellular cyclic AMP by blocking its hydrolysis by phosphodiesterase type III (Kimura et al., 1985). Recently, evidence emerged that cilostazol inhibited the lipopolysaccharide-induced apoptosis in human umbilical vein endothelial cells; a reversal by cilostazol of the lipopolysaccharide-induced decrease in Bcl-2 protein and increases in Bax protein production and cytochrome c release (Kim et al., 2002). Choi et al. (2002) have observed the neuroprotective effect of cilostazol against cerebral ischemic infarct in rats. Most recently, cilostazol showed an increase in the K⁺ currents in SK-N-SH cells (human neuroblastoma) by activating the maxi-K channels without affecting the ATP-sensitive K⁺ channel (Hong et al., 2003). The maxi-K channels, large conductance calcium-activated K⁺ channels, are reported to be activated by depolarization and increased intracellular calcium (Latorre et al., 1989), and K⁺ channel opener reduces neurotransmitter release by suppressing accumulation of pathological levels of Ca²⁺ and attenuates the ischemic injury (Robitaille and Charlton, 1992).

Protein kinase CK2 (CK2, formerly known as casein kinase 2) is a ubiquitous protein Ser/Thr kinase, localized in the cell cytoplasm and nucleus, and its potential role is known to regulate the cell functions, including cell growth and proliferation by catalyzing the phosphorylation of a large number of proteins (Pinna, 1990; Allende and Allende, 1995).

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ABBREVIATIONS: MCAO, middle cerebral artery occlusion; MCA, middle cerebral artery; CK2, casein kinase 2; CREB, cyclic AMP response element-binding protein; PTEN, phosphatase and tensin homolog deleted on chromosome 10; p-PTEN, phosphorylated PTEN; p-Akt and p-CREB, phosphorylated Akt and CREB; BBB, blood-brain barrier; BMS 204352, (3S)-(+)-(5-chloro-2-methoxyphenyl)-2H-indole-2-one.
apoptosis, negatively regulating the phosphatidylinositol 3-kinase pathway by catalyzing degradation of the phosphatidylinositol (3,4,5)-triphosphate to phosphatidylinositol (4,5)-diphosphate (Stambolic et al., 1998; Cantley and Neel, 1999). Overexpression of phosphatidylinositol 3-kinase and its downstream Akt (serine/threonine kinase) have been documented to up-regulate the Bcl-2 promoter activity associated with increased Bcl-2 protein through enhanced CREB activation (Finkbeiner, 2000; Pugazhenthi et al., 2000; Walton and Dragunow, 2000). Bcl-2 was emphasized to protect against the ischemic damage in the cerebral focal ischemia (Martinou et al., 1994) and global ischemic injury (Antonawich et al., 1999). Nevertheless, little information is known regarding the signaling pathway, including activation of CK2, PTEN, Akt, and CREB in relation with brain ischemic injury.

Our hypothesis is that, if activation/phosphorylation of protein kinase CK2 inactivates the PTEN phosphorylation, the results consequently increase the Akt/CREB phosphorylation and Bcl-2 protein formation; these may provide the favorable signals for cell survival. In this study, rats were subjected to 2-h occlusion of middle cerebral artery (MCA) followed by 24-h reperfusion. We determined the cerebral infarct lesion area. Furthermore, we verified DNA fragmentation, and changes in phosphorylations of CK2/PTEN/Akt/CREB (p-CK2/p-PTEN/p-Akt/p-CREB) levels in the tissue samples corresponding to the penumbral zone under treatment with cilostazol in the absence and presence of iberiotoxin, a maxi-K channel blocker.

Materials and Methods

Animal Preparation. The experimental designs, including all procedures, were conducted in accordance with the Animal Care Procedures Guidelines of the Animal Experimental Committee of College of Medicine, Pusan National University. Adult male Sprague-Dawley rats weighing 280 to 320 g were anesthetized with sodium thiopental (50 mg/kg intraperitoneally). Core body temperature was maintained at 37 ± 0.5°C on the heating pad.

Middle Cerebral Artery Occlusion. MCA occlusion was induced by the procedure of Longa et al. (1989). Briefly, surgical nylon suture thread (3-0 in size) with a round tip was advanced from the external carotid artery into the lumen of the internal carotid artery to block the flow of MCA. Two hours after the completion of MCA occlusion, reperfusion was allowed by withdrawal of the suture thread until the tip cleared the internal carotid artery. Rats received two oral administrations of 20–20, 30–30, and 50–30 mg/kg cilostazol (dissolved in dimethyl sulfoxide, 30%) at 5 and 4 h after the completion of 2-h MCA occlusion. Vehicle-treated rats received 1 ml of 30% dimethyl sulfoxide solution orally. Sham-operated animals underwent the same surgical procedure without insertion of suture thread into the internal carotid artery.

Neurological Deficit Examination. Neurological dysfunction was evaluated just before the animals were sacrificed. The neurological findings were based on a five-point scale: a score of 0 indicated no neurological deficit; score 1 is failure to extend right forepaw fully; score 2 is circling to the right; score 3 is falling to the right after a slight push; score 4 is no spontaneous walk and exhibition of a depressed level of consciousness.

Determination of Edema Formation. Brain edema was assessed by measuring the hemispheric water content. Fresh tissue samples of the contralateral and the ipsilateral hemispheres were weighed separately. They were weighed on preweighed aluminum foils immediately to yield the wet weight. The dry weight of each hemisphere was measured after it was dried at 100°C for 24 h. The water content in the hemispheres was calculated as follows: water content (% = (wet weight − dry weight)/wet weight × 100.

Measurement of Evans Blue Extravasation. A quantitative assay of Evans blue was based on the method described previously by Gorgulu et al. (2000). After 24-h reperfusion, 0.1 ml of 4% Evans blue (Sigma-Aldrich, St. Louis, MO) in 0.9% saline was intravenously administered. At 24 h after injection, rats were perfused with heparinized saline (10 U/ml heparin in 0.9% saline) to wash out the blood and isolated. Brain samples were weighed and homogenized in 50% trichloroacetic acid solution. After centrifugation at 15,000 rpm for 20 min, its absorption was spectrophotometrically measured at 610 nm. The tissue content of Evans blue was expressed as micrograms of dye per gram of wet weight.

Cerebral Infarct Area. At 24-h of reperfusion after 2-h MCA occlusion, rats were given an overdose of thiopental sodium and decapitated. The brain was cut into 2-mm-thick coronal block slices. The brain slices were immersed in 2% solution of 2,3,5-triphenyltetrazolium chloride in normal saline at 37°C for 30 min and then fixed in 10% phosphate-buffered formalin at 4°C. The size of infarct was calculated with the image analysis system (Image-Pro Plus; Media Cybernetics, Silver Spring, MD) and expressed as the percentage of infarcted area in reference to the ipsilateral hemisphere.

DNA Fragmentation Assay. Sections obtained at the level of bregma +0.7 to −1.3 mm (section 3) at the ipsilateral and contralateral control sites were used for determination of the laddering feature of DNA. For oligonucleosomal fragmentation of genomic DNA, cells were lysed in 1 ml of lysis buffer (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 1% sodium dodecyl sulfate, and 0.5 mg/ml proteinase K). Digestion was continued for −1 to 3 h at 55°C, followed by addition of RNase A, 0.1 mg/ml and running dye (10 mM EDTA, 0.25% bromophenol blue, 50% glycerol). Equivalent amounts of DNA (~15–20 μg) were loaded into wells of 1% agarose gel and electrophoresed in 0.5× TAE buffer (40 mM Tris- acetate, 1 mM EDTA) for 2 h at 6 V/cm. DNA was visualized by ethidium bromide staining. Gel pictures were taken by the UV transillumination with the Polaroid camera. Bands were quantified by the Molecular Analyst Software using the Bio-Rad’s Image Analysis System (Bio-Rad, Hercules, CA). Iberiotoxin (0.1 μM, 100 μl in volume) was applied via cisterna magna 30 min before application of cilostazol or BMS 204352.

Western Blot Assays. The penumbral zone of coronal sections 4 and 5 (at bregma levels approximating −1.3 to −3.3 and −3.3 to −5.3 mm) was used for the Western blot assays. After MCA occlusion and reperfusion, the samples corresponding to the penumbral zone were harvested, and cells were lysed in lysis buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.1% sodium azide, 100 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1% Triton X-100. After centrifugation at 12,000 rpm, 50 μg of total protein of each sample was loaded into a 12% SDS-PAGE gel and transferred to nitrocellulose membrane (Amersham Biosciences Inc., Piscataway, NJ). The blocked membranes were then incubated with the indicated antibody, and the immunoreactive bands were visualized using chemiluminescent reagent as recommended by the Supersignal West Dura Extended Duration Substrate kit (Pierce Chemical, Rockford, IL). The signals of the bands were quantified using the GS-710 calibrated imaging densitometer (Bio-Rad). The results were expressed as a relative density. Polyclonal antibodies against CREB, p-CREB, Bcl-2, and CK2 were from the Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and polyclonal antibodies against PTEN, p-PTEN (Ser380/Thr382/383), Akt, and p-Akt (Ser473) were from the Cell Signaling Technology (Beverly, MA). Polyclonal antibodies against p-CK2 were from the Calbiochem (San Diego, CA). Equal protein loading in each lane was confirmed by hybridization with a 1:2000 dilution of β-actin antibody (Santa Cruz Biotechnology, Inc.). The protein concentration of the lysate was determined using Bio-Rad DC assay kit (Bio-Rad).

Chemicals. Cilostazol, 6-(4-[(1-cyclohexyl-1H-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1H)-quinoxaline, was generously donated from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan). BMS 204352
was generously donated from the Korea Research Institute of Chemical Technology (Daejon, Korea).

**Statistical Analysis.** Results were expressed as mean ± S.E.M. The comparison of the results of hemispheric infarct areas between groups was analyzed by repeated measures analysis of variance followed by Tukey’s multiple comparison tests as a post hoc comparison. The edematous swelling was analyzed by William’s test (lower tailed) among each dose of cilostazol and vehicle groups. The neurological deficit scores were analyzed by Mann-Whitney U test. The differences were considered statistically significant at **P** < 0.05.

**Results**

**Physiological Variables.** There was no significant difference in the physiological variables: mean arterial blood pressure, blood pH, PaCO₂, PaO₂, and rectal (core) temperature that were measured before, during ischemia, and after 24-h reperfusion between vehicle and cilostazol groups (Table 1).

**Neurological Deficit.** Cilostazol effectively improved the neurological deterioration. The average neurological deficit scores were 1.17 ± 0.20 (n = 12) in the cilostazol-treated group, whereas they were 2.25 ± 0.18 in the vehicle group. The neurological deterioration such as circling to the right and falling to the right by a slight push were significantly improved under treatment with 30 mg/kg cilostazol administered two times at 5 min and 4 h after the completion of ischemia, and the scores of neurological deficit largely shifted to low level (Table 2).

**Cerebral Water Content and Blood-Brain Barrier Permeability.** The cerebral ischemic hemisphere of the vehicle group showed significantly increased water content (85.7 ± 0.5%; **P** < 0.01) in comparison with the ipsilateral hemisphere of sham group (79.9 ± 0.3%). Cilostazol-treatment significantly reduced the increased cerebral water content of the ipsilateral hemisphere to 80.5 ± 0.3% (**P** < 0.01) (Table 3).

The permeability of BBB of ipsilateral hemisphere of sham group (6.4 ± 1.7 µg dye/g wet tissue) was significantly increased to 17.9 ± 2.2 µg dye/g wet tissue (**P** < 0.01) in the brain subjected to the MCA occlusion and reperfusion, which was markedly reduced to 11.7 ± 1.8 µg dye/g wet tissue (**P** < 0.05) under treatment with cilostazol 30 mg/kg orally two times (Table 3).

**Effect of Cilostazol on Infarct Size.** The infarct area identified in the cortex and striatum of the left cerebral hemisphere was significantly reduced when the animals received 30–30 and 50–30 mg/kg cilostazol two times at 5 min and 4 h after the completion of 2-h ischemia (Fig. 1).

**DNA Fragmentation Assay.** The cortical samples corresponding to the penumbral zone obtained after 24-h reperfusion showed prominent DNA fragmentation. The DNA was fragmented at 180- to 200-base pair intervals, reflecting the increased endonuclease cleavage of DNA at internucleosomal sites. Treatment with cilostazol (30–30 mg/kg and 50–30 mg/kg orally) concentration dependently suppressed the laddering feature of DNA fragmentation (Fig. 2, A and C). Suppression of DNA laddering feature by cilostazol (30–30 mg/kg orally) was fully antagonized under pretreatment with iberiotoxin (0.1 µM intracranially), a maxi-K channel blocker. Iberiotoxin (0.1 µM) alone was without effect (Fig. 2, B and D).

**Expression of Phosphorylated CK2 and PTEN.** Samples from sham-operated group showed a considerable amount of CK2 and p-CK2 protein levels constitutively, but a trace level of p-PTEN in contrast to PTEN level. In the cortical samples of the penumbral zone obtained after 24-h reperfusion, p-CK2 level (p-CK2/CK2 ratio = 1) was largely reduced to 0.57 ± 0.01-fold (**P** < 0.001). The reduced p-CK2 level was concentration dependently elevated by cilostazol treatment (20–20, 30–30, and 50–30 mg/kg orally two times) up to 1.24 ± 0.03-fold (**P** < 0.001), which was antagonized by iberiotoxin (0.1 µM, intracranially) (Fig. 3, A and B). The constitutively expressed PTEN and CK2 protein levels showed little change throughout the experiment.

On the other hand, the p-PTEN/PTEN ratio was markedly increased in the vehicle-treated samples to 3.92 ± 0.12 (**P** < 0.001), and it was concentration dependently reduced in the cilostazol-treated samples. In the densitometric analysis, suppression of p-PTEN to PTEN ratio by 30–30 mg/kg cilostazol (2.06 ± 0.08; **P** < 0.001) was significantly reversed to 2.90 ± 0.11-fold (**P** < 0.001) by iberiotoxin (Fig. 3, A and C). p-CK2/CK2 and p-PTEN/PTEN ratios shown in the samples from the penumbral zone were little influenced by iberiotoxin alone.

Additionally, we examined effect of BMS 204352, a known maxi-K channel opener. Treatment with BMS 204352 (10–10 mg/kg, orally) showed significantly increased p-CK2 level, and in contrast decreased p-PTEN levels, both of which were significantly reversed by iberiotoxin (0.1 µM intracranially) (Fig. 4).

**Expression of PTEN and Akt Phosphorylation.** In contrast to the p-PTEN levels (Fig. 5, A and B), p-Akt protein level was modestly decreased in the vehicle-treated samples, and it was largely elevated to 3.78 ± 0.42-fold (**P** < 0.001) by 30–30 mg/kg cilostazol, which was reversed by iberiotoxin (0.1 µM intracranially) (Fig. 5, A and C). Nevertheless, the constitutively expressed PTEN and Akt protein levels remained unchanged.

**TABLE 1**

<table>
<thead>
<tr>
<th>Physiological variables measured before and during the MCAO and after 24-h reperfusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>During the observation period, all variables were in the normal ranges and they did not significantly change. Each value represents mean ± S.E.M. (n = 12).</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>MABP</th>
<th>pH</th>
<th>pCO₂</th>
<th>pO₂</th>
<th>Rectal Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-MCAO Vehicle</td>
<td>115 ± 9</td>
<td>7.45 ± 0.01</td>
<td>34.9 ± 1.3</td>
<td>86.7 ± 3.7</td>
</tr>
<tr>
<td>Cilostazol (30–30 mg/kg)</td>
<td>110 ± 10</td>
<td>7.43 ± 0.03</td>
<td>37.3 ± 1.5</td>
<td>87.4 ± 5.2</td>
</tr>
<tr>
<td>During 2-h ischemia Vehicle</td>
<td>107 ± 9</td>
<td>7.40 ± 0.02</td>
<td>35.7 ± 2.1</td>
<td>88.5 ± 3.4</td>
</tr>
<tr>
<td>Cilostazol (30–30 mg/kg)</td>
<td>105 ± 11</td>
<td>7.42 ± 0.01</td>
<td>37.9 ± 2.3</td>
<td>89.7 ± 3.5</td>
</tr>
<tr>
<td>After 24-h reperfusion Vehicle</td>
<td>114 ± 12</td>
<td>7.43 ± 0.02</td>
<td>34.5 ± 1.5</td>
<td>90.7 ± 4.2</td>
</tr>
<tr>
<td>Cilostazol (30–30 mg/kg)</td>
<td>109 ± 11</td>
<td>7.42 ± 0.02</td>
<td>37.0 ± 2.1</td>
<td>91.2 ± 3.5</td>
</tr>
</tbody>
</table>
TABLE 2
Neurological deficit scores in the vehicle and cilostazol-treated groups
Each value represents mean ± S.E.M. of neurological deficit scores.

<table>
<thead>
<tr>
<th>Score</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>Average Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle (n = 12)</td>
<td>1</td>
<td>7</td>
<td>4</td>
<td>1</td>
<td></td>
<td>2.25 ± 0.18</td>
</tr>
<tr>
<td>Cilostazol (n = 12) (30–30 mg/kg)</td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>1</td>
<td></td>
<td>1.17 ± 0.20***</td>
</tr>
</tbody>
</table>

***P < 0.001 versus vehicle (Mann-Whitney U test).

TABLE 3
Cerebral water content (%) and blood-brain barrier permeability in the ischemic ipsilateral and contralateral hemispheres
The water content and blood brain barrier permeability were compared between the ischemic ipsilateral and contralateral hemispheres for the group treated with cilostazol or vehicle in comparison with sham group. Values are mean ± S.E.M.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cerebral Water Content (n = 9, each)</th>
<th>Blood-Brain Barrier Permeability (n = 6, each)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Contralateral hemisphere</td>
<td>Ipsilateral hemisphere</td>
</tr>
<tr>
<td>Sham</td>
<td>79.4 ± 0.1</td>
<td>79.9 ± 0.3**</td>
</tr>
<tr>
<td>Vehicle</td>
<td>80.3 ± 0.4</td>
<td>83.7 ± 0.5**</td>
</tr>
<tr>
<td>Cilostazol (30–30 mg/kg)</td>
<td>79.1 ± 0.2</td>
<td>80.5 ± 0.3**</td>
</tr>
</tbody>
</table>

***P < 0.01 vs. ipsilateral hemisphere of sham group; **P < 0.01 and *P < 0.05 vs. ipsilateral hemisphere of vehicle group.

Fig. 1. Effect of cilostazol on the reduction in infarct size in the vehicle- and cilostazol-treated rats. Rats received cilostazol, 20–20, 30–30, and 50–30 mg/kg for two times at 5 min and 4 h after the completion of 2-h ischemia, respectively. Results are expressed as means ± S.E.M. from five animals. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus vehicle group.

Western Blot for p-CREB and Bcl-2 Protein. Figure 6 shows Western blot assay for p-CREB and Bcl-2 protein in the ischemic cortical sample corresponding to the penumbral zone. Vehicle-treated samples obtained from rats subjected to 2-h MCA occlusion and 24-h reperfusion showed low levels of the p-CREB (0.89 ± 0.12-fold) and Bcl-2 (sham; 0.48 ± 0.35-fold). The p-CREB and Bcl-2 levels in the ipsilateral ischemic brain were markedly increased to 5.47 ± 0.67-fold and 4.43 ± 0.43-fold, respectively, by treatment with cilostazol (30–30 mg/kg orally).

Discussion
In the present study, the major findings were that cilostazol 1) showed a potent protection against cerebral infarct area in association with decrease in BBB permeability, leading to improvement of the neurological dysfunction; 2) suppressed DNA fragmentation in the ischemic brain tissue corresponding to the penumbral zone; 3) significantly reduced the increased PTEN phosphorylation and elevated the decreased phosphorylation of CK2/Akt/CREB in the ipsilateral brain lesion with increased Bcl-2 protein; and 4) that these cilostazol effects were significantly antagonized by iberiotoxin, a maxi-K channel blocker.
As suggested by the report of Yang et al. (1999), disruption of BBB in the ipsilateral hemisphere was associated with focal cerebral ischemia, which was significantly suppressed by cilostazol. Gotoh et al. (2000) reported a risk reduction in patients with recurrent cerebral infarction by cilostazol treatment. Yao et al. (2001) emphasized the importance of apoptosis in the development of ischemic infarction in the penumbral zone with moderately reduced cerebral blood flow. Nevertheless, the protective effect of cilostazol seems to be independent of cerebral blood flow, because the cerebral blood flow was unchanged by cilostazol throughout the experiment (data not shown).

Recently, Choi et al. (2002) have provided in vivo evidence that intravenously administered cilostazol (10 mg/kg) inhib-
Protein, and decreased Bax protein and cytochrome c release in association with increased Bcl-2 protein, and tumor necrosis factor-α-induced decreased cell viability in association with suppression of DNA fragmentation via reducing PTEN phosphorylation and in turn via increasing the phosphorylation of Akt/CREB levels, which were blocked by iberiotoxin, a maxi-K channel blocker. In accordance with these results, our in vivo data also showed that increased p-PTEN and decreased p-CK2/p-Akt/p-CREB levels in the vehicle-treated ischemic brain samples were reversed by cilostazol (30–30 mg/kg) treatment. On the other hand, CK2 as a physiologically relevant PTEN kinase is known to mediate phosphorylation of PTEN and its phosphorylation inhibits PTEN function (Vazquez et al., 2000; Torres and Puelles, 2001). Most recently, Hong et al. (2003) have shown that cilostazol prevents tumor necrosis factor-α-induced decreased cell viability in association with suppression of DNA fragmentation via reducing PTEN phosphorylation and in turn via increasing the phosphorylation of Akt/CREB levels, which were blocked by iberiotoxin, a maxi-K channel blocker.

Considering these in vitro results (Kim et al., 2003), our in vivo data indicate that increase in p-CK2 activity suppresses the p-PTEN level, consequently leading to increase in the phosphorylation of PTEN, Akt, and CREB, in the action of cilostazol to explain the underlying mechanism of DNA fragmentation in the ischemic brain samples. PTEN is known to negatively regulate several cellular functions, including cell cycle progression, cell migration, and survival from apoptosis by suppression of Akt activation via dephosphorylating the phosphatidylinositol (3,4,5)-triphosphate to phosphatidylinositol (4,5)-diphosphate, an inactive state (Stambolic et al., 1998; Cantley and Neel, 1999; Walton et al., 1999; van Golen et al., 2001). Most recently, Hong et al. (2003) have shown that cilostazol prevents tumor necrosis factor-α-induced decreased cell viability in association with suppression of DNA fragmentation via reducing PTEN phosphorylation and in turn via increasing the phosphorylation of Akt/CREB levels, which were blocked by iberiotoxin, a maxi-K channel blocker. In accordance with these results, our in vivo data also showed that increased p-PTEN and decreased p-CK2/p-Akt/p-CREB levels in the vehicle-treated ischemic brain samples were reversed by cilostazol (30–30 mg/kg) treatment. On the other hand, CK2 as a physiologically relevant PTEN kinase is known to mediate phosphorylation of PTEN and its phosphorylation inhibits PTEN function (Vazquez et al., 2000; Torres and Puelles, 2001; Miller et al., 2002). Kim et al. (2003) have further assessed the functional relevance of p-PTEN to p-CK2 in the SK-N-SH cells, in that suppressed p-CK2 expression and CK2 activity after treatment with emodin (10 μM, CK2-selective inhibitor; Battistutta et al., 2000) were significantly elevated by cilostazol, and in contrast, increased p-PTEN level was concentration dependently suppressed by cilostazol. Moreover, they documented that the selective depletion of CK2 subunits with antisense oligodeoxynucleotide in the SK-N-SH cells resulted in the marked DNA fragmentation and in these cells cilostazol was without effect on the p-PTEN, p-Akt, and p-CREB levels and DNA fragmentation, indicative of essential role of p-CK2 and CK2.

Considering these in vitro results (Kim et al., 2003), our in vivo data indicate that increase in p-CK2 activity suppresses the p-PTEN level, consequently leading to increase in the p-Akt/p-CREB. These are well consistent with the results reported by Guo et al. (2001) and Ruzzene et al. (2002), in that CK2 counteracts apoptosis and apoptotic cell death.

Bcl-2 mRNA expression (in reverse transcription-polymerase chain reaction) was significantly increased under cilostazol treatment (data not shown). Huang et al. (2001) have demonstrated that transient transfection of PTEN into the PTEN-null cells results in decrease in Bcl-2 mRNA and protein, and loss of PTEN leads to up-regulation of the Bcl-2 gene. Overexpression of phosphatidyl-3 kinase and its downstream effector Akt mediate growth factor-induced neuronal survival, and Akt in turn activates the Bcl-2 promoter activity in association with increased Bcl-2 protein production through enhanced CREB activation (Bonni et al., 1999; Pugazhenthi et al., 2000). Tanaka (2001) demonstrated the coexpression of Bcl-2 in the 80% of the phosphorylated CREB-positive neurons in the ischemic area of brain. In our results of Western blot assay, decreased p-CREB level in the
ischemic region after MCA occlusion was markedly elevated in the cilostazol-treated brain samples. In the previous electrophysiological results (Hong et al., 2003), cilostazol increased the K⁺ currents in SK-N-SH cells by activating the iberiotoxin-inhibitable maxi-K channels, and concentration dependently decreased the cytosolic Ca²⁺, which was significantly suppressed by iberiotoxin (a peptidyl toxin; Galvez et al., 1990). Evidence emerges that activation of maxi-K channels protect neurons against glutamate release and excitotoxicity, and reduce the pathological consequences of ischemia (Lawson, 2000). It was demonstrated that the maxi-K channel opener BMS 204352 (Cheney et al., 2001) elicited neuroprotection against acute ischemic stroke by blocking Ca²⁺ entry and by minimizing the neuronal depolarization in neurons at risk (Gribkoff et al., 2001). In the line with this report, our result also showed that BMS 204352 significantly increased p-CrkII level, and in contrast decreased p-Pten levels as did cilostazol. In the preliminary study, we observed that cilostazol significantly decreased the elevated cytosolic Ca²⁺ level evoked by tumor necrosis factor-α (50 ng/ml) in the SK-N-SH cells, which was reversed by iberiotoxin. Thus, it was speculated that maxi-K channel opening by cilostazol might regulate the intracellular Ca²⁺ increase and membrane potential via activation of cyclic AMP protein kinase, thereby suppressing the PTEN phosphorylation. Intriguingly, in our results the blockade by iberiotoxin was prominently evident in both cilostazol-stimulated p-CrkII and p-Akt levels and cilostazol-suppressed p-PTEN level, strongly suggesting that cilostazol regulates this signal pathway. It remains, however, undefined as to the molecular mechanism how the maxi-K channel opening increases the p-CrkII level. So far, it goes beyond the scope of our present in vivo study to illustrate the mechanism(s) by which p-CrkII controls the p-PTEN levels. We did not determine the relationships between changes in membrane potential and p-CrkII level.

In conclusion, it is suggested that suppression of cerebral ischemic injury with DNA fragmentation by cilostazol is associated with the maxi-K channel opening-coupled up-regulation of CrkII phosphorylation and down-regulation of PTEN phosphorylation with resultant increase in the Akt and CREB phosphorylation and increased Bcl-2 protein. These biochemical findings may contribute to the reduction of BBB disruption, amelioration of edema formation, and thereby improvement of neurological deterioration. Together, it is considered that CK2 may serve as a target for inhibiting neuronal cell apoptosis in the cell injury and thus may offer cell survival strategy.

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References


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