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Cilostazol Prevents Tumor Necrosis Factor-α-Induced Cell Death by Suppression of Phosphatase and Tensin Homolog Deleted from Chromosome 10 Phosphorylation and Activation of Akt/Cyclic AMP Response Element-Binding Protein Phosphorylation

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ABSTRACT

This study examines the signaling mechanism by which cilostazol prevents neuronal cell death. Cilostazol (~0.1–100 μM) prevented tumor necrosis factor-α (TNF-α)-induced decrease in viability of SK-N-SH and HCN-1A cells, which was antagonized by 1 μM iberiotoxin, a maxi-K channel blocker. TNF-α did not suppress the viability of the U87-MG cell, a phosphatase and tensin homolog deleted from chromosome 10 (PTEN)-null glioblastoma cell, but it did decrease viability of U87-MG cells transfected with expression vectors for the sense PTEN, and this decrease was also prevented by cilostazol. Cilostazol as well as 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one (NS-1619) and (3*S*)-(+)-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2*H*-indole-2-one (BMS 204352), maxi-K channel openers, prevented increased DNA fragmentation evoked by TNF-α, which were antagonizable by iberiotoxin. TNF-α-induced increased PTEN phosphorylation and decreased Akt/cyclic AMP response element-binding protein (CREB) phosphorylation were significantly prevented by cilostazol, those of which were antagonized by both iberiotoxin and paxilline, maxi-K channel blockers. The same results were evident in U87-MG cells transfected with expression vectors for sense PTEN. Cilostazol increases the K^+ current in SK-N-SH cells by activating maxi-K channels without affecting the ATP-sensitive K^+ channel. Thus, our results for the first time provide evidence that cilostazol prevents TNF-α-induced cell death by suppression of PTEN phosphorylation and activation of Akt/CREB phosphorylation via mediation of the maxi-K channel opening.

Recent research has shown that the phosphatase and tensin homolog deleted from chromosome 10 (PTEN) is implicated in the regulation of several cellular functions, including cell viability from apoptosis (Li et al., 1998; Stambolic et al., 1998; Cantley and Neel, 1999). PTEN is capable of dephosphorylating both phospho-tyrosine and phospho-serine/threonine-containing substrates (Myers et al., 1997) and also of dephosphorylating the phosphatidylinositol-3,4,5-triphosphate [PI(3,4,5)P_3], a direct product of phosphatidylinositol 3-kinase (PI3-K) activity, thereby converting the PI(3,4,5)P_3 to phosphatidylinositol 3,4-diphosphate [PI(3,4)P_2], an inactive state (Maehama and Dixon, 1998; Stambolic et al., 1998). 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 PI3-K and its downstream effector Akt (serine/threonine kinase) have been documented to mediate growth factor-induced neuronal survival (Crowder and Freeman, 1998) and to up-regulate Bcl-2 promoter activity associated with increased Bcl-2

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ABBREVIATIONS: PTEN, phosphatase and tensin homolog deleted from chromosome 10; PI(3,4,5)P_3, phosphatidylinositol 3,4,5-triphosphate; PI3-K, phosphatidylinositol 3-kinase; PI(3,4)P_2, phosphatidylinositol 3,4-diphosphate; CREB, cyclic AMP response element-binding protein; p-PTEN, phosphorylated PTEN; p-Akt and p-CREB, phosphorylated Akt and CREB; sPTEN, transfected with expression vectors for sense PTEN; MEM, minimal essential medium; bp, base pair(s); MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; NS-1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one; BMS 204352, (3*S*)-(+)-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2*H*-indole-2-one.

protein through enhanced cyclic AMP response element-binding protein (CREB) activation (Pugazhenthi et al., 2000).

On the other hand, potassium channel is one of the key players in the control of neuronal excitability. The maxi-K channels, large conductance calcium-activated K^+ channels, are activated by depolarization and increased intracellular calcium (Latorre et al., 1989). During ischemia, K^+ channel opener was reported to reduce neurotransmitter release by suppressing accumulation of pathological levels of Ca^{2+}, thereby significantly attenuating the ischemic injury (Robitaille and Charlton, 1992). Recent studies have documented that BMS 204352, a maxi-K channel opener, protect neuronal cells from acute damage under conditions that cause excessive depolarization and accumulation of intracellular Ca^{2+}, such as brain ischemia (Gribkoff et al., 2001).

Cilostazol was first introduced to increase the intracellular level of cyclic AMP by blocking its hydrolysis by type III phosphodiesterase (Kimura et al., 1985). Recently, Kim et al. (2002) have addressed the in vitro inhibition of lipopolysaccharide-induced apoptosis by cilostazol in human umbilical vein endothelial cells, in that they demonstrated a reversal by cilostazol of the lipopolysaccharide-induced decrease in Bcl-2 protein and the increase in Bax protein and cytochrome *c* release. Furthermore, Choi et al. (2002) have confirmed the in vivo preventive effect of cilostazol against cerebral infarct evoked by middle cerebral artery occlusion and reperfusion via its antiapoptotic action.

Given that cilostazol electrophysiologically increases the calcium-activated K^+ currents in the SK-N-SH cells, we assessed in this study the suppressive effect of cilostazol on the PTEN phosphorylation in relation to cell viability in the absence and presence of iberiotoxin, a maxi-K channel blocker, in the SK-N-SH (human neuroblastoma) and HCN-1A cells (human cortical neuron). Furthermore, we simulate the interaction of cilostazol and iberiotoxin with respect to changes in p-PTEN and p-CREB levels in response to the introduction of TNF-α in U87-MG cells (human brain PTEN-null glioblastoma) transfected with expression vectors for sense PTEN (sPTEN).

Materials and Methods

Cell Cultures. SK-N-SH cells (KCLB 30011, human brain neuroblastoma), HCN-1A cells (ATCC CRL-10442, human brain cortical cells) and U87-MG (KCLB 30014, human brain PTEN-null glioblastoma) cells were cultured in Eagle's minimal essential medium (MEM) with 2 mM L-glutamine and 1.0 mM sodium pyruvate supplemented with 10% heat-inactivated fetal bovine serum. Cells were grown to confluence at 37°C in 5% CO_2.

Plasmid Construction. The expression of plasmid encoding the human PTEN protein was cloned by reverse transcription-polymerase chain reaction using the total RNA of SK-N-SH cells. Sequence analysis was performed to confirm the nucleotide sequences. The following sequences of oligodeoxynucleotides were used as primers containing linker recognizable by *Xho*I as underlined: sense, 5′-GCGCTCGAGATGACAGCCATCAAA G-3′. Amplified 1264-bp fragments containing the human PTEN coding region were ligated into the *Xho*I site of pcDNA3.1 HisC (Invitrogen, San Diego, CA). pcDNA3.1-sPTEN is transcripted sense nucleotide.

DNA Transfection and Transfection Efficiency Assay. U87-MG cells were seeded for 24 h before transfection in tissue culture dishes. At 50 to 70% confluence, the dishes were washed twice with Opti-MEM medium, to remove the fetal bovine serum, and a transfection cocktail containing 10 μg of DNA and 10 μl of LipofectAMINE reagent (Invitrogen) per 100-mm dish was added. The medium was removed and then 7 ml of MEM medium containing 10% fetal bovine serum was added to each dish. The β-galactosidase assay was performed 36 h after transfection using a commercially available β-Gal staining kit (Invitrogen). Under microscope (200× total magnitude), the blue-colored cells were counted in 5 to 10 random fields of view and the transfection efficiency was estimated. In the U87-MG cells transfected with expression vectors for sPTEN, the efficiency of transfection was estimated to be over 70% with enhanced expression of PTEN protein.

Cell Viability Assay. According to the mitochondrial tetrazolium assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; MTT) procedure, cells were seeded 1×10^4 cells/well in 96-well tissue culture plates. The confluent cells received MEM medium with 1% fetal bovine serum plus drugs 3 h before stimulation with TNF-α and then were exposed to TNF-α for 24 h. After incubation, 20 μl/well of MTT solution (5 mg/ml phosphate-buffered saline) was added and incubated for 2 h. The medium was aspirated and replaced with 150 μl/well of ethanol/dimethyl sulfoxide solution (1:1). The optical density was measured at 570 to 630 nm using ELISA reader (Bio-Tek Instruments, Inc., Winooski, VT).

DNA Fragmentation Assay. After incubation in the absence and presence of the drugs for 3 h, cells ($1–5 \times 10^6$) were exposed to TNF-α (50 ng/ml) for 24 h. At harvest, trypsinized cells were pelleted by centrifugation. 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 to 0.1 mg/ml and running dye (10 mM EDTA, 0.25% bromphenol blue, and 50% glycerol). Equivalent amounts of DNA (15–20 μg) were loaded into wells of 1.6% agarose gel and electrophoresed in 0.5× TAE buffer (40 mM Tris-acetate and 1 mM EDTA) for 2 h at 6 V/cm. DNA was visualized by ethidium bromide staining. Gel pictures were taken by UV transillumination with a Polaroid camera.

Western Blot Analysis. The confluent cells received MEM medium with 1% fetal bovine serum plus cilostazol 3 h before stimulation with TNF-α and then were exposed to TNF-α for 1 h. The cells were lysed in lysis buffer containing 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 0.02% sodium azide, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1% Triton X-100. After centrifugation at 12,000 rpm, 50 μg of total protein was loaded into 8 or 10% SDS-polyacrylamide gel electrophoresis 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, Hercules, CA). The results were expressed as a relative density. Polyclonal antibodies against maxi-K channel α-subunit, CREB, p-CREB, and monoclonal antibodies against Bcl-2 and Bax were from the Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Polyclonal antibodies against PTEN, p-PTEN (Ser380/Thr382/383), Akt, and p-Akt (Ser473) were from the Cell Signaling Technology, Inc. (Beverly, MA).

Recording of the Whole-Cell K^+ Current. Experiments were performed in the small bath (0.5 ml) mounted on the stage of an inverted microscope (model TE300; Nikon, Tokyo, Japan) perfused continuously at a flow rate of 1 ml/min. Using the whole-cell configuration of the patch-clamp technique, the K^+ currents were recorded at room temperature (20–22°C) with the Axopatch-200B patch-clamp amplifier (Axon Instruments, Inc., Foster City, CA). Currents were sampled at 1 to 10 kHz after anti-alias filtering at 0.5 to 5 kHz. Data acquisition and command potentials were controlled by pClamp 6.0.3 software (Axon Instruments, Inc.). To ensure voltage-clamp quality, electrode resistance was kept below 3 MΩ. Junction potentials were zeroed with the electrode in the standard bath solution. Gigaohm seal formation was achieved by suction and, after estab-

lishing the whole-cell configuration, the capacitive transients elicited by symmetrical 10-mV voltage-clamp steps from −80 mV were recorded at 50 kHz for calculation of cell capacitance. The normal bath solution (millimolar) for the whole-cell recordings was 130 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.8 mM $CaCl_2$ 1.8, 10 mM HEPES, and 5.2 mM glucose; pH was adjusted to 7.4 with NaOH. Pipettes were filled with 140 mM KCl, 0.5 mM $MgCl_2$, 0.1 mM $CaCl_2$, 0.09 mM EGTA, 10 mM HEPES, and 10 mM glucose; pH was adjusted to 7.4 with KOH.

Drugs. Cilostazol (OPC-13013) [6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)butoxy]-3,4-dihydro-2-(1*H*)-quinolinone], generously donated from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan), was dissolved in dimethyl sulfoxide as a 10 mM stock solution. TNF-α (Upstate Biotechnology, Lake Placid, NY) was dissolved in the phosphate-buffered saline as a 10 μg/ml stock solution. Others were NS-1619 (Sigma/RBI, Natick, MA), iberiotoxin, paxilline, and clotrimazole (Upstate Biotechnology). Glibenclamide and MTT were from the Sigma-Aldrich (St. Louis, MO). BMS 204352 was generously donated from the Korea Research Institute of Chemical Technology (Daejon, Korea) and dissolved in dimethyl sulfoxide as a 10 mM stock solution.

Statistical Analysis. The results are expressed as means ± S.E.M. The comparison of changes in TNF-α-induced cell viability between wild-type U87-MG and U87-MG cells of sPTEN groups was analyzed by repeated measures analysis of variance, followed by Tukey's multiple comparison tests as a post hoc comparison. Student's t test was used for analyzing values between the data of vehicle and inhibitor-treated groups of other results. $P < 0.05$ was considered to be significant.

Results

Cell Viability in SK-N-SH and HCN-1A Cells. Both SK-N-SH and HCN-1A cells showed significant reduction in viability in response to TNF-α (~1–100 ng/ml). The reduced viability was 55.3 ± 5.7 and 60.7 ± 7.4%, respectively, in response to 50 ng/ml TNF-α, which was fully prevented by cilostazol (~0.1–100 μM) in a concentration-dependent manner (Fig. 1, A and B). The increased cell viability stimulated by cilostazol (10^{-5} M) was significantly antagonized by 1 μM iberiotoxin ($P < 0.01$) in both SK-N-SH and HCN-1A cells. Iberiotoxin (1 μM) alone was without effect (Fig. 1C).

On the other hand, SK-N-SH, HCN-1A, and U87-MG cells of sPTEN, but not wild-type U87-MG cells, showed PTEN protein expression in the Western blot, whereas Akt protein expression remained unchanged in all four cell types (Fig. 2A). The viability of U87-MG cells was little changed in response to TNF-α, whereas U87-MG cells of sPTEN showed concentration-dependent decreases in viability to TNF-α (~1–100 ng/ml) (analysis of variance, $P < 0.001$) (Fig. 2B). In the latter cells, TNF-α (50 ng/ml)-induced reduction in viability (64.1 ± 5.6%, $P < 0.01$) was fully prevented by cilostazol (~1–100 μM, $P < 0.05$) (Fig. 2C).

Antiapoptotic Effect. Increased DNA fragmentation under application of TNF-α (50 ng/ml) was concentration dependently suppressed by treatment with cilostazol (~0.1–10 μM) (Fig. 3A). The suppression of oligonucleosomal DNA laddering by cilostazol (10 μM) was fully antagonized by iberiotoxin (~0.3–3 μM) (Fig. 3B). Iberiotoxin (1 μM) alone was without effect. Figure 3C shows that the suppressed DNA fragmentation by cilostazol (lane 1) was blocked by pretreatment with iberiotoxin (1 μM, lane 2), but not by clotrimazole, an intermediate conductance of Ca^{2+}-activated K^+ channel blocker (10 μM, lane 3), and not by glibenclamide, an ATP-sensitive K^+ channel blocker (10 μM, lane 4).

When other reported maxi-K channel openers, NS-1619 (10 μM) and BMS 204352 (10 μM), were used instead of cilostazol in the SK-N-SH cells, increased DNA fragmentation induced by TNF-α was also strongly suppressed by NS-1619 and BMS 204352, which were also reversed by pretreatment with iberiotoxin (1 μM), but not by clotrimazole and not by glibenclamide (Fig. 4).

Effects on p-PTEN, p-Akt, and p-CREB Levels. TNF-α (~1–100 ng/ml) concentration dependently increased the p-

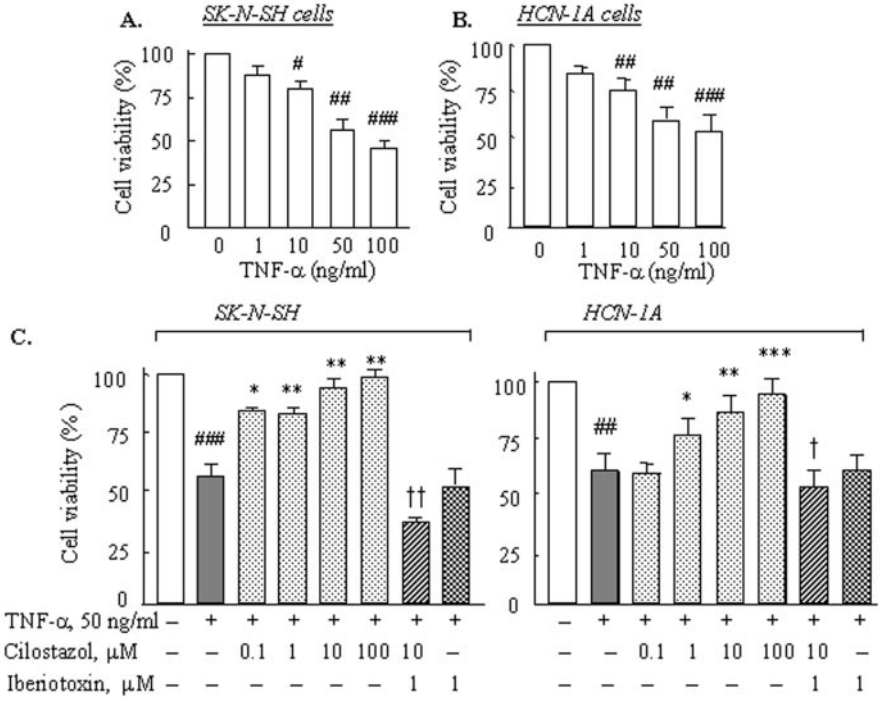

Fig. 1. Concentration-dependent decreases in cell viability of SK-N-SH (A) and HCN-1A cells (B) in response to TNF-α (~1–100 ng/ml). C, rescuing effect of cilostazol (~0.1–100 μM) on the TNF-α (50 ng/ml)-induced reduction in viability and the antagonizing effect of iberiotoxin (1 μM) in the SK-N-SH and HCN-1A cells. Values are means ± S.E.M. of three different preparations with quadruplicate experiments. #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$ versus none; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus TNF-α alone; †, $P < 0.05$; ††, $P < 0.01$ versus 10 μM cilostazol alone.

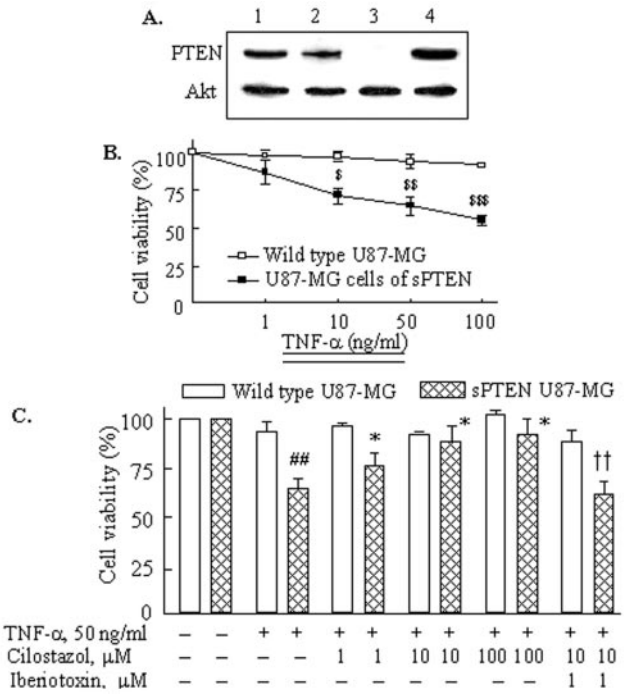

Fig. 2. A, representative Western blot of PTEN and Akt protein expression in the SK-N-SH (lane 1), HCN-1A (lane 2), wild-type of U87-MG cells (lane 3) and U87-MG cells transfected with expression vectors for sPTEN (lane 4). U87-MG cells of sPTEN, but not wild-type U87-MG cells, showed a prominent expression of PTEN protein. B, concentration-dependent decrease in cell viability to TNF-α (~1–100 ng/ml) in the U87-MG cells of sPTEN, but not in the wild-type U87-MG cells. Significant differences were shown between groups of the wild-type U87-MG cells and U87-MG cells of sPTEN by two-way repeated measures analysis of variance ($P < 0.001$). C, rescuing effect of cilostazol (~0.1–100 μM) on the reduced viability induced by TNF-α (50 ng/ml), and the antagonizing action of iberiotoxin (1 μM). Values are means ± S.E.M. of three different preparations with quadruplicate experiments in B and C. \$, $P < 0.05$; \$\$, $P < 0.01$; \$\$\$, $P < 0.001$ versus corresponding result of wild-type U87-MG cells; ##, $P < 0.01$ versus absence of TNF-α; *, $P < 0.05$ versus TNF-α alone; ††, $P < 0.01$ versus 10 μM cilostazol alone in the U87-MG cells of sPTEN.

PTEN levels, showing a maximum level with 50 ng/ml TNF-α. TNF-α (50 ng/ml)-stimulated p-PTEN was significantly attenuated by cilostazol (~1–100 μM) in the SK-N-SH cells in a concentration-dependent manner. Cilostazol (10 μM)-induced suppression of p-PTEN level was antagonized by iberiotoxin (0.3, 1, and 3 μM) concentration dependently. In the densitometric analysis, p-PTEN to PTEN ratio was significantly elevated 4.1 ± 0.4-fold ($P < 0.01$) by 50 ng/ml TNF-α, which was concentration dependently decreased 2.6 ± 0.3-fold by 10 μM cilostazol ($P < 0.05$), and its suppression was significantly reversed 3.3 ± 0.6-fold by iberiotoxin (1 μM) ($P < 0.05$) (Fig. 5, A and B).

The expression of p-Akt and p-CREB protein, in contrast, was marginally suppressed under treatment with TNF-α (50 ng/ml). However, the p-Akt and p-CREB protein levels markedly and concentration dependently elevated up to 3- to 4-fold under treatment with 10 μM cilostazol ($P < 0.01$), which were suppressed by iberiotoxin (0.3, 1, and 3 μM) (Fig. 5, A, C, and D). The unphosphorylated PTEN and Akt protein levels were little changed in the presence of TNF-α (50 ng/ml) and cilostazol. In the absence of TNF-α, cilostazol (~1–100 μM) did not show any change in the p-PTEN and p-Akt levels in the Western blot (data not shown).

We simulated the actions of cilostazol and iberiotoxin on the changes in p-PTEN and p-CREB levels in response to TNF-α in the U87-MG cells of sPTEN. U87-MG cells of sPTEN showed the same responsiveness to cilostazol and iberiotoxin as demonstrated in SK-N-SH cells (Fig. 6).

In addition, increased PTEN phosphorylation by TNF-α (50 ng/ml) was significantly suppressed by cilostazol (10 μM, $P < 0.001$), and the decreased PTEN phosphorylation was antagonized under paxilline (1, 3, and 10 μM), a maxi-K channel blocker, in SK-N-SH cells (Fig. 7, A and B). In contrast, decreased CREB phosphorylation under TNF-α (50 ng/ml) was elevated by cilostazol (10 μM, $P < 0.001$), which was antagonized by paxilline (1, 3, and 10 μM) (Fig. 7, A and C). Paxilline (10 μM) itself was without effect.

Effects on Bcl-2 and Bax Proteins. Decreased Bcl-2 protein expression under TNF-α (50 ng/ml) was elevated by cilostazol (10 μM, $P < 0.001$), which was antagonized by iberiotoxin (1 μM, $P < 0.001$). In contrast, increased Bax protein by TNF-α was significantly prevented by cilostazol (10 μM, $P < 0.01$), and the decreased Bax protein was elevated under iberiotoxin (1 μM, $P < 0.05$). However, clotrimazole (10 μM) and glibenclamide (10 μM) showed little effects (Fig. 8).

Activation of I_{KCa} by Cilostazol. Cilostazol significantly increased the outward K^+ current beginning about 3 min after application, and this effect was recovered to the control level by washout (Fig. 9, A–C). Cilostazol (3 μM) increased the steady state of outward K^+ current approximately 4-fold ($P < 0.01$) of the control level at 60 mV. Glibenclamide alone did not affect the K^+ current. In the presence of glibenclamide, a selective ATP-sensitive potassium channel blocker, cilostazol (3 μM) markedly increased the K^+ current 6-fold ($n = 12$, $P < 0.01$) of the control current at 60 mV (Fig. 9, D–F). Iberiotoxin alone inhibited K^+ current in the SK-N-SH cells to 68.5 ± 17.3% ($n = 12$, $P < 0.01$). However, in the presence of iberiotoxin (100 nM), a selective maxi-K channel blocker, cilostazol (3 μM) had little effect on the K^+ current (Fig. 9, G–I). These results indicate that cilostazol increases the K^+ current in SK-N-SH cells by activating the maxi-K channels.

Discussion

Using an in vitro model, we show that activation of maxi-K channels with cilostazol rescue TNF-α-induced cell death in the SK-N-SH and HCN-A cells in association with suppression of p-PTEN and elevation of p-Akt/p-CREB, all of which were reversed with iberiotoxin, a maxi-K channel blocker. TNF-α-induced reduction in viability was fully prevented by cilostazol in the U87-MG cells of sPTEN, whereas the wild type of U87-MG cells did not show any change in viability in response to TNF-α. Cilostazol increased the K^+ current in SK-N-SH cells by activating mainly maxi-K channels.

TNF-α, a deleterious cytokine, mediates inflammatory, thrombogenic, and vascular changes in association with brain injury (Kochanek and Hallenbeck, 1992). Increased level of TNF-α in the brain tissue after cerebral ischemia (Buttini et al., 1996) is known to cause neuronal cell death via induction of free radicals in glial cells (Hu et al., 1997). TNF-α-induced neuronal apoptosis was noted to be implicated in the ceramide-generating pathway (Sortino et al., 1999). Wu et al. (2001) have shown that ceramide decreased the opening probability of maxi-K channels in GH3 cells, which depolarizes cell membrane and activation of voltage-

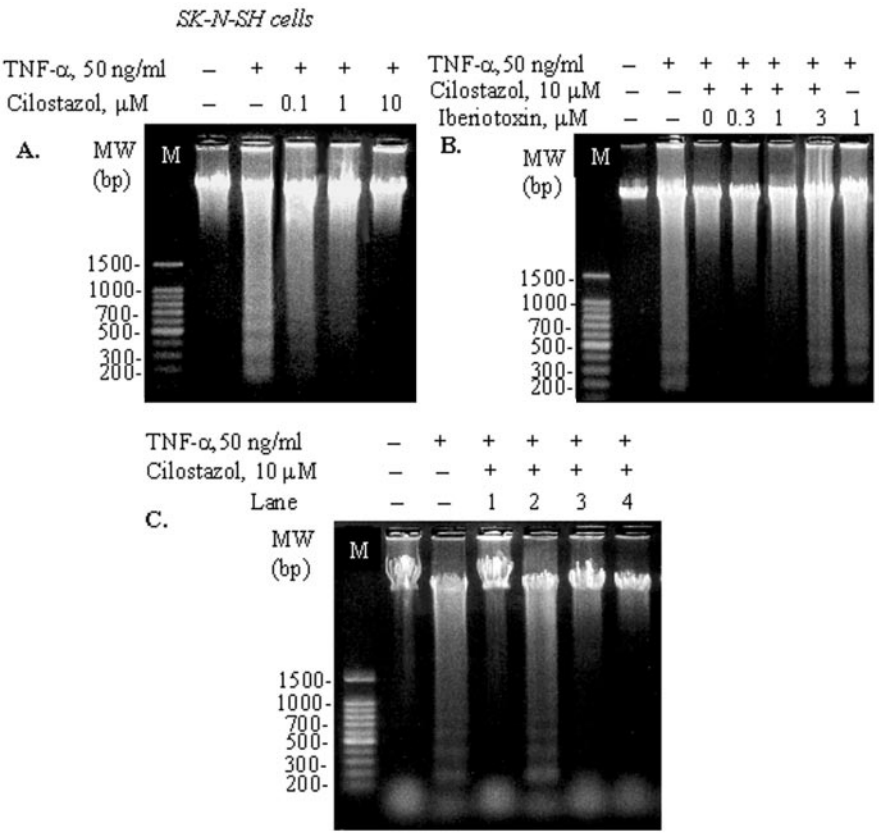

Fig. 3. Preventive effect of cilostazol on the DNA fragmentation and its reverse by iberiotoxin in the SK-N-SH cells. A, representative agarose gel electrophoresis showing DNA laddering after exposure of SK-N-SH cells to 50 ng/ml TNF-α and suppression by cilostazol (~0.1–10 μM). B, blockade by iberiotoxin (~0.3–3 μM) of the cilostazol-induced suppression. C, cilostazol effect (10 μM) on the DNA fragmentation (lane 1) under pretreatment with iberiotoxin (1 μM, lane 2), clotrimazole (10 μM, lane 3), and glibenclamide (10 μM, lane 4). M represents the 100-bp (base pair) DNA Ladder markers. Each was confirmed with four different preparations.

gated calcium channels. Our results showed that the cell viability was significantly decreased in response to TNF-α in both SK-N-SH and HCN-1A cells. TNF-α-induced cell death was prevented by cilostazol in a concentration-dependent manner, and the increased cell viability by cilostazol was fully antagonized by iberiotoxin. Increased DNA fragmentation under TNF-α was suppressed by treatment with cilostazol, which was reversed by iberiotoxin (maxi-K channel blocker), but not by clotrimazole (intermediate conductance of Ca^{2+}-activated K^+ channel blocker; Ishii et al., 1997) and not by glibenclamide (ATP-sensitive K^+ channel blocker; Schmid-Antomarchi et al., 1987). Maxi-K channel opening effect of cilostazol was further verified by using NS-1619 (Olesen et al., 1994) and BMS 204352 (Cheney et al., 2001) in the SK-N-SH cells, in that increased DNA fragmentation induced by TNF-α was suppressed by NS-1619 and BMS 204352, maxi-K channel openers, and the suppression was reversed by iberiotoxin, but not by clotrimazole and not by glibenclamide.

On the other hand, PTEN was found to dephosphorylate PI(3,4,5)P_3 to PI(4,5)P_2, acting as an antagonist of PI3-K (Maehama and Dixon, 1998). PTEN is implicated in the regulation of several cellular functions, including cell cycle progression, cell migration, and survival from apoptosis through suppression of Akt activation (Stambolic et al., 1998; Li et al., 1998; Cantley and Neel, 1999; Van Golen et al., 2001). In the present study, the viability of both SK-N-SH (human brain neuroblastoma) and HCN-1A (human brain cortical neuron) cells was significantly reduced ~55 to 60% in response to TNF-α (50 ng/ml). HCN-1A cells demonstrated the characteristics as neurons in the human central nervous system (Ronnett et al., 1990). Interestingly, TNF-α-induced

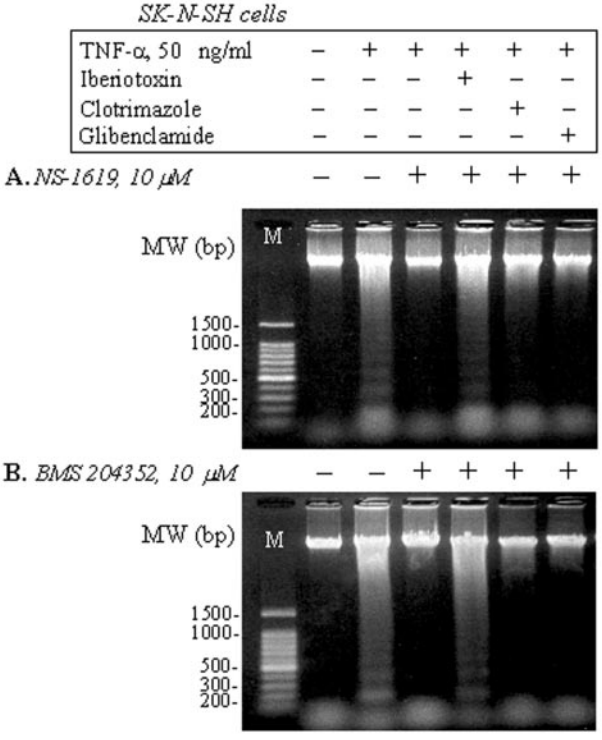

Fig. 4. Preventive effect of maxi-K channel openers on the DNA fragmentation and the reverse by iberiotoxin in the SK-N-SH cells. Representative agarose gel electrophoresis showing DNA laddering after exposure of SK-N-SH cells to 50 ng/ml TNF-α under pretreatment with iberiotoxin (1 μM), clotrimazole (10 μM), and glibenclamide (10 μM) with either NS-1619 (10 μM, A) or BMS 204352 (10 μM, B), respectively. M represents the 100-bp (base pair) DNA Ladder markers. Each was confirmed with four different preparations.

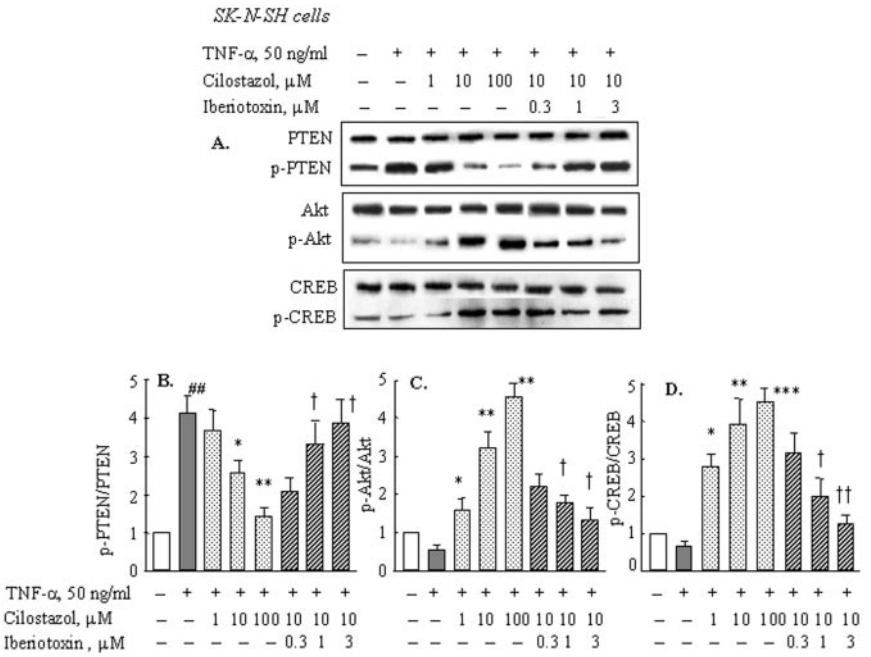

Fig. 5. A, representative Western blot of cilostazol effect (~1–100 μM) on the p-PTEN/PTEN, p-Akt/Akt, and p-CREB/CREB ratio after exposure of the SK-N-SH cells to 50 ng/ml TNF-α, and their reverses by iberiotoxin (~0.3–3 μM). Densitometric analyses of p-PTEN/PTEN (B), p-Akt/Akt values (C), and p-CREB/CREB (D). Values are means ± S.E.M. of four different experiments. None was normalized to 1. ##, $P < 0.01$ versus none; *, $P < 0.05$, **, $P < 0.01$, **≱, $P < 0.001$ versus TNF-α alone; †, $P < 0.05$; ††, $P < 0.01$ versus 10 μM cilostazol alone.

reduction in cell viability was prevented by cilostazol, which was blocked by iberiotoxin in both SK-N-SH and HCN-1A cells. In this study, wild-type U87-MG cells did not express the PTEN mRNA in reverse transcription-polymerase chain reaction as was also reported by Haas-Kogan et al. (1998), but did show increased Akt and p-Akt protein. Consequently, the viability remained unchanged in response to the TNF-α. The interactions of cilostazol and iberiotoxin shown in SK-N-SH cells were similarly evident in U87-MG cells of sPTEN, but not in wild-type U87-MG cells (human brain PTEN-null glioblastoma). In line with these data, the changes in p-CREB/p-Akt levels showed a strong inverse correlation with changes in p-PTEN in the U87-MG cells of sPTEN as well as in SK-N-SH and HCN-1A cells, when plotted in the presence of cilostazol and iberiotoxin. These findings have highlighted the fact that decreases in PTEN phosphorylation and activation of p-Akt/p-CREB signaling pathways by cilostazol may prevent neuronal cell death. In the present study, the effect of cilostazol on the PI3-K phosphorylation was not evident (data not shown), indicating that cilostazol affects the downstream of PI3-K activation.

Maxi-K channels when activated conduct an outward K^+ current that accelerates the action potential repolarization in hippocampal pyramidal cells (Shao et al., 1999) and contribute to negative feedback regulation of the Ca^{2+} influx, thus limiting the neurotransmitter release (Gribkoff et al., 2001; Hu et al., 1997). The use of maxi-K channel openers was suggested for the neuronal cell survival against acute isch-

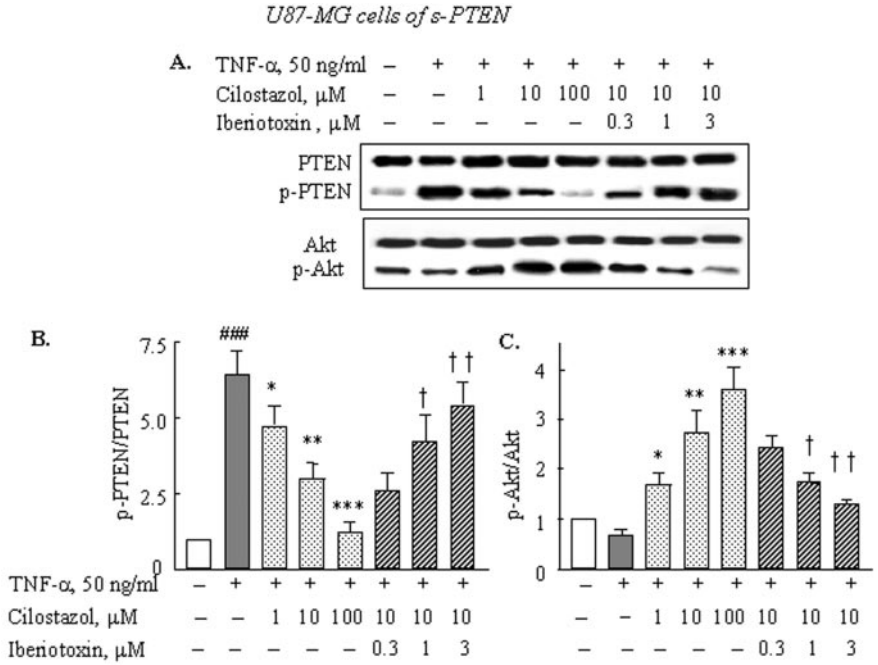

Fig. 6. A, representative Western blot of cilostazol effect (~1–100 μM) on the PTEN, p-PTEN, Akt, and p-Akt expression after exposure of the U87-MG cells of sPTEN to 50 ng/ml TNF-α, and their reverses by iberiotoxin (~0.3–3 μM). Bottom graphs showing densitometric analyses of p-PTEN/PTEN (B) and p-Akt/Akt ratio (C). Values are means ± S.E.M. of three different experiments. None was normalized to 1. ###, $P < 0.001$ versus none; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$ versus TNF-α alone; †, $P < 0.05$, ††, $P < 0.01$ versus 10 μM cilostazol alone.

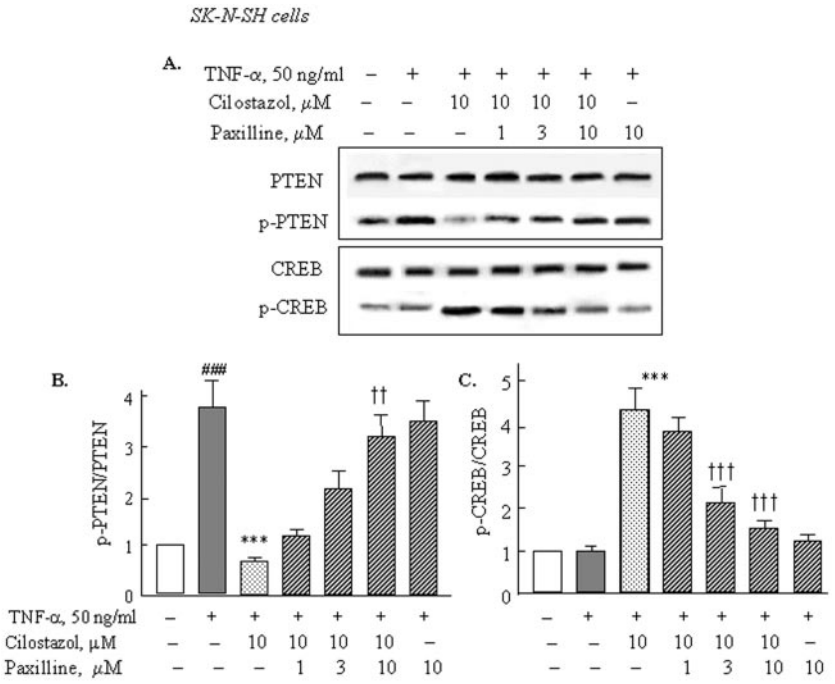

Fig. 7. A, representative Western blot of cilostazol effect (~1–100 μM) on the p-PTEN/PTEN and p-CREB)/CREB ratio after exposure of the SK-N-SH cells to 50 ng/ml TNF-α, and their reverses by paxilline (~1–10 μM). B and C, densitometric analysis of p-PTEN/PTEN and p-CREB/CREB, respectively. Values are means ± S.E.M. of four different experiments. None was normalized to 1. ###, $P < 0.001$ versus none; ***, $P < 0.001$ versus TNF-α alone; ††, $P < 0.01$, †††, $P < 0.001$ versus 10 μM cilostazol alone.

emic stroke in neurons at risk (Gribkoff et al., 2001). Recently, Rundén-Pran et al. (2002) observed that maxi-K channel blockers, including paxilline and iberiotoxin, augmented cell death induced by oxygen-glucose deprivation in the hippocampus and suggested a protective role for maxi-K channels in the neuronal cells. In the present study, cilostazol increased the outward K^+ current approximately 4-fold ($P < 0.01$), which was not inhibitable by glibenclamide, but was by iberiotoxin in SK-N-SH cells.

Intriguingly, we confirmed that NS-1619 and BMS 204352, the maxi-K channel openers, significantly suppressed TNF-α-stimulated p-PTEN, similar to cilostazol, indicating that the maxi-K channel opening by cilostazol might contribute to the anti-p-PTEN effect. This hypothesis was further supported by the results that both suppressed p-PTEN and elevated p-CREB levels induced by cilostazol were antagonized by paxilline (a mycotoxin naturally produced by the fungus *Penicillium*; Sanchez and McManus, 1996), as well as by

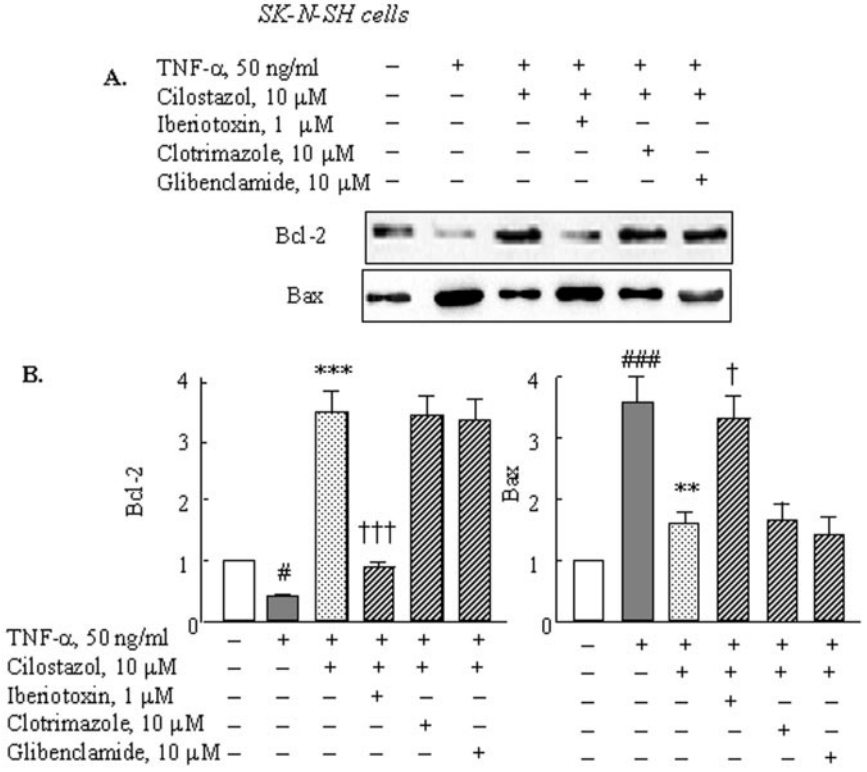

Fig. 8. Representative immunoblotting for effect of cilostazol (10 μM) on the Bcl-2 and Bax levels after exposure of the SK-N-SH cells to 50 ng/ml TNF-α. Cilostazol (10 μM) increased Bcl-2 and, in contrast, decreased Bax protein levels, both of which were reversed by iberiotoxin (1 μM), but not by clotrimazole (10 μM) and glibenclamide (10 μM). The results were confirmed by four different experiments. #, $P < 0.05$, ###, $P < 0.001$ versus none; **, $P < 0.01$, ***, $P < 0.001$ versus TNF-α alone; †, $P < 0.05$, †††, $P < 0.001$ versus 10 μM cilostazol alone.

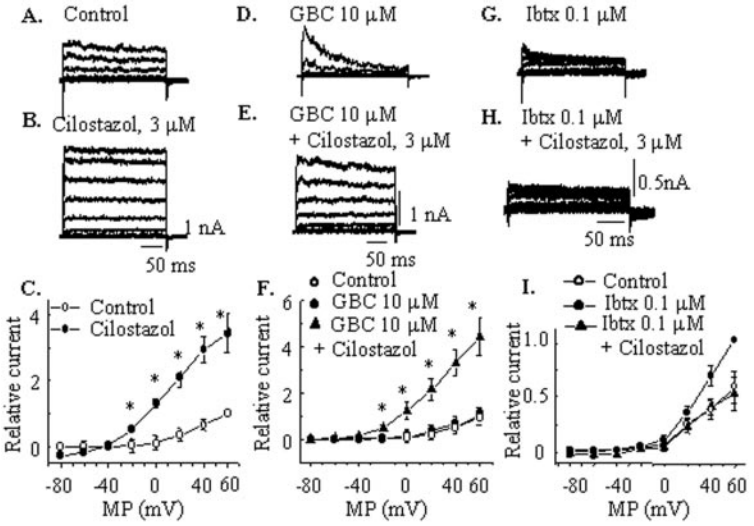

Fig. 9. Activation of the outward K^+ current in SK-N-SH cells by cilostazol. Representative current tracings evoked by depolarizing pulses in the absence (A) and presence (B) of cilostazol (3 μM). C, averaged current-voltage plots of steady-state currents in the absence and presence of cilostazol (3 μM) ($n = 8$). D and E, representative current tracings showing effect of cilostazol (3 μM) on the K^+ current in the presence of glibenclamide (GBC, 10 μM). F, averaged current-voltage plots in the absence and presence of glibenclamide (10 μM) ($n = 6$). G and H, representative current tracings showing effect of cilostazol (3 μM) on the K^+ current in the presence of iberiotoxin (Ibtx, 100 nM). I, averaged current-voltage plot in the absence and presence of iberiotoxin (100 nM) ($n = 6$). Each point denotes mean ± S.E.M. *, $P < 0.05$ versus each control value.

iberiotoxin (a peptidyl scorpion toxin) (Galvez et al., 1990). Although data are not shown, cilostazol significantly decreased the elevated cytosolic Ca^{2+} level evoked by TNF-α in SK-N-SH cells, which was reversed by iberiotoxin. It is apparently suggested that maxi-K channel opening by cilostazol may control the intracellular Ca^{2+} increase and membrane potential, thereby initiating the suppression of PTEN phosphorylation. So far, we could not find any information regarding the relationship between PTEN phosphorylation and change in membrane potential or intracellular calcium. Cilostazol did not affect the expression of maxi-K channel α-subunit in the Western blot (data not shown). Given these results, we were not able to determine the molecular mechanism by which cilostazol, as a maxi-K channel opener, decreased the PTEN phosphorylation.

Recently, a number of reports have documented that the Bcl-2 protein protects neurons against ischemia-induced cell death (Martinou et al., 1994; Bredesen, 1995) via preventing loss of the mitochondrial membrane potential and release of cytochrome *c* to cytosol (Gross et al., 1999). In contrast, Bax, as a cell-death effector protein, promotes apoptosis by triggering the release of cytochrome *c* and activation of caspase cascade (Jürgensmeier et al., 1998), and its activity is neutralized by Bcl-2 (Sato et al., 1994). Recently, Riccio et al. (1999) and Pugazhenthi et al. (2000) have addressed that enhanced CREB activity by Akt signaling leads to increased Bcl-2 promotor activity and up-regulation of Bcl-2 expression, thereby promoting cell survival. Based on the report that Bcl-2 is known to prevent generation of reactive oxygen species and reduction in mitochondrial membrane potential induced under TNF-α (Gottlieb et al., 2000), the findings that decreased Bcl-2 and elevated Bax protein expression under application of TNF-α were fully reversed by cilostazol well coincide with the impressive neuronal cell-protective effect of cilostazol.

Cilostazol was introduced to increase the intracellular level of cyclic AMP by blocking its hydrolysis by type III phosphodiesterase (Kimura et al., 1985). In our study, cilostazol showed an increase in cAMP level (data not shown). Gonzalez and Montminy (1989) have emphasized the importance of cAMP stimulation of somatostatin transcription by CREB phosphorylation at Ser-133. Franke et al. (2000) have further documented that dibutylyl cAMP enhances the survival-promoting effect of brain-derived neurotrophic factor or neurotrophin-3. At present time, it is not clear whether increase in cAMP directly related with the down-regulation of PTEN phosphorylation in the neuronal cells.

It is suggested that under cilostazol, the maxi-K channel opening-linked down-regulation of p-PTEN and up-regulation of p-Akt/p-CREB increase the Bcl-2 protein and decrease the Bax protein, leading to prevention of the cell death.

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