Cilostazol Enhances Casein Kinase 2 Phosphorylation and Suppresses Tumor Necrosis Factor-α-Induced Increased Phosphatase and Tensin Homolog Deleted from Chromosome 10 Phosphorylation and Apoptotic Cell Death in SK-N-SH Cells

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ABSTRACT

This study shows the signaling pathway by which cilostazol suppresses tumor necrosis factor-α (TNF-α)-induced the phosphatase and tensin homolog deleted from chromosome 10 (PTEN) phosphorylation and apoptosis via casein kinase 2 (CK2) phosphorylation in the SK-N-SH cells (neuroblastoma cells). Cilostazol (10 μM) fully restored cell proliferation with suppression of DNA fragmentation induced by TNF-α and emodin, a CK2 inhibitor, which were antagonized by iberiotoxin, a maxi-K channel blocker. Under application of TNF-α or emodin, increased PTEN phosphorylation and decreased phosphorylation of CK2/Akt/cyclic AMP response element-binding protein (CREB), and CK2 activity were significantly reversed by cilostazol (1–100 μM), all of which were antagonized by iberiotoxin. 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS-1619) and (3S)-(–)-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2H-indol-2-one (BMS 204352) maxi-K channel openers significantly elevated CK2 activities that were reversible by iberiotoxin. SK-N-SH cells treated with antisense CK2 oligodeoxynucleotide showed a prominent DNA fragmentation with little responsiveness to TNF-α in the phosphorylation of PTEN, indicative of the essential role of p-CK2/CK2 in cell proliferation, and the decreased cell viability of these cells was not restored by cilostazol. It is suggested that the action of cilostazol promoting cell survival is ascribed to the maxi-K channel opening-coupled up-regulation of CK2 phosphorylation and down-regulation of PTEN phosphorylation with resultant increased phosphorylation of Akt and CREB.

Cilostazol is known to increase the intracellular level of cyclic AMP by inhibiting its hydrolysis by type III phosphodiesterase (Kimura et al., 1985). Recently, Kim et al. (2002) 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 increases in Bax protein production and cytochrome c release. The neuroprotective effect of cilostazol was documented against cerebral ischemic infarct in rats (Choi et al., 2002).

The phosphatase and tensin homolog deleted from chromosome 10 (PTEN) has both protein phosphatase (phosphoserine/threonine and phospho-tyrosine) and phosphoinositide 3-phosphatase activities (Myers et al., 1997; Maehama and Dixon, 1998), and negatively regulates the phosphatidylinositol-3 kinase pathway by catalyzing degradation of the phosphatidylinositol (3,4,5)-triphosphate (Stambolic et al., 1998). Increasing numbers of reports have shown that a signaling cascade mediated by phosphatidylinositol-3 kinase/Akt (PKB, serine/threonine kinase) up-regulates cyclic AMP response element-binding protein (CREB), and in turn Bcl-2 promoter activity in association with enhanced Bcl-2 protein expression, and promotes cell survival by growth factors (Dudek et al., 1997; Crowder and Freeman, 1998; Walton et al., 1999; Pugazhenthi et al., 2000). Most recently,

ABBREVIATIONS: PTEN, phosphatase and tensin homolog deleted from chromosome 10; CREB, cyclic AMP response element-binding protein; CK2, casein kinase 2; p-CK2, phosphorylated casein kinase 2; p-PTEN, phosphorylated phosphatase and tensin homolog deleted from chromosome 10; AS, antisense; ODN, oligodeoxynucleotide; MEM, minimal essential medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; TNF-α, tumor necrosis factor-α; NS-1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one; BMS 204352, (3S)-(–)-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2H-indol-2-one.
Hong et al. (2003) have shown that cilostazol increases the K\(^+\) current in SK-N-SH cells by activating the maxi-K channels, and increase in neuronal cell survival by cilostazol is linked to its ability to decrease PTEN phosphorylation and to increase Akt/CREB phosphorylation.

Protein kinase CK2 (CK2, formerly known as casein kinase 2) has been extensively studied in recent years for its potential role in multiple functional activities, including regulation of cell growth and proliferation. It is a ubiquitous protein Ser/Thr kinase, localized in the cell cytoplasm and nucleus, existing as a heterotetramer consisting of \(\alpha\), \(\alpha'\), and \(\beta\) subunits. CK2 catalyzes the phosphorylation of a large number of proteins, and its phosphorylation modulates their activities positively or negatively (Pinna, 1990; Allende and Alende, 1995). Nevertheless, little information is known regarding implication of CK2 activation and the signaling pathway including PTEN, Akt, and CREB phosphorylation in relation with cell survival.

Therefore, it is inferred that, if activation/phosphorylation of protein kinase CK2 regulates increase in Akt/CREB phosphorylation via inactivation of PTEN, these may provide the favorable signals for cell survival. In this study, we assessed the protective effect of cilostazol as a maxi-K channel opener against TNF-\(\alpha\)- and emodin (CK2 inhibitor)-induced reduction in proliferation in the SK-N-SH cells (human neuroblastoma). Here, to identify whether cilostazol regulates the CK2 phosphorylation (p-CK2) and PTEN phosphorylation (p-PTEN) as a maxi-K channel opener, we observed these parameters in the absence and presence of iberiotoxin, a maxi-K channel blocker. We further assessed the changes in p-CK2, p-PTEN, Akt phosphorylation (p-Akt), and CREB phosphorylation (p-CREB) levels in the wild-type and antisense (AS) CK2 oligodeoxynucleotide (ODN)-treated SK-N-SH cells by Western blot analysis in the absence and presence of TNF-\(\alpha\) and emodin, a CK2 inhibitor.

Materials and Methods

Neuronal Cell Cultures. SK-N-SH (KCLB 30011, human brain neuroblastoma) 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\(_2\).

Sense and Antisense Oligodeoxynucleotides. The AS and sense are phosphorothioate analogs of ODN to the 5’ end of the different subunits of CK2. These were synthesized commercially (Bioneer Corporation, Daejon, South Korea). AS phosphorothioate ODN had the following sequences: complimentary to CK2 \(\alpha\)-subunit mRNA (CK2\(a\), target sequence site 702–721): 5’-CCAAACTCTCAGATATACCCAA, complimentary to CK2 \(\alpha\) mRNA (CK2\(a\), target sequence site 144–163): 5’-GTCCCGCATGTCGACAGGG, complimentary to CK2 \(\beta\) mRNA (CK2\(\beta\), target sequence site 124–143): 5’-GAACGTGTCTATCGACGG. Sense phosphorothioate ODN had the following sequences: complimentary to CK2 \(\alpha\) mRNA (target sequence site 144–163): 5’-CTCTCCTCGATGTCGAGAC.

Oligodeoxynucleotide Treatment of SK-N-SH Cells. SK-N-SH cells were rendered quiescent by a low concentration of fetal bovine serum (0.5%) for 15 h. ODN was added to the medium (final concentration, 100 \(\mu\)g/ml) 2 h before treatment of drugs.

Cell Proliferation Assay. For mitochondrial tetrazolium assay [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; MTT], cells were seeded 1 \(\times\) 10\(^4\) cells/well in 96-well tissue culture plates. The confluent cells were incubated in the MEM medium with 1% fetal bovine serum plus drugs and then exposed to TNF-\(\alpha\) for 24 h. Thereafter, 20 \(\mu\)l/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 \(\mu\)l/well of ethanol/dimethyl sulfoxide solution (1:1). The plates were shaken for 20 min and the optical density was measured at a wavelength 570 nm and a reference wavelength 630 nm using enzyme-linked immunosorbent assay (Bio-Tek Instruments, Winooski, VT).

DNA Fragmentation Assay. After incubation of the cells in the absence and presence of the drugs for 3 h, cells (1–5 \(\times\) 10\(^4\)) were exposed to TNF-\(\alpha\) (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 protease 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, 50% glycerol). Equivalent amounts of DNA (15–20 \(\mu\)g) were loaded into wells of 1.6% agarose gel and electrophoresed in 0.5 \(\times\) 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.

Western Blot Analysis. The confluent cells received MEM medium with 1% fetal bovine serum plus cilostazol 3 h before stimulation with TNF-\(\alpha\) and then were exposed to TNF-\(\alpha\) 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 \(\mu\)g/ml phenylmethylsulfonyl fluoride, 1 \(\mu\)g/ml aprotinin, and 1% Triton X-100. After centrifugation at 12,400 \(g\) for 15 min, the supernatant was collected. Protein concentrations were determined by Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). A total of 20 \(\mu\)g of protein was loaded onto each lane, and separated on 12.5% SDS-PAGE gel. The separated proteins were transferred to nitrocellulose membranes. After blocking in 5% non-fat dried milk for 60 min at room temperature, the blots were incubated overnight at 4°C with primary antibodies against Akt, CREB, p-Akt, p-CK2, p-PTEN, and GAPDH. The blots were then incubated with HRP-conjugated goat anti-rabbit or mouse IgG secondary antibody. The blots were developed with ECL Western Blotting Detection Reagents (Amersham, Piscataway, NJ) and exposed to X-ray film. The relative intensity of the bands was measured with densitometric analysis software (Quantity One, Bio-Rad Laboratories).

Results

Effect of Cilostazol on the TNF-\(\alpha\) and Emodin-Induced Decreased Cell Proliferation. The confluent SK-N-SH cells were cultured in Eagle’s MEM medium with 1% fetal bovine serum plus drugs. Cilostazol and emodin were added to the medium, and the cells were incubated for 24 h in the absence and presence of the drugs. The medium was aspirated and replaced with 150 \(\mu\)l/well of ethanol/dimethyl sulfoxide solution (1:1). The plates were shaken for 20 min and the optical density was measured at a wavelength 570 nm and a reference wavelength 630 nm using enzyme-linked immunosorbent assay (Bio-Tek Instruments, Winooski, VT)

Fig. 1. A. effect of cilostazol on the TNF-\(\alpha\) and emodin-induced decreased cell proliferation measured by MTT conversion in SK-N-SH cells. Values are means \(\pm\) S.E.M. from three experiments performed in quadruplicate. Significant differences were shown between control and TNF-\(\alpha\)/emodin-treated groups by two-way repeated measures analysis of variance followed by Tukey’s multiple comparison tests as a post hoc comparison (P < 0.001). B, amelioration of the TNF-\(\alpha\) and emodin-induced DNA fragmentation by cilostazol (1–100 \(\mu\)M) in the SK-N-SH cells. Representative agarose gel electrophoresis showing DNA fragmentation by cilostazol (~1–100 \(\mu\)M) in the SK-N-SH cells. Representative agarose gel electrophoresis showing DNA ladder feature after exposure of the SK-N-SH cells to TNF-\(\alpha\) (50 ng/ml) and emodin (10 \(\mu\)M) in the absence and presence of iberiotoxin (1 \(\mu\)M). M represents the 100-base pair DNA ladder markers. The results were confirmed with four different preparations.
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 CREB, p-CREB, 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 PTEN, p-PTEN were from the Santa Cruz Biotechnology Inc. (Santa Cruz, CA), and polyclonal antibodies against PTEN, p-PTEN were from the Santa Cruz Biotechnology Inc. (San Diego, CA).

**CK2 and PTEN mRNA Expression.** The expression of CK2 and PTEN mRNA was determined by reverse transcription-polymerase chain reaction. Polymerase chain reaction primers for amplification of CK2 and PTEN mRNA were designed based on the sequences obtained (sense, 5′-CCT CGA GAA TAC TGG GAT TAC GAG-3′; antisense, 5′-AGC CAT CCT CGA GAA TAC TGG GAT TAC GAG-3′). Reverse transcription-polymerase chain reaction was carried out in a total volume of 50 μl containing reverse transcription reaction, 10× Taq polymerase buffer (500 mM KCl, 100 mM Tris-HCl, pH 9.0 at 25°C, 1.0% Triton X-100, 25 mM MgCl2, 100 pmol of primers, and 1 U of Taq polymerase; Promega, Madison, WI). The conditions were 35 cycles of denaturation at 94°C (30 s), annealing at 55°C (30 s), and extension at 72°C (90 s), followed by a 10-min extension reaction at 72°C. The primer for sense PTEN was 5′-TCT ACT CCT CCA ACT CAG GAC-3′, and for antisense PTEN was 5′-CAT TAT CCC CAC GCT CTA TAC-3′ (Konu et al., 2001). The conditions were 35 cycles of denaturation at 94°C (1 min), annealing at 60°C (1 min), and extension at 72°C (1 min), followed by a 10-min extension reaction at 72°C.

**Endogenous CK2 Activity in Cell Lysate.** CK2 activity was assayed by using casein kinase 2 assay kit (Upstate Biotechnology, Lake Placid, NY). The 2.5 to 5 μg of proteins from the cleared lysate was used for the assay of CK2 activity, measured by addition of the specific peptide R_D_S_D (1 mM) in the presence of 20 mM MOPS, pH 7.2, 25 mM β-glycerophosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol, and 10 μl of [γ-32P]ATP (100 μCi) in a total volume of 50 μl, and the radioactivity was measured by scintillation counting. CK2 activity value represents pmol phosphate incorporated into CK2 substrate peptide per min per nanogram of CK2 according to the manual of the product company.

**Drugs.** Cilostazol (OPC-13013), (6-[4-(1-cyclohexyl-1H-tetrazol-5-yl) butoxy]-3,4-dihydro-2(1H)-quinolinone), generously donated from Otsuka Pharmaceutical Co. Ltd. (Tokushima, Japan), and dissolved in dimethyl sulfoxide as a 10 mM stock solution. TNF-α (Upstate Biotechnology) was dissolved in the phosphate-buffered saline as a 10 μg/ml stock solution. Others were NS-1619 (Sigma/ RBI, Natick, MA) and iberiotoxin (Upstate Biotechnology). MTT and...
containing 50 ng/ml TNF-α present study, SK-N-SH cells were incubated in the medium (7.9% at 24 h, respectively), which were effectively restored by treatment with cilostazol (1–10 μM) concentration dependently increased the p-CK2/CK2 ratio, whereas the p-PTEN/PTEN ratio was suppressed by cilostazol. Application of iberiotoxin (1 μM) reversed the whole cilostazol effects. Values are means ± S.E.M. from four experiments. #, P < 0.05; ##, P < 0.01 versus none; *, P < 0.05; †, P < 0.01 versus emodin alone; ††, P < 0.01 versus emodin plus 10 μM cilostazol.

emodin 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. Results are expressed as means ± S.E.M. The changes in cell proliferation between control and emodin/TNF-α/AS CK2 ODN-treated groups in the absence and presence of cilostazol were 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. P < 0.05 was accepted as statistically significant.

Results

Prevention of Cell Death and Apoptosis. In our previous study, we observed that SK-N-SH cells showed a significant reduction in viability in response to TNF-α (~1–100 ng/ml). When cells were incubated for 1h under TNF-α (~1–100 ng/ml), the maximum value of PTEN phosphorylation was obtained at 50 ng/ml TNF-α (Hong et al., 2003). In the present study, SK-N-SH cells were incubated in the medium containing 50 ng/ml TNF-α or 10 μM emodin. Cell proliferation was time dependently decreased (33.1 ± 6.1 and 58.45 ± 7.9% at 24 h, respectively), which were effectively restored by pretreatment with cilostazol (10 μM) (Fig. 1A).

DNA fragmentation evoked by exposure to TNF-α (50 ng/ml) or emodin (10 μM) was largely suppressed under treatment with cilostazol (~1–100 μM) in a concentration-dependent manner, those of which were antagonized by iberiotoxin (1 μM) (Fig. 1B). Iberiotoxin (1 μM) alone was without effect (data not shown).

Effect of Cilostazol on the p-PTEN, p-Akt, and p-CREB. When cells were incubated in the medium containing TNF-α (50 ng/ml), the maximum value of PTEN phosphorylation was obtained at 1 h of incubation. Thus, Western blot assay for PTEN and Akt/CREB were determined after incubation with TNF-α for 1 h. In Western blot, p-CK2 expression from SK-N-SH cells modestly increased at ~1 to 10 ng/ml TNF-α and then decreased in response to high concentration of TNF-α (~30–100 ng/ml), whereas CK2 protein levels were little changed (Fig. 2A). TNF-α (50 ng/ml)-induced decreased p-CK2 expression was concentration dependently increased by cilostazol (~1–100 μM). In accordance with these results, decreased p-Akt and p-CREB levels were increased, whereas p-PTEN level was markedly decreased by cilostazol (~1–100 μM). Intriguingly, cilostazol (10 μM)-induced increased p-CK2, p-Akt, and p-CREB and decreased p-PTEN levels were all antagonized by iberiotoxin (~0.3–3 μM) concentration dependently (Fig. 2, B and C).

p-CK2 and p-PTEN Expression in the Emodin-Applied Cells. Decreased p-CK2/CK2 ratio under emodin (10 μM) was significantly elevated with increasing concentration of cilostazol (~1–100 μM), which was antagonized by iberiotoxin (1 μM; P < 0.01). In contrast, markedly increased p-PTEN/PTEN ratio by emodin (10 μM) was significantly and concentration dependently suppressed by cilostazol (~1–100 μM), which was also antagonized by iberiotoxin (1 μM) (Fig. 3, A and B).

Effect of Cilostazol on the CK2 Activity. Cilostazol significantly increased CK2 activity concentration dependently (~1–100 μM) in the SK-N-SH cells (Fig. 4A). CK2 activity was little influenced by TNF-α (50 ng/ml), but significantly decreased by emodin (10 μM) from 1.39 ± 0.01 to 0.51 ± 0.04 pmol/min/ng (P < 0.01). In the presence of TNF-α (50 ng/ml) or emodin (10 μM), CK2 activity was significantly and concentration dependently elevated by cilostazol (~1–100 μM), which was antagonized by iberiotoxin (~0.3–3 μM) (Fig. 4B).

Maxi-K Channel Openers NS-1619 and BMS 204352 Enhanced the CK2 Activity. When NS-1619 and BMS 204352, other maxi-K channel openers, were used instead of cilostazol in the SK-N-SH cells, both NS-1619 and BMS 204352 (~1–100 μM) concentration dependently increased CK2 activity in the presence of TNF-α (50 ng/ml) (Fig. 5, A and B). The increased CK2 activity by either NS-1619 or BMS 204352 (10 μM) was concentration dependently antagonized by pretreatment with iberiotoxin (~0.3–3 μM) (Fig. 5, C and D).

Experiments with CK2 AS-Treated SK-N-SH Cells. Three kinds of AS CK2 ODN were used. SK-N-SH cells treated with AS CK2 α subunit target sequence site 144–163 (α144–163), a subunit target sequence site 702–721 (α702–721), and β subunit target sequence site 124–143 (β124–143) ODN did not show the CK2 mRNA expression in contrast to wild-type cells, whereas they all expressed the PTEN mRNA intact (Fig. 6A). The levels of p-PTEN, p-Akt, and p-CREB little differ between the cells treated with AS CK2 α144–163, AS CK2 α702–721, or AS CK2 β124–143 ODN, and remained in relatively low levels. Cilostazol (~1–100 μM) and iberiotoxin (1 μM) could not exert any effect on the p-PTEN, p-Akt, and p-CREB levels in the AS CK2 ODN α144–163-treated SK-N-SH cells in the absence (Fig. 6, B and C) and presence of TNF-α (Fig. 7, A and B). The p-PTEN expression of these cells was characteristically augmented in the presence of TNF-α.
but were not influenced by pretreatment with cilostazol (10 μM) in the SK-N-SH cells. B, effect of cilostazol (1–100 μM) on the CK2 activity of the SK-N-SH cells in the presence of 50 ng/ml TNF-α or 10 μM emodin without and with iberiotoxin (~0.3–3 μM). Values are means ± S.E.M. from three experiments performed in triplicate. †‡, P < 0.01 versus none; †, P < 0.05; ††, P < 0.01 versus emodin or TNF-α alone; †‡, P < 0.05; †††, P < 0.01 versus emodin or TNF-α plus 10 μM cilostazol.

The cells treated with AS CK2 ODN, including α144–163, α270–271, and β124–143, showed marked DNA fragmentation, but were not influenced by pretreatment with cilostazol (10 μM). The feature of DNA fragmentation was, however, not elicited in the sense CK2 ODN-treated cells (Fig. 8A). The proliferation of the SK-N-SH cells treated with AS CK2 α144–163 was decreased with time, which was not recovered under pretreatment with cilostazol (10 μM) (Fig. 8B).

Discussion

The present study shows that cilostazol as a maxi-K channel opener elevates p-CK2 level and suppresses TNF-α-induced increased p-PTEN with inhibition of apoptotic cell death in the SK-N-SH cells, which are reversible by iberiotoxin, a maxi-K channel blocker. Both CK2 and p-CK2 were constitutively expressed in high levels in baseline state. The levels of p-CK2/p-Akt/p-CREB showed a close inverse correlation with the levels of p-PTEN in SK-N-SH in the presence of cilostazol and iberiotoxin.

Protein kinase CK2 is a highly conserved, ubiquitously expressed Ser-Thr kinase that phosphorylates a variety of substrates involved in essential cell processes, including cell cycle and growth (Pinna, 1990; Allende and Allende, 1995). 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 Pulido, 2001; Miller et al., 2002).

On the other hand, PTEN exerts both protein phosphatase (phospho-serine/threonine and phospho-tyrosine) and phosphoinositide 3-phosphatase activities (Myers et al., 1997; Maehama and Dixon, 1998). Recently, it has been demonstrated that most cellular PTEN is constitutively phosphorylated, and phosphorylation sites are located in a cluster of the C-terminal tail of PTEN, possibly at amino acid Ser370, Ser380, Thr382, Thr383, Ser385 (Vazquez et al., 2000; Torres and Pulido, 2001). In the present experiment, the polyclonal antibodies against PTEN phosphorylated at amino acid Ser370–143, showed marked DNA fragmentation, and presence of iberiotoxin (50 ng/ml) in the absence of TNF-α inhibited the viability of U87-MG cell, a PTEN-null glioblastoma cell line, but when transfected with expression vectors for the sense PTEN, it decreased the viability of
U87-MG cells. In these cells, cilostazol significantly reduced TNF-α-induced increased p-PTEN level in accordance with marked elevation of p-Akt and p-CREB protein levels (up to ~3–4-fold) as was shown in the SK-N-SH cells. The PTEN level remained unaltered. Although data are not shown, in Western blot assay cilostazol showed a modest effect on the Akt and CREB phosphorylation in the wild type of U87-MG cells (human brain PTEN-null glioblastoma), as contrasted with the cells transfected with expression vectors for the sense PTEN. In the light of these data, it seems that effects of cilostazol on the Akt and CREB phosphorylation are closely related with phosphorylated PTEN.

To assess the functional relevance of p-PTEN by p-CK2, cells were treated with emodin, a CK2-selective inhibitor. The replacement of ATP by the competitive inhibitor emodin was reported to induce the conformational modifications in the catalytic site of CK2 (Battistutta et al., 2000). Emodin, a CK2 inhibitor, potently inhibited both p-CK2 expression and CK2 activity. Nevertheless, both decreased p-CK2 and CK2 activity under emodin (10 μM) application was significantly elevated by cilostazol, and in contrast, markedly increased p-PTEN level was concentration dependently suppressed by cilostazol. Hence, it was conceivable that cilostazol effect might be mediated through an action site distinct from the

Fig. 6. A, reverse transcription-polymerase chain reaction showing a lack of CK2 mRNA expression in the SK-N-SH cells treated with antisense CK2 α subunit target sequence site 144–163 (α144–163), α subunit target sequence site 702–721 (α702–721), and β subunit target sequence site 124–143 (β124–143) oligodeoxynucleotides, whereas these cells expressed the PTEN mRNA intact. B and C, Western blot assay and densitometric analysis showing effect of cilostazol and iberiotoxin on the levels of p-PTEN, p-Akt, and p-CREB proteins in the SK-N-SH cells treated with antisense CK2 α144–163 oligodeoxynucleotide.

Fig. 7. Western blot assay (A) and densitometric analysis (B) showing effect of cilostazol and iberiotoxin on the levels of p-PTEN, p-Akt, and p-CREB proteins in the SK-N-SH cells treated with antisense CK2 α144–163 oligodeoxynucleotide in the presence of TNF-α. PTEN, Akt, and CREB protein levels were little changed. The p-PTEN expression of these cells was characteristically augmented in the presence of TNF-α. Values are mean ± S.E.M. from four experiments. ##, P < 0.01; ###, P < 0.001 versus none.
cilostazol increased the \( K^+ / H^+ \) entry and by minimizing the neuronal depolarization in neurons at risk. These facts strongly indicate that the maxi-K channel opening by cilostazol contributes to the anti-p-PTEN effect via increases in p-CK2 expression/CK2 activity. So far, it goes beyond the scope of our present study to illustrate the mechanism(s) how p-CK2 controls the p-PTEN levels. We did not determine the relationship between changes in membrane potential and p-CK2 expression/CK2 activity. To confirm the role of p-CK2, we used the AS CK2 ODN to inhibit the expression of the genes encoding for the CK2 (Agrawal, 1992). Selective depletion of both two AS CK2 catalytic \( \alpha \) subunits (target sequence sites 144–163 and 702–721) and one regulatory \( \beta \) subunit (target sequence site 124–143) resulted in the marked DNA fragmentation accompanied by much reduced p-PTEN expression, indicative of pivotal role of p-CK2/CK2 in the cell viability, and cilostazol was without effect on the DNA fragmentation in the absence of changes in p-PTEN/p-Akt/p-CREB expressions.

Based on these results, it is suggested that endogenous CK2 can regulate the p-PTEN, p-Akt, and p-CREB expressions in the cells such that the cell viability (survival) is greatly modulated by the p-CK2-activating agent such as cilostazol. In the light of the finding that p-PTEN remained in relatively low levels in the cells treated with AS CK2 ODN, it seems likely that endogenous CK2 regulates phosphorylation of PTEN. However, the p-PTEN level was characteristically augmented in the presence of TNF-\( \alpha \) in the cells treated with AS CK2 ODN, reflecting the activation of p-PTEN by TNF-\( \alpha \) being independent of p-CK2.

Currently, it remains unclear whether increase in cyclic AMP directly relates with the up-regulation of CK2 phosphorylation and down-regulation of PTEN phosphorylation in the neuronal cells. Although data are not shown, cilostazol significantly decreased the elevated cytosolic Ca\(^{2+} \) level evoked by TNF-\( \alpha \) (50 ng/ml) in the SK-N-SH cells, which was reversed by iberiotoxin. Thus, it is speculated that maxi-K channel opening by cilostazol may regulate intracellular Ca\(^{2+} \) increase and membrane potential via activation of cyclic AMP protein kinase, thereby initiating the suppression of PTEN phosphorylation. So far, we do not have any critical evidence regarding the implication of cyclic AMP protein kinase in the phosphorylation of PTEN related with the change in membrane potential or intracellular calcium. Together, it is considered that CK2 may serve as a target for inhibiting neuronal cell apoptosis in cell injury and thus may offer a novel cell survival strategy. Recently, Choi et al. (2002) provided in vivo evidence that postischemic treatment

**Fig. 8.** A, representative agarose gel electrophoresis showing DNA laddering in the SK-N-SH cells treated with CK2 sense \( \alpha \) subunit target sequence site 144–163 (\( \alpha_{144-163} \)) and antisense \( \alpha \) subunit 144–163 (\( \alpha_{144-163} \)), \( \alpha \) subunit 702–721 (\( \alpha_{702-721} \)), and \( \beta \) subunit 124–143 (\( \beta_{124-143} \)) oligonucleotides (100 \( \mu \)g/ml, each) under treatment with cilostazol (10 \( \mu \)M). Cilostazol could not exert any effect on the DNA fragmentation in the cells treated with antisense oligodeoxynucleotide. M represents the 100-base pair DNA ladder markers. The results were confirmed with four different preparations. B, decreased cell proliferation in the SK-N-SH cells treated with antisense CK2 \( \alpha_{144-163} \) oligonucleotide. Cilostazol was without effect on these cells. Values are means \( \pm \) S.E.M. from three experiments performed in quadruplicate.
with cilostazol reduces cerebral infarct size in association with decreased DNA fragmentation accompanied by up-regulation of Bcl-2 and down-regulation of Bax protein and cytochrome c release from mitochondria.

In conclusion, it is suggested that inhibition of TNF-α-induced cell death by cilostazol is ascribed to the maxi-K channel opening-coupled up-regulation of CK2 phosphorylation and down-regulation of PTEN phosphorylation with increased Akt and CREB phosphorylation.

References


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