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Anti-apoptotic action of (2*S*,3*S*,4*R*)-*N*″-cyano-*N*-(6-amino-3,4-dihydro-3-hydroxy-2-methyl-2-dimethoxymethyl-2H-benzopyran-4-yl)-*N*′-benzylguanidine (KR-31378) by suppression of the phosphatase and tensin homolog deleted from chromosome 10 phosphorylation and increased phosphorylation of casein kinase2/Akt/ cyclic AMP response element binding protein via maxi-K channel opening in neuronal cells☆

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Abstract

This study shows the signaling pathway by which (2*S*,3*S*,4*R*)-*N*″-cyano-*N*-(6-amino-3,4-dihydro-3-hydroxy-2-methyl-2-dimethoxymethyl-2H-benzopyran-4-yl)-*N*′-benzylguanidine (KR-31378) prevents tumor necrosis factor (TNF)-α-induced neuronal cell death. KR-31378 restored TNF-α-induced decreased cell viability of SK–N–SH. U87-MG cells (PTEN-null glioblastoma cell line) transfected with expression vectors for sense PTEN (phosphatase and tensin homolog deleted from chromosome 10) showed significantly decreased cell viability, which was restored by KR-31378. TNF-α-induced increased PTEN phosphorylation and decreased phosphorylation of Akt/cyclic AMP response element-binding protein (CREB) in SK–N–SH cells were concentration-dependently reversed by KR-31378, those of which were antagonized by iberiotoxin, a maxi-K channel blocker. TNF-α and apigenin, a casein kinase2 (CK2) inhibitor, showed decreased CK2 phosphorylation and increased PTEN phosphorylation, which were reversed by KR-31378. KR-31378 increased K^+ currents by activating the maxi-K channels in SK–N–SH cells, with suppression of TNF-α-induced increase in cytosolic Ca^{2+} and elevation of suppressed mitochondrial membrane potential, all of which were antagonized by iberiotoxin. It is suggested that increase in cell viability by KR-31378 is ascribed to the maxi-K channel opening-coupled upregulation of CK2/Akt/CREB phosphorylation and downregulation of PTEN phosphorylation in association with increased Bcl-2 and decreased Bax levels.

Keywords: KR-31378; PTEN phosphorylation; Maxi-K channel; CREB; Akt; Apoptosis

1. Introduction

Recently, it has been demonstrated that (2*S*,3*S*,4*R*)-*N*″-cyano-*N*-(6-amino-3,4-dihydro-3-hydroxy-2-methyl-2-dimethoxymethyl-2H-benzopyran-4-yl)-*N*′-benzylguani-

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dine (KR-31378) potently suppressed lipopolysaccharide-induced cell death and DNA fragmentation in human umbilical vein endothelial cells in association with significant reduction in the intracellular reactive oxygen species and tumor necrosis factor (TNF)-α. Lipopolysaccharide-induced decrease in Bcl-2 and increase in Bax protein and cytochrome c release were totally reversed by KR-31378 (Kim et al., 2002). Hong et al. (2002) have shown the in vivo results, in that the infarct area of rats subjected to 2 h occlusion of the left middle cerebral artery followed by 24 h reperfusion was significantly reduced after treatment with KR-31378, after the completion of 2 h ischemia. Treatment with KR-31378 significantly reduced the TUNEL-positive cells and suppressed the DNA fragmentation in the cortical tissue associated with increased Bcl-2 protein and decreased Bax protein and cytochrome c release.

PTEN (the phosphatase and tensin homolog deleted from chromosome 10) that has both protein phosphatase (phospho-serine/threonine and phospho-tyrosine) and phosphoinositide 3-phosphatase activities (Myers et al., 1997; Maehama and Dixon, 1998) negatively regulates the phosphatidylinositol 3-kinase by catalyzing degradation of the phosphatidylinositol(3,4,5)-triphosphate (PI(3,4,5)P_3) to phosphatidylinositol(3,4)-diphosphate (PI(3,4)P_2) (Stambolic et al., 1998). Evidence has accumulated that a signaling cascade mediated by activated Akt/cyclic AMP response element-binding protein (CREB) regulates Bcl-2 expression and promotes cell survival by growth factors against apoptotic stimuli (Dudek et al., 1997; Kulik et al., 1997). Overexpression of phosphatidylinositol 3-kinase and its downstream effector Akt (serine/threonine kinase) mediate growth factor-induced neuronal survival, and they in turn up-regulate Bcl-2 promoter activity, in association with Bcl-2 protein expression through enhanced CREB activation (Crowder and Freeman, 1998; Walton et al., 1999; Pugazhenthi et al., 2000). Protein kinase CK2 (CK2, formerly known as casein kinase 2) has a role in the regulation of cell growth and proliferation, catalysis of the phosphorylation of a number of proteins and in the modulation of the activities of proteins (Pinna, 1990; Allende and Allende, 1995).

On the other hand, maxi-K channels, a large conductance Ca^{2+}-activated K^+ channels, are activated by depolarization and increase in intracellular Ca^{2+} (Latorre et al., 1989). Gribkoff et al. (2001) introduced the usefulness of the maxi-K channel opener, (3*S*)-(+)-(5-chloro-2-methoxyphenyl)-1,3-dihydro-3-fluoro-6-(trifluoromethyl)-2H-indole-2-one (BMS 204352), for neuroprotection against acute ischemic stroke by restricting Ca^{2+} entry in neurons at risk. Robitaille and Charlton (1992) early demonstrated the limited accumulation of pathological levels of Ca^{2+} by K^+ channel opening during brain ischemia, thereby reducing the neurotransmitter release and attenuating the ischemic injury. Most recently, Rundén-Pran et al. (2002) showed a protective role of maxi-K channels, in that treatment with maxi-K channel blocker, paxilline (a mycotoxin naturally produced by the fungus *Penicillium*) and iberiotoxin (a peptidyl scorpion toxin) enhanced cell death of the hippocampus during and after oxygen–glucose deprivation. However, little information is known regarding the relationships between activation of maxi-K channels and regulation of PTEN phosphorylation (P-PTEN) and/or CK2/Akt/CREB phosphorylation (P-CK2, P-Akt, P-CREB) in the cell viability.

In the present study, to elucidate the signaling pathway by which KR-31378 increases the cell viability, we examined the protective effect of KR-31378 against TNF-α-induced reduction in viability in the SK–N–SH cells (neuroblastoma cells). To verify implication of PTEN, we employed U87-MG cells (PTEN-null glioblastoma cell line) and U87-MG cells transfected with expression vectors for sense PTEN (sPTEN). To further identify the mechanism by which KR-31378 ameliorates cell injury, we determined the changes in P-CK2, P-PTEN, P-Akt, P-CREB, Bcl-2 and Bax protein levels by Western blot analysis under treatment with KR-31378, in the absence and presence of iberiotoxin, a maxi-K channel blocker. Finally, we electrophysiologically confirmed the effect of KR-31378 on the K^+ current in SK–N–SH cells.

2. Materials and methods

2.1. Neuronal cell cultures

SK–N–SH (KCLB 30011, human brain neuroblastoma) and U87-MG (KCLB 30014, human brain PTEN-null glioblastoma) cells were cultured in Eagle's minimum essential medium (MEM) with 2.0 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.

2.2. Cell viability assay

For mitochondrial tetrazolium assay (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 from 3 h prior to 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 plates were shaken for 20 min and the OD was measured at 570–630 nm using the microplate reader (Bio-Tek Instruments, Winooski, VT).

2.3. Plasmid construction

The expression of plasmid encoding the human PTEN protein was cloned by reverse transcription–polymerase chain reaction (RT-PCR) 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′–GCG CTC GAG ATG ACA GCC ATC AAA G–3′; antisense, 3′–ACT CCC TAT TTT GTG GTA CGA GCT CGC G–5′. 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.

2.4. DNA transfection and transfection efficiency assay

U87-MG cells were seeded for 24 h before transfection in tissue culture dishes. At 50–70% confluency, the dishes were washed twice with Opti-MEM medium, in order to remove the fetal bovine serum; and a transfection cocktail containing 10 μg DNA and 10 μl LipofectAMINE reagent (Invitrogen, San Diego, CA) 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 by using a commercially available β-Gal staining kit (Invitrogen, San Diego, CA). Under microscope (200× in magnitude), the blue colored cells were counted in 5–10 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.

2.5. Western blot analysis

The confluent cells received MEM medium with 1% fetal bovine serum plus KR-31378 3 h prior to 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 phenylmethylsulflonyl fluoride, 1 μg/ml aprotinin and 1% Triton X-100. Following centrifugation at 12,000 rpm, 50 μg of total protein was loaded into 8% or 10% SDS-PAGE gel and then transferred to nitrocellulose membrane (Amersham Pharmacia Biotech, 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, Rockford, IL). The signals of the bands were quantified using the GS-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA). The results were expressed as a relative density. Polyclonal antibodies against CREB, P-CREB, and CK2 and monoclonal antibodies against Bcl-2 and Bax were from the Santa Cruz Biotechnology (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).

2.6. Sense and antisense oligodeoxynucleotides

The antisense and sense phosphorothioate analogs of oligodeoxynucleotide to the 5′ end of the different subunits of CK2 were synthesized commercially (Bioneer, Daejon, Korea). Antisense phosphorothioate oligodeoxynucleotide had the following sequences: complimentary to CK2 α-subunit mRNA ($CK2\alpha_{144–163}$, 144–163 target sequence site): 5′–GTCCCGACATGTCAGACAGG.

2.7. 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. Oligodeoxynucleotide was added to the medium (final concentration, 100 μg/ml) 2 h prior to treatment of drugs.

2.8. Recording of the whole cell K^+ current

Using the whole cell configuration of the patch clamp technique, the K^+ currents were recorded at room temperature (20–22 °C) with an Axopatch-200B patch clamp amplifier (Axon Instruments, Foster City, CA). Currents were sampled at 1–10 kHz after antialias filtering at 0.5 to 5 kHz. Data acquisition and command potentials were controlled by pClamp 6.0.3 software (Axon Instruments, Foster City, CA). To ensure the 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 establishing 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 for the whole cell recordings was: 130 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM HEPES, 5.2 mM glucose and the 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 ethylenebis (oxonitrilo) tetra-acetic acid (EGTA), 10 mM HEPES, 10 mM glucose and the pH was adjusted to 7.4 with KOH. After establishment of whole cell recordings and collecting control recordings for approximately 5 min until the current elicited by depolarization stabilized, KR-31378 was applied into the bath. In the experiments using glibenclamide or iberiotoxin, KR-31378 was applied to the bath about 20 min after administering glibenclamide or iberiotoxin.

2.9. Measurement of cytosolic Ca^{2+} concentration and mitochondrial membrane potential ($\Delta\Psi_m$)

Cells were seeded 5×10^4 cells/well in 12-well tissue culture plates. The confluent cells received MEM medium with 1% fetal bovine serum plus iberiotoxin 30 min prior to

stimulation with KR-31378, and then were exposed to KR-31378 for 3 h. Thereafter, TNF-α was added for 24 h. Cytosolic Ca^{2+} concentration was measured by using the fluo-3 AM (Molecular Probes, Eugene, OR). The cell pellets were then incubated with fluo-3 AM (2 μM, final concentration) in phosphate-buffered saline for 1 h at 37 °C. The residual-free fluo-3 AM was eliminated. The fluorescent dye was excited at 488 nm, and the fluorescence intensities of fluo-3 AM molecules were recorded at 525 nm with the Microspectrofluorometer (SPF-500C, SLM Inst., Urbana, IL).

The mitochondrial membrane potential was measured by using the 5,5′,6,6′-tetrachloro-1,1′,3, 3′-tetraethylbenzimidazolylcarbocyanine (JC-1; Molecular Probes, Eugene, OR). Cells were stained with 1 μg/ml of JC-1 for 30 min at 37 °C. JC-1 was excited at 488 nm and the monomer signal (green) was analyzed at 535 nm on a Microspectrofluorometer (SPF-500C, SLM Inst., Urbana, IL). Simultaneously, the aggregate signal (red) was analyzed at 595 nm.

2.10. DNA fragmentation assay

After incubation of the cells in the absence and presence of the drugs for 3 h, cells ($1–5\times10^6$) 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 1–3 h at 55 °C, followed by addition of RNAse A to 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 1.6% 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 ultraviolet transillumination with the Polaroid camera.

2.11. Drugs

KR-31378 and BMS 204352 were obtained from the Korea Research Institute of Chemical Technology (Daejon, Korea) and were dissolved in dimethyl sulfoxide as a 100-mM stock solution. TNF-α (Upstate Biotechnology, Lake Placid, NY) was dissolved in the phosphate-buffered saline as a 10-μg/ml stock solution. Iberiotoxin and clotrimazole were from the Upstate Biotechnology (Lake Placid, NY). Glibenclamide was from Sigma-Aldrich (St. Louis, MO).

2.12. 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 and 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.

3. Results

3.1. Cell viability in SK–N–SH and U87-MG cells

SK–N–SH cells and U87-MG cells of sPTEN, but not wild-type U87-MG cells, showed PTEN expression in Western blot, whereas all three cell types showed maxi-K channel α-subunit and Akt expression, suggestive of PTEN-null glioblastoma cell line (Fig. 1A). The cell viability was concentration-dependently decreased in response to TNF-α (approximately 1–100 ng/ml) in the SK–N–SH cells and U87-MG cells with sPTEN (ANOVA, $P<0.001$), but not in the wild-type U87-MG cells. The viability of SK–N–SH cells was significantly reduced to 55.3±5.7% and 49.1±3.2% in response to 50 and 100 ng/ml of TNF-α, respectively. The reduced viability induced by 50 ng/ml of TNF-α in SK–N–SH and in U87-MG cells with sPTEN was fully reversed by KR-31378 (approximately 0.1–100 μM) in a concentration-dependent manner. The restored viability by KR-31378 (10 μM) was significantly suppressed by pretreatment with 1 μM iberiotoxin ($P<0.01$; Fig. 1C). The cell viability of wild-type U87-MG cells was barely changed in response to TNF-α and KR-31378.

3.2. Western blot assay for PTEN, Akt and CREB phosphorylation

In the absence of TNF-α, KR-31378 (approximately 1–100 μM) and iberiotoxin (approximately 0.3–1 μM) showed little effect on the P-PTEN and P-Akt levels (data not shown). TNF-α (approximately 1–100 ng/ml) concentration-dependently increased the P-PTEN level, showing a maximum effect with 50 ng/ml TNF-α, whereas constitutive PTEN levels remained unchanged (data not shown). TNF-α (50 ng/ml)-stimulated P-PTEN level (4.13±0.55-fold) was significantly and concentration-dependently suppressed by KR-31378 (1, 10 and 100 μM) in the SK–N–SH cells. KR-31378 (10 μM)-induced suppression of P-PTEN level was antagonized by iberiotoxin (0.3, 1 and 3 μM) concentration-dependently (Fig. 2A and B). The reduced P-Akt level (0.24±0.08-fold) induced by TNF-α (50 ng/ml) was, in contrast, largely increased with increasing concentration of KR-31378 (1, 10 and 100 μM), which was reversed by iberiotoxin (0.3, 1 and 3 μM; Fig. 2A and C). The interactions of KR-31378 and iberiotoxin on the changes in P-CREB levels were similarly manifested. These results were evident in the U87-MG with sPTEN cells (data not shown). The constitutive PTEN, Akt, and CREB levels were little changed throughout in the presence of either TNF-α (50 ng/ml) or KR-31378. TNF-α

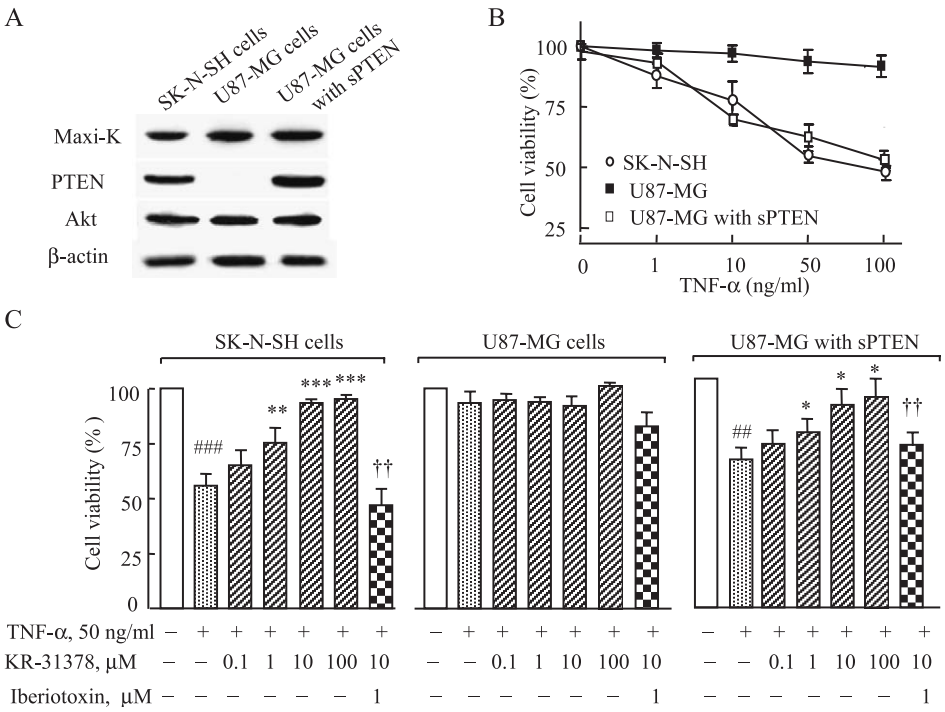

Fig. 1. (A) Representative Western blot of maxi-K channel α subunit, PTEN and Akt protein expression in the SK–N–SH, wild-type U87-MG and U87-MG cells transfected with expression vectors for sPTEN (sPTEN). (B) Cell viability in the SK–N–SH (○), wild-type U87-MG cells (■) and U87-MG cells with sPTEN (□). Concentration-dependent decreases in cell viability in response to TNF-α (approximately 1–100 ng/ml) were observed in the SK–N–SH cells and U87-MG cells with sPTEN (two-way repeated-measures analysis of variance, $P<0.001$), but not in the wild-type U87-MG cells. (C) KR-31378 (approximately 1–100 μM) significantly restored TNF-α (50 ng/ml)-induced reduced viability in the SK–N–SH, and U87-MG cells with sPTEN, but not in the wild-type U87-MG cells. Each control was normalized to 100%. Values are means±S.E.M. of three different preparations with four experiments. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. the absence of TNF-α; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. TNF-α alone; $^{\dagger\dagger}P<0.01$ vs. TNF-α plus 10 μM KR-31378.

receptor expression in the SK–N–SH cells was barely altered by TNF-α, KR-31378 or iberiotoxin.

3.3. Phosphorylation of CK2 and PTEN

P-CK2 of SK–N–SH cells concentration-dependently decreased in response to TNF-α (approximately 1–100 ng/ml), whereas constitutive CK2 protein levels were barely changed. TNF-α (50 ng/ml)-induced decreased P-CK2 (0.23±0.09-fold) and elevated P-PTEN (5.12±0.49-fold) were concentration-dependently reversed by KR-31378 (1, 10 and 100 μM), respectively. The effects of KR-31378 were significantly antagonized by iberiotoxin (1 μM; Fig. 3A).

Both decreased P-CK2/CK2 ratio (0.34±0.12-fold) and increased P-PTEN/PTEN ratio (4.26±0.25-fold) under apigenin treatment (100 μM) were significantly reversed by KR-31378 (approximately 1–100 μM), which were antagonized by iberiotoxin (1 μM; Fig. 3B). Apigenin alone concentration-dependently decreased P-CK2 and P-Akt level and increased the P-PTEN level in the absence of TNF-α (data not shown).

3.4. Experiments with CK2 antisense oligodeoxynucleotide-treated cells

SK–N–SH cells treated with antisense CK2 (α subunit target sequence site 144–163) oligodeoxynucleotide showed no expression of CK2 mRNA, while they expressed the PTEN mRNA intact in reverse transcription–polymerase chain reaction (Fig. 4A). KR-31378 (approximately 1–100 μM) and iberiotoxin (1 μM) did not show any distinct effect on the P-PTEN levels in the SK–N–SH cells treated with antisense CK2α oligodeoxynucleotide. However, the P-PTEN expression was fivefold higher in the presence of TNF-α (Fig. 4C), whereas it remained relatively in low levels in the absence of TNF-α (Fig. 4B).

3.5. Bcl-2 and Bax protein levels

TNF-α (50 ng/ml)-induced decreased Bcl-2 protein and increased Bax protein were concentration-dependently reversed by KR-31378 (approximately 1–100 μM), and both were significantly antagonized by iberiotoxin (approximately 0.3–3 μM; Fig. 5).

3.6. Activation of K^+ current by KR-31378

Addition of KR-31378 (3 μM) significantly increased the outward K^+ current beginning at around 3 min after application, and this effect was recovered to 72.3±8.0% (n=12) of the control level by washout for 20 min. KR-31378 of 3 μM at +60 mV increased the steady-state

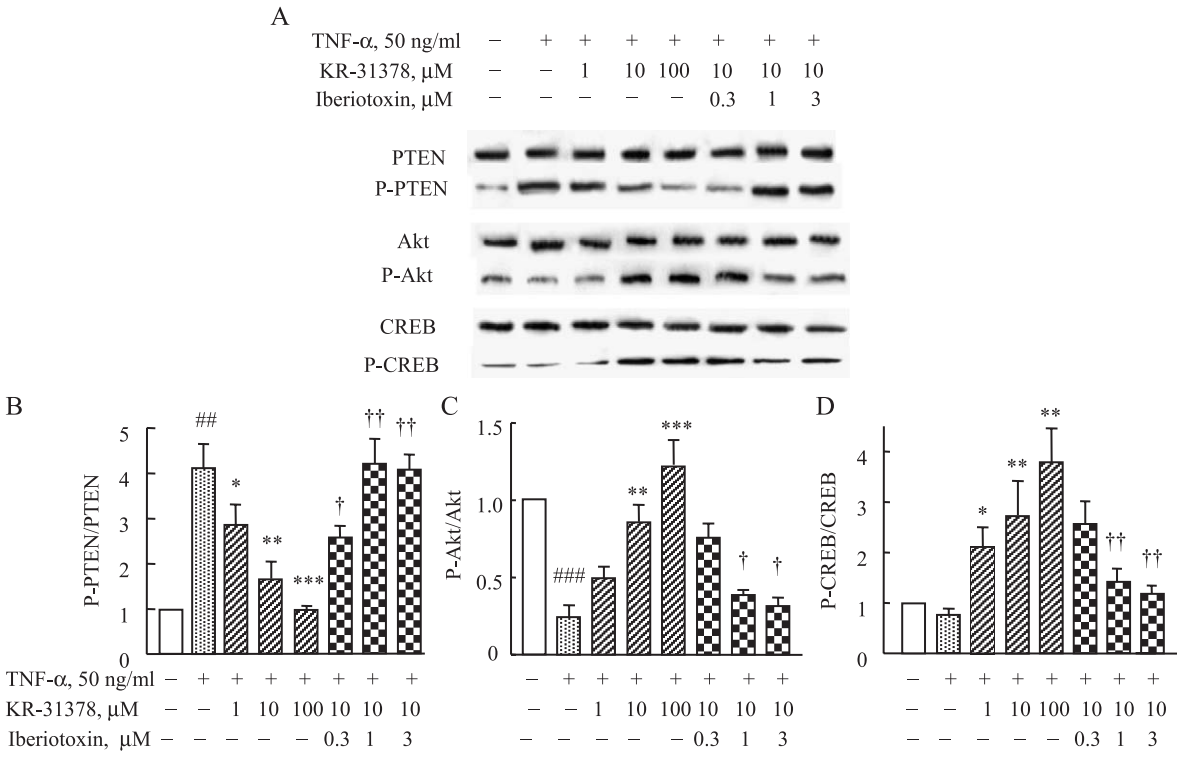

Fig. 2. Effect of KR-31378 on the phosphorylation of PTEN, Akt and CREB. (A) Representative Western blot showing effects of KR-31378 (approximately 1–100 μM) to suppress phosphorylated PTEN (P-PTEN) and to elevate phosphorylated Akt (P-Akt) and phosphorylated CREB (P-CREB) levels, and antagonism by iberiotoxin (approximately 0.3–3 μM) in the SK–N–SH cells. (B–D) Densitometric analyses. Values are means±S.E.M. approximately from 4 to 5 individual experiments. $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ vs. the absence of TNF-α; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ vs. TNF-α alone; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs. TNF-α plus 10 μM KR-31378.

outward K^+ current to 391.5±70.2 (n=8; p<0.01) of the control level (Fig. 6A, B and C). However, in the presence of iberiotoxin (100 nM), a selective large conductance calcium-activated K^+ channel (maxi-K^+) blocker, KR-31378 (3 μM) at +60 mV did not affect the K^+ current (62.6±21.0% of control current; n=6),

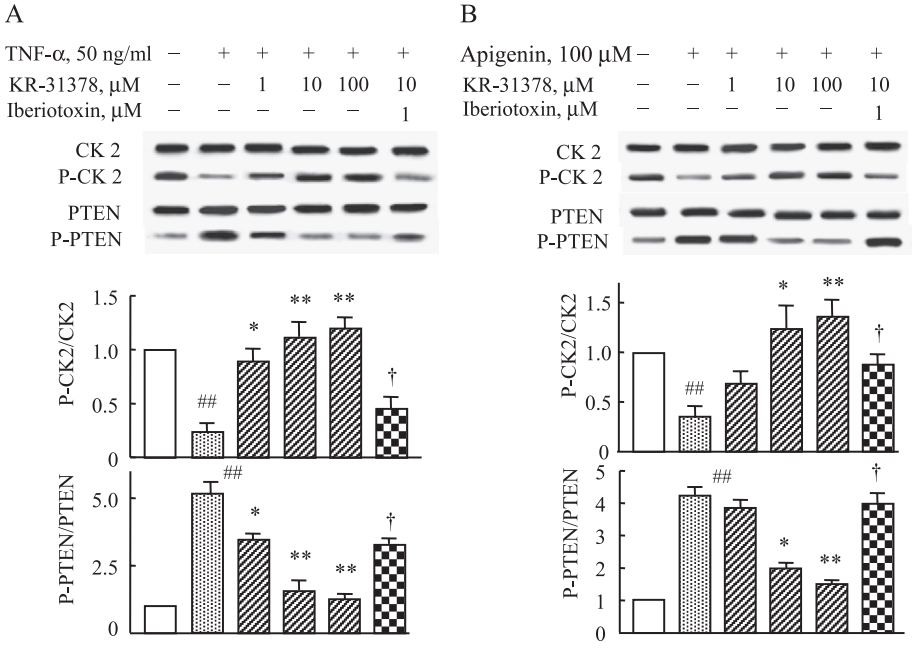

Fig. 3. Western blot assay showing effect of KR-31378 on the phosphorylated CK2 (P-CK2) and phosphorylated PTEN (P-PTEN) levels in the presence of TNF-α (50 ng/ml; A) and apigenin (100 μM; B) in SK–N–SH cells, and the densitometric analyses. Values are means±S.E.M. from 4 individual experiments. $^{\#\#}$ P<0.01 vs. the absence of TNF-α; * P<0.05, ** P<0.01 vs. the presence of TNF-α alone; † P<0.05 vs. TNF-α plus 10 μM KR-31378.

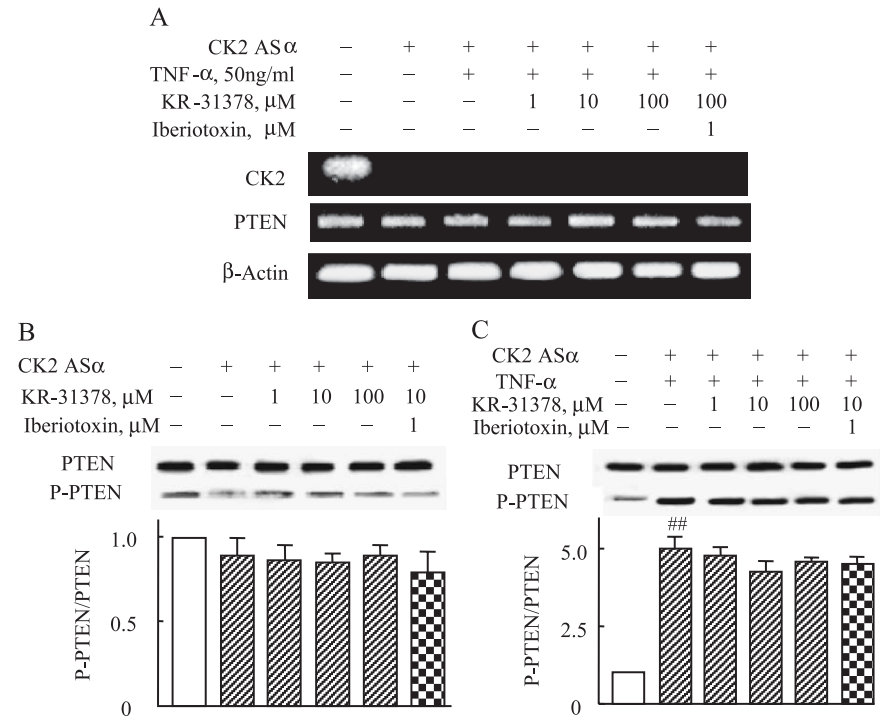

Fig. 4. (A) Representive reverse transcription-polymerase chain reaction showing expression of CK2 and PTEN mRNA (A) and Western Blot showing expression of CK2/P-CK2 and PTEN/P-PTEN protein (B) in the SK-N-SH cells treated with CK2α antisense oligodeoxynucleotide in the presence of TNF-α, KR-31378 and iberiotoxin. Western blot assay and densitometric analyses showing effect of KR-31378 on the phosphorylated PTEN (P-PTEN) levels in the absence (B) and presence of TNF-α (50 ng/ml) (C) in the SK–N–SH cells treated with CK2α antisense oligodeoxynucleotide. Values are means±S.E.M. from four experiments. $^{\#\#}P<0.01$ vs. the absence of TNF-α.

indicating that KR-31378 increases the K^+ current in SK–N–SH cells by activating mainly maxi-K^+ channels. Glibenclamide, a selective ATP-sensitive potassium channel (K^+_{ATP}) blocker, did not affect on the KR-31378 effect (data not shown).

3.7. Suppression of cytosolic Ca^{2+} and increase of mitochondrial membrane potential by KR-31378

Cytosolic Ca^{2+} concentration was significantly and concentration-dependently elevated by TNF-α (approxi-

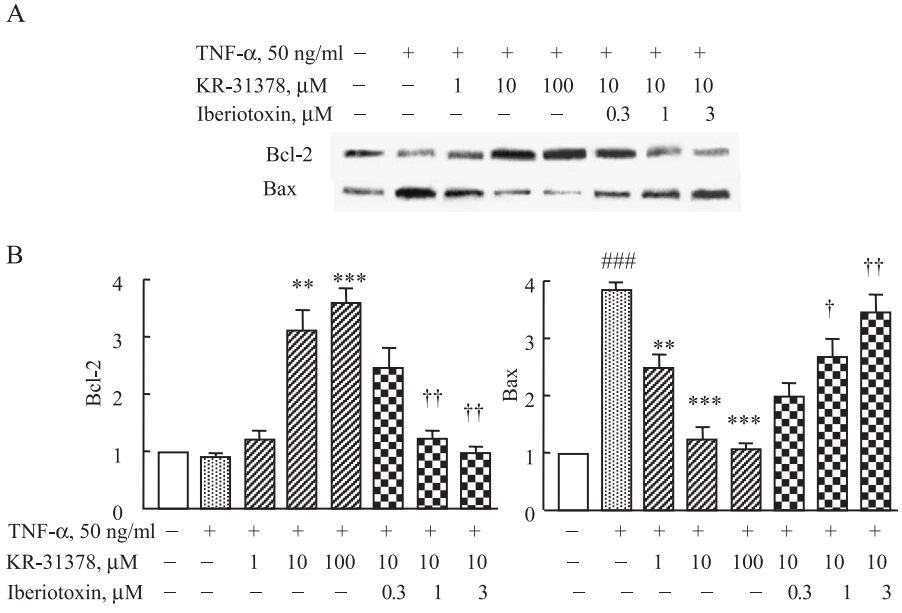

Fig. 5. (A) Effect of KR-31378 on the Bcl-2 and Bax protein expression in the SK–N–SH cells. KR-31378 (approximately 1–100 μM) concentration-dependently increased Bcl-2 and, in contrast, decreased Bax protein levels, both of which were concentration-dependently reversed by iberiotoxin (0.3, 1 and 3 μM). (B) The densitometric analyses. Values are means±S.E.M. from four to five experiments. $^{\#\#\#}P<0.001$ vs. the absence of TNF-α; $^{**}P<0.01$, $^{***}P<0.001$ vs. the presence of TNF-α alone; $^{\dagger}P<0.05$, $^{\dagger\dagger}P<0.01$ vs. TNF-α plus 10 μM KR-31378.

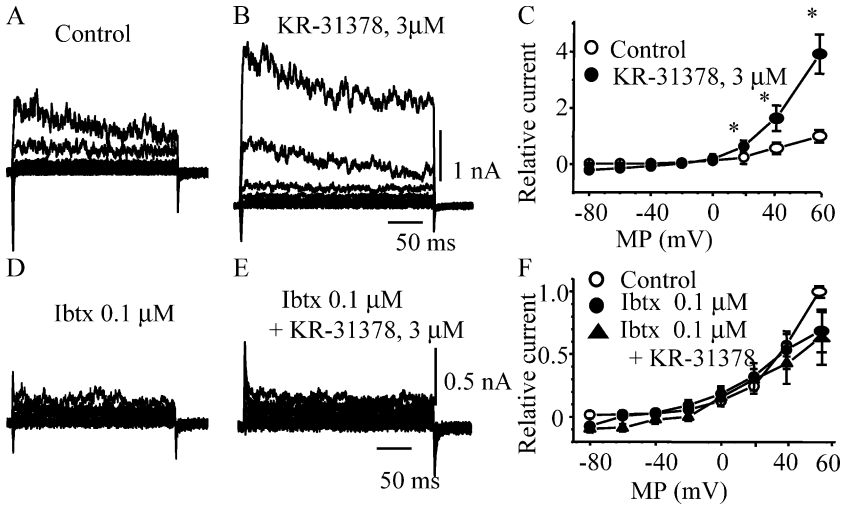

Fig. 6. Activation of the outward K^+ current by KR-31378 in SK–N–SH cells. Representative current tracings evoked by depolarizing pulses in the absence (A) or presence (B) of KR-31378 (3 μM). (C) Averaged current–voltage plots of steady-state currents in the absence or presence of KR-31378 (3 μM). (D and E) Tracings showing the effect of iberiotoxin (Ibtx, 100 nM) alone and Ibtx plus KR-31378 (3 μM) on the K^+ current. (F) Current–voltage plot of steady-state currents in the presence of Ibtx and Ibtx plus KR-31378. Each point with vertical bar denotes means±S.E.M. from six to eight cells. *P<0.05 vs. each control.

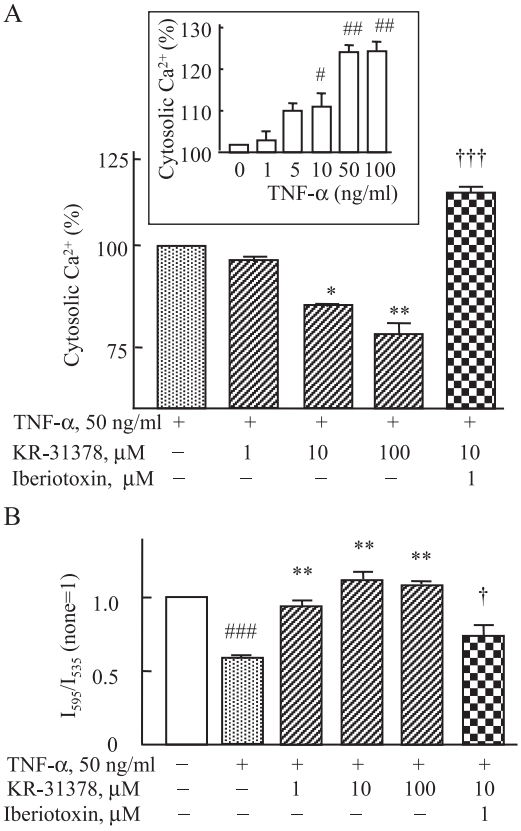

Fig. 7. (A) Effect of KR-31378 on the changes in cytosolic Ca^{2+} in SK–N–SH cells. Cells were treated with KR-31378 and iberiotoxin from 3 h before and during incubation in TNF-α solution. Inset: TNF-α-induced (approximately 1–100 ng/ml) increases in cytosolic Ca^{2+}. (B) Effect of KR-31378 on the changes in the mitochondrial membrane potential in SK–N–SH cells. Values are means±S.E.M. from four different preparations with triplicate experiments. None was normalized to 100%. $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 vs. None; *P<0.05, **P<0.01 vs. TNF-α alone; $^{\dagger}P$<0.05, $^{\dagger\dagger\dagger}P$<0.001 vs. TNF-α plus 10 μM KR-31378.

mately 1–100 ng/ml). Increased cytosolic Ca^{2+} (24±3%) induced by TNF-α (50 ng/ml) was concentration-dependently decreased under treatment with KR-31378 (approx-

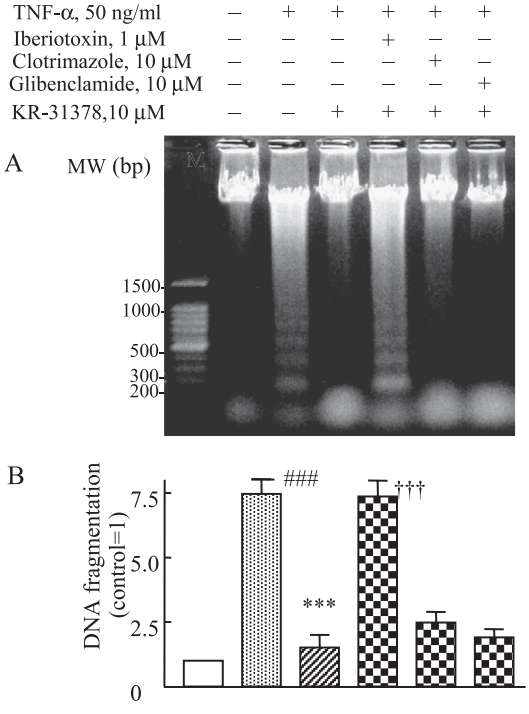

Fig. 8. (A) Preventive effect of KR-31378 on the DNA fragmentation. Representative agarose gel electrophoresis showing DNA laddering after exposure of SK–N–SH cells to 50 ng/ml TNF-α. KR-31378 (10 μM) suppressed DNA fragmentation, which was reversed by iberiotoxin (1 μM), but not by clotrimazole (10 μM) and glibenclamide (10 μM). (B) The densitometric analyses. M represents the 100 bp (base pair) DNA ladder markers. Each was confirmed with four different preparations. Values are means±S.E.M. from four different experiments. None was normalized to 1. $^{\#\#\#}P$<0.001 vs. None; ***P<0.001 vs. TNF-α alone; $^{\dagger\dagger\dagger}P$<0.001 vs. TNF-α plus 10 μM KR-31378.

imately 1–100 μM), which was antagonized by 1 μM of iberiotoxin (Fig. 7A). Moreover, decreased mitochondrial membrane potential (0.58±0.02-fold) under application of TNF-α (50 ng/ml, for 24 h) was in turn elevated with increasing concentration of KR-31378, which was antagonized by iberiotoxin (1 μM).

3.8. Anti-apoptotic effect of KR-31378

Augmented DNA fragmentation under application of TNF-α (50 ng/ml) was suppressed by treatment with KR-31378 (3 μM) in the SK–N–SH cells (Fig. 8). The degree of DNA fragmentation induced by KR-31378 was reversed by pretreatment with iberiotoxin (1 μM), but not by clotrimazole (10 μM), an intermediate conductance of Ca^{2+}-activated K^+ channel blocker, and glibenclamide (10 μM), a K_{ATP} channel blocker, indicating that KR-31378 inhibited TNF-α-induced DNA fragmentation in SK–N–SH cells by activating the iberiotoxin-inhibitable maxi-K channels.

4. Discussion

In the present study, elevation of phosphorylated CK2 by KR-31378 was accompanied by decreased P-PTEN levels in association with increased P-Akt and P-CREB levels, and all these effects of KR-31378 were well antagonized by iberiotoxin, a maxi-K channel blocker. These findings have highlighted the hypothesis that the maxi-K channel opening-linked increase in P-CK2 and that suppression of P-PTEN lead to activation of Akt/CREB/Bcl-2 and repression of Bax production, thereby preventing the neuronal cell death.

In the present results, the viability of SK–N–SH cells and U87MG cells transfected with expression vectors for sense sPTEN to TNF-α was significantly reduced in response to TNF-α, whereas the wild-type U87-MG cells showed a high resistance to TNF-α. U87-MG cells transfected with expression vectors for sense PTEN showed increased PTEN protein expression with high P-PTEN levels in response to TNF-α (Myers et al., 1997; Haas-Kogan et al., 1998), suggesting that P-PTEN is closely related with TNF-α-induced cell death.

Accumulating evidence have shown that PTEN negatively regulates the phosphatidylinositol 3-kinase pathway by dephosphorylation of the $PI(3,4,5)P_3$ (Maehama and Dixon, 1998; Stambolic et al., 1998). Some reports demonstrated that most cellular PTEN is constitutively phosphorylated, and phosphorylation sites are located in a cluster of the C-terminal tail of PTEN (Vazquez et al., 2000; Torres and Pulido, 2001). In our results, the findings showing that TNF-α-induced increases in P-PTEN and decreases in P-Akt/P-CREB levels were significantly and concentration-dependently reversed by KR-31378, and these variables were inhibited by iberiotoxin, a maxi-K channel blocker, indicating that KR-31378 has a potential in regulation of PTEN/Akt/CREB phosphorylation as a maxi-K channel opener. When effects of KR-31378 and NS-1619, a maxi-K channel opener (Olesen et al., 1994) were examined on the vascular endothelial growth $factor_{165}$-stimulated expression of phosphorylated phosphatidylinositol 3-kinase protein, both agents showed no influence on the phosphorylated phosphatidylinositol 3-kinase levels. Thus, it is suggested that KR-31378 acts on the downstream of phosphatidylinositol 3-kinase (data not shown).

Protein kinase CK2 is a highly conserved, ubiquitously expressed Ser–Thr kinase that phosphorylates a wide variety of substrates involved in essential cell processes including cell cycle and growth (Pinna, 1990; Allende and Allende, 1995). It has been reported that CK2 mediates phosphorylation of PTEN as a physiologically relevant PTEN kinase (Vazquez et al., 2000; Torres and Pulido, 2001; Miller et al., 2002). When cells were treated with either TNF-α or apigenin (CK2-selective inhibitor) to assess the functional relevance of P-CK2 to P-PTEN, P-CK2 level was significantly suppressed, and the suppressed P-CK2 was elevated by KR-31378, and the increased P-PTEN was in contrast suppressed by KR-31378. These KR-31378 effects were antagonized by iberiotoxin, suggesting that P-CK2 and P-Akt/P-CREB levels showed inverse correlation with the changes in P-PTEN level in SK–N–SH cells.

A question arises as to whether P-CK2 modulates the change in P-PTEN. To explain this query, we employed the SK–N–SH cells treated with antisense CK2 (α subunit target sequence site 144–163) oligodeoxynucleotide. Both KR-31378 (approximately 1–100 μM) and iberiotoxin (1 μM) could not exert any effect on the P-PTEN levels in the SK–N–SH cells treated with antisense CK2α oligodeoxynucleotide regardless of the absence and presence of TNF-α, suggesting that the phosphorylated CK2 has a pivotal role in the regulation of P-PTEN.

Evidence has accumulated that enhanced CREB activity by Akt signaling leads to increased Bcl-2 promoter activity and upregulation of Bcl-2 expression (Du and Montminy, 1998; Pugazhenthi et al., 1999). A decrease in Bcl-2 and increase in Bax protein were identified in neurons within ischemic cortex and thalamus (Gillardon et al., 1996). It was also documented that overexpression of Bcl-2 in transgenic mice protects neurons from ischemia-induced cell death (Martinou, 1999). The findings that decreased Bcl-2 and elevated Bax protein expression under application of TNF-α were fully reversed by KR-31378 indicate that increased cell survival under KR-31378 coincided with the ability of cells to maintain high levels of Bcl-2 protein and low Bax protein level.

Maxi-K channels are known to be activated by depolarization and by an increase in intracellular Ca^{2+}. Increase in K^+ currents through maxi-K channels hyperpolarizes the cell membrane (Latorre et al., 1989; Gribkoff et al., 2001). Cytoplasmic K^+ levels in excitable and nonexcitable cells play an important role in maintaining intracellular ion homeostasis (Bortner et al., 1997) and in inhibiting apoptotic enzymes in the cytosol and nucleus (Hughes et al., 1997). Activation of maxi-K channels was reported to

protect neurons against glutamate release and excitotoxicity and to reduce the pathological consequences of ischemia (Lawson, 2000). Activation of this channel in the ischemic cells was demonstrated to block the Ca^{2+} entry and to minimize the neuronal depolarization (Gribkoff et al., 2001). Zhu et al. (1999) suggested the role of mitochondrial Ca^{2+} homeostasis for cell survival, in that decrease of mitochondrial Ca^{2+} preceded activation of caspase cascade and DNA fragmentation, and in contrast, overexpression of Bcl-2 protein led to increase in mitochondrial Ca^{2+} load and increased mitochondrial membrane potential with inhibition of apoptosis. Considering that Bcl-2 is to prevent generation of reactive oxygen species and reduction in mitochondrial membrane potential induced by TNF-α (Gottlieb et al., 2000), the ability of cells to maintain high levels of Bcl-2 protein and low Bax protein under KR-31378 may also have a potential to modulate the mitochondria Ca^{2+} homeostasis for cell survival. Nevertheless, it remains unexplained how the maxi-K channel opening by KR-31378 could increase the phosphorylation of CK2 protein kinase.

KR-31378 strongly suppressed TNF-α-induced DNA fragmentation in SK–N–SH cells. Interestingly, the reduction in DNA fragmentation by KR-31378 was wholly reversed by pretreatment with iberiotoxin, a maxi-K channel blocker, but not by clotrimazole, an inhibitor of intermediate conductance of Ca^{2+}-activated K^+ channel blocker (Ishii et al., 1997) and glibenclamide, a K_{ATP} channel blocker (Schmid-Antomarchi et al., 1987). These facts indicate the maxi-K channel opening being the specific action site of KR-31378 to suppress the DNA fragmentation.

Taken together, it is suggested that maxi-K channel opening by KR-31378 is coupled to the increased P-CK2 and decreased P-PTEN, thereby consequently up-regulating the P-Akt/P-CREB with increased Bcl-2 protein and reduced Bax protein, and thereby improving cell viability.

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