Suppression of CLIC4/mtCLIC enhances hydrogen peroxide-induced apoptosis in C6 glioma cells

YE XU[1,2*], JINSONG KANG[1*], ZHAOXIN YUAN[1,3], HONGYAN LI[1], JING SU[1], YANG LI[1], XIAOXIA KONG[1], HONGYU ZHANG[1], WEIWEI WANG[1] and LIANKUN SUN[1]

[1]Department of Pathophysiology, Norman Bethune College of Medicine, Jilin University, Changchun 130021; [2]Medical Research Laboratory, Jilin Medical College, Jilin 132013; [3]Changchun Medical College, Changchun 130013, P.R. China

Received August 24, 2012; Accepted November 2, 2012

DOI: 10.3892/or.2013.2265

Abstract. CLIC4/mtCLIC (referred to here as CLIC4) is one of the seven-member family of chloride intracellular channels (CLIC). CLIC4 localizes to the mitochondria, nucleus, cytoplasm and other organellular compartments and participates in the apoptotic response to stress. However, the role of CLIC4 in oxidative stress and apoptosis is not well understood. In this study, we showed the important role of CLIC4 in apoptosis of C6 glioma cells induced by hydrogen peroxide (H_2O_2). Our results showed that CLIC4 protein expression was upregulated following H_2O_2-induced C6 cell apoptosis. The upregulation of CLIC4 protein expression was paralleled with an increased Bax/Bcl-2 ratio, cytochrome *c* and cleaved caspase-3 protein expression upon H_2O_2-induced C6 cell apoptosis. Suppression of CLIC4 expression by RNA interference enhanced cell apoptosis, but the ratio of Bax/Bcl-2 was not involved in this process. Dissipation of mitochondrial membrane potential and nuclear translocation of CLIC4 were involved in the activation of apoptosis induced by H_2O_2. Our data indicate that CLIC4 protein may be a key element in the apoptotic response to oxidative stress.

Introduction

Oxidative stress is thought to play a critical role in various pathological conditions such as stroke, Parkinson's disease, Alzheimer's disease, AIDS and cancer, and has been demonstrated to be related with apoptosis. Hydrogen peroxide (H_2O_2), which is a type of reactive oxygen species (ROS), is often used to induce apoptosis and cell injury (1,2). One important pathway of H_2O_2-induced apoptosis appears to involve the mitochondrial pathway (3,4), while the ion channel protein was found to be involved in the regulation of apoptosis following intensive research of the mitochondrial pathway (5,6).

Recently, members of the chloride intracellular channel (CLIC) protein family have been implicated in apoptosis. CLIC4 is a 28.6-kDa protein corresponding to a cDNA cloned from rat brain and also known in the mouse as mtCLIC. CLIC is a novel p64-related protein family with seven members (p64, CLIC1-5 and parchorin) that are localized in various cellular compartments and expressed in multiple tissue types (7,8). Unlike most membrane channels, CLIC proteins are present in cells both as an integral membrane protein form and as soluble protein forms, suggesting that CLICs may have non-channel functions (9,10). CLIC4 is a newly identified effector of apoptosis which is capable of altering mitochondrial function, leading to caspase activation and cell death (11-13). However, reduction of CLIC4 proteins by antisense expression enhances TNFα-mediated apoptosis independent of p53 (14). It was also reported that CLIC4 translocates to the nucleus in cells following induction of apoptosis by a variety of stress inducers, and nuclear-targeted CLIC4 is strongly pro-apoptotic even when the mitochondrial apoptotic pathway is inhibited by the genetic deletion of Apaf1 (15). These data imply that the role of CLIC4 may vary depending on cell type, subcellular localization and intensity of stress. Based on these research findings we hypothesed that CLIC4 may play an important role in the apoptotic response in H_2O_2-induced C6 cell death.

As there are no specific inhibitors for chloride channels available at the present time, we used the DNA vector-based RNAi approach for establishing a long-term siRNA strategy to block CLIC4 expression in glioma C6 cells. We found that H_2O_2 induced the mitochondrial apoptosis pathway and increased expression of CLIC4 in glioma C6 cells. To further determine whether CLIC4 is associated with the mitochondrial pathway that causes H_2O_2-induced C6 cell death, MMP and expression levels of Bcl-2, Bax, cleaved caspase-3 were also examined after treatment with different concentrations of H_2O_2 in mock and siRNA groups. Western blot assay showed that the modulating effect of CLIC4 during H_2O_2-induced glioma cell injury was not correlated with the Bcl-2 family.

Correspondence to: Dr Liankun Sun, Department of Pathophysiology, Norman Bethune College of Medicine, Jilin University, 126 Xinmin Street, Changchun 130021, P.R. China
E-mail: sunlk@jlu.edu.cn

*Contributed equally

Key words: hydrogen peroxide, glioma cells, apoptosis, chloride intracellular channel, RNA interference

We also found that reduction in expression of CLIC4 increased its translocation in C6 cells treated with H_2O_2.

Materials and methods

Construction of siRNA expression vectors. pSilencer™ neo3.1-H1 siRNA and pSilencer™ neo3.1-H1-scramble siRNA (Ambion Inc., Austin, TX, USA) were used for DNA vector-based siRNA synthesis under the control of the H1 promoter *in vitro*. Three CLIC4 mRNA-targeted hairpin siRNAs were designed from different positions of the rat glioma cell mtCLIC sequence (GenBank™ accession number EF397567). The specific siRNA sequences for mtCLIC are shown in Table I. These oligonucleotides contain a sense strand of 19 or 20 nucleotides followed by a short spacer (TTCAAGAGA), the antisense strand, and five terminators (Biotechnic, Inc., Shanghai, China). A scrambled siRNA (Ambion) which did not match any rat sequences in a BLAST search was used as a negative control. After annealing, the double-stranded complementary oligonucleotides were cloned into the *Bam*HI and *Hin*dIII sites of the pSilencer 3.1-H1 plasmids, followed by amplification in *E. coli* and DNA sequencing.

Cell culture and transfection. The rat glioma C6 cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (CACAS) (Shanghai, China) and cultured in monolayers in plastic flasks in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 kU/l penicillin and 100 mg/l streptomycin) in a 95% air, 5% CO_2 incubator at 37˚C. For cell transfection, Lipofectamine 2000 (Invitrogen) was used for transfecting the plasmids following the manufacturer's instructions. The resulting recombinant pSilencer 3.1-H1 constructs were then introduced into the C6 cells, and the expression of CLIC4 mRNA and protein was examined by RT-PCR and western blotting, respectively. Cells were cultured for another 24 h after transfection and used for experiments.

Reverse transcription-PCR. Total cellular RNA was prepared with TRIzol (Invitrogen) and was reverse-transcribed with Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega) according to the manufacturer's instructions. The PCR reaction was performed following standard procedures. The primers used for PCR amplification are shown in Table II. Typical PCR parameters were: 94˚C for 3 min, followed by 30 cycles of 94˚C for 30 sec, 55-60˚C for 30 sec, 72˚C for 45 sec, followed by a final extension step at 72˚C for 10 min. After amplification, the products were resolved by electrophoresis on 1.0% agarose gel, stained with ethidium bromide and photographed under ultraviolet light. The amplification of the PCR products was monitored to assure that the PCR was within the range of linearity.

Western blotting. Anti-C terminus CLIC4 antibodies were generated as previously described (14) and were kindly provided by Professor Stuart H. Yuspa of the National Institutes of Health, Bethesda, MD. The affinity-purified anti-C terminus CLIC4 antibodies are mono-specific for CLIC4 and were used in all experiments, including immunocytochemical and western blot analysis. Bcl-2 (sc-492), Bax (sc-7480), cleaved caspase-3 (sc-22171), cytochrome *c* (sc-13156), β-actin (sc-47778), and lamin A/C (sc-6215) antibodies were purchased from Santa Cruz Biotechnology. SDS-PAGE and western blotting were performed using standard techniques (16). Briefly, cells were harvested 72 h after transfection and lysed on ice in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1% Na_3VO_4, 0.1% SDS and 10 μg/ml each of aprotinin, pepstatin and leupeptin (Sigma). Following centrifugation at 12,000 x g for 5 min, equivalent amounts of proteins (30 μg) were subjected to SDS-PAGE using 10-12% polyacrylamide gels and transferred onto nitrocellulose membranes (Whattman). The membranes were blocked with 5% bovine serum albumin (BSA) in PBS for 1 h and then incubated overnight at 4˚C with primary antibodies against CLIC4, Bcl-2, Bax, cleaved caspase-3 and β-actin. After extensive washing, a horseradish peroxidase-coupled anti-rabbit (or anti-mouse) antibody (Pierce) diluted in PBS containing 0.1% Tween-20 was added for 1 h. Protein loading was analyzed by the immunodetection of β-actin antibody and binding was detected by DAB stain (Sigma).

Cell viability assay. The viability of the C6 cells was determined by MTT assays (17). Briefly, C6 cells were seeded in 96-well plates at a density of $5x10^3$ cells/well and incubated. The cells were then exposed to medium containing H_2O_2 in different concentrations (0, 62.5, 125, 250, 500, 750 and 1,000 μM), or treated with 250, 500 and 750 μM H_2O_2 and/or transfected with the pSH1Si-scrambled plasmid or pSH1Si-CLIC4 plasmid-2. Each treatment was repeated in 5 wells. The cells were incubated for 20 h in a 95% air, 5% CO_2 incubator at 37˚C. Then 10 μl of MTT reagent (5 mg/ml in PBS; Sigma, USA) was added to each well and incubated for another 4 h. The supernatants were discarded from the wells, and 150 μl of DMSO was added and mixed thoroughly to dissolve the dark blue crystals of formazan. Absorbance was recorded at a wavelength of 570 nm. Results are expressed as the percentage of MTT reduction, assuming that the absorbance of the control cells was 100%.

Immunocytochemistry. The C6 cells were plated on coverslips and incubated overnight, transfected with pSilencer neo3.1-H1-CLIC4 siRNA or pSilencer neo3.1-H1-scrambled siRNA or pSilencer empty vector and then treated with 250, 500 and 750 μM H_2O_2 for 24 h. The cells were then fixed in 4% paraformaldehyde/PBS for 30 min and extensively washed with PBS. Fixed cells were permeabilized with 1% Triton X-100 for 10 min, washed with PBS, the endogenous peroxidase activity was quenched after incubation in methanol containing 3% hydrogen peroxide for 10 min, and then blocked for 1 h in 5% bovine serum albumin/PBS. The cells were then incubated simultaneously with anti-C terminus CLIC4 antibodies at room temperature. As a negative control, rabbit immunoglobulins were used to replace the primary antibody. Goat anti-rabbit IgG conjugated with horseradish peroxidase was used as a second antibody. Immunohistochemical staining was carried out manually at room temperature, using an avidin-biotin-peroxidase complex method. The criteria for immunohistochemical assay results are as follows: cells exhibiting brown particle staining in the nucleus or cytoplasm were considered positive.

Table I. siRNA primer sequences for CLIC4.

Gene	Primers	5'→3' sequence
CLIC4-siRNA-1	Forward	GATCCGCCTTCAACAGCGAAGTCAATTCAAGAGATTGACTTCGCTGTTGAAGGTTTTTTGGAAA
	Reverse	AGCTTTTCCAAAAAACCTTCAACAGCGAAGTCAATCTCTTGAATTGACTTCGCTGTTGAAGGCG
CLIC4-siRNA-2	Forward	GATCCGAACAGCATGGAGGACATCTTCAAGAGAGATGTCCTCCATGCTGTTCTTTTTTGGAAA
	Reverse	AGCTTTTCCAAAAAAGAACAGCATGGAGGACATCTCTCTTGAAGATGTCCTCCATGCTGTTCG
CLIC4-siRNA-3	Forward	GATCCGGCTCAAGACCAGAGGCTAATTTCAAGAGAATTAGCCTCTGGTCTTGAGTTTTTTGGAAA
	Reverse	AGCTTTTCCAAAAAACTCAAGACCAGAGGCTAATTCTCTTGAAATTAGCCTCTGGTCTTGAGCCG

Table II. Primer sequences and conditions used for semi-quantitative RT-PCR analysis.

Gene	Primers	5'→3' sequence	Amplicon size (bp)	Annealing temperature (˚C)
CLIC4	Forward	AGCAGAAGCAGCAGCAG	762	57
	Reverse	ATACCTTGTCTATCCTTGATCCTA		
Bcl-2	Forward	AACACCAGAATCAAGTGTTCG	447	58
	Reverse	TCAGGTGGACCACAGGTGGC		
Bax	Forward	AGGGTTTCATCCAGGATCGAGC	468	58
	Reverse	AGGCGGTGAGGACTCCAGCC		
GAPDH	Forward	GGGTGATGCTGGTGCTGAGTATGT	617	58
	Reverse	AAGAATGGGTGTTGCTGTTGAAGTC		

Nuclear protein extraction. Nuclear extracts were prepared as previously described with some modifications (17). Briefly, ~10^7 cells were homogenized in 4 ml of cold solution A (0.6% NP-40, 150 mM NaCl, 10 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM PMSF) using a homogenizer for 10 strokes. The cell suspension was incubated on ice for 5 min, then centrifuged at 4˚C for 10 min at 3,000 x g. The supernatant was collected and stored at -70˚C for western blot analysis of cytosolic proteins. The pellets were resuspended in 200 μl cold solution B (25% glycerol, 420 mM NaCl, 20 mM HEPES, pH 7.9, 1.2 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 5 mg/ml pepstain, 5 mg/ml leupetin, 5 mg/ml aprotinin), incubated for 30 min on ice and centrifuged at 4˚C for 30 min at 20,000 x g. The supernatants containing nuclear proteins were collected, aliquoted and stored at -70˚C. The protein concentration was determined using the Bio-Rad protein reagent.

Measurement of apoptosis by flow cytometry. C6 cells were transfected with pSH1Si-scramble plasmid or pSH1Si-CLIC4 plasmid-2 and then treated with 250, 500 and 750 μM H_2O_2 for 24 h. For quantification of apoptosis, culture supernatants were collected and washed two times with PBS and fixed with 70% ethanol, centrifuged and adjusted to a concentration of 1x10^6 cells/ml. Incubation with 50 μg/ml propidium iodide (Sigma, USA) was carried out in the dark at 4˚C for 30 min. In the DNA histogram, the amplitude of the sub-G1 DNA peak represents the number of apoptotic cells.

Mitochondrial membrane potential measurement. The mitochondrial membrane potential (MMP) was measured using the fluorescent probe Rhodamine 123 as previously described (18,19). C6 cells transfected with pSH1Si-scramble plasmid or pSH1Si-CLIC4 plasmid-2 were then treated with 250, 500 and 750 μM H_2O_2 for 24 h, respectively, followed by incubation with 10 μM Rhodamine 123. The samples were then analyzed by a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). Ten thousand cells/sample were analyzed and the mean fluorescence intensity (MFI) in the positive cells was taken as an index of the level of MMP.

Statistical analysis. All experiments were repeated at least three times with different batches of cell preparations. Data are represented as the means ± SD. Statistical analysis was carried out with the SPSS 11.0 program using ANOVA. Results with values of $P<0.05$ were considered to be statistically significant.

Results

CLIC4 protein expression is upregulated during H_2O_2-induced C6 cell injury. C6 cells treated with different concentrations of H_2O_2 for 24 h exhibited attenuated cell viability in a dose-dependent manner (Fig. 1). Following treatment with 250, 500 and 750 μM H_2O_2, the cell viability was 82.8±4.78, 65.68±4.22 and 52.83±3.63%, respectively. H_2O_2 also induced upregulation of CLIC4 protein expression in a dose-dependent manner (Fig. 2), suggesting that CLIC4 was involved in the H_2O_2-induced C6 cell injury process.

H_2O_2 induces C6 cell apoptosis through the mitochondrial pathway. Several genes related to apoptosis were also

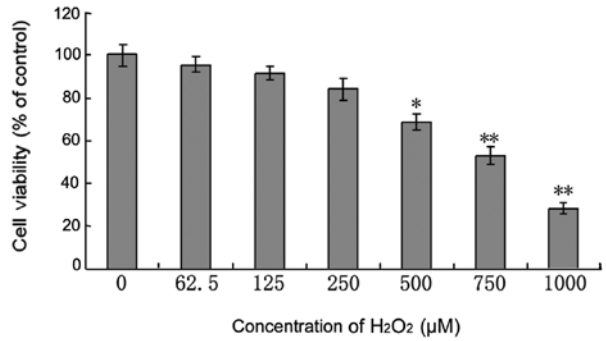

Figure 1. Effect of H_2O_2 on cell viability of C6 cells. C6 cells were seeded in 96-well plates at a density of $5x10^3$ cells/well, incubated overnight and then exposed to 0, 62.5, 125, 250, 500, 750 and 1,000 μM H_2O_2 for 24 h. The viability of the C6 cells was determined by MTT assay. Values are the means ± SD (n=3). *P<0.05, **P<0.01 vs. the control group.

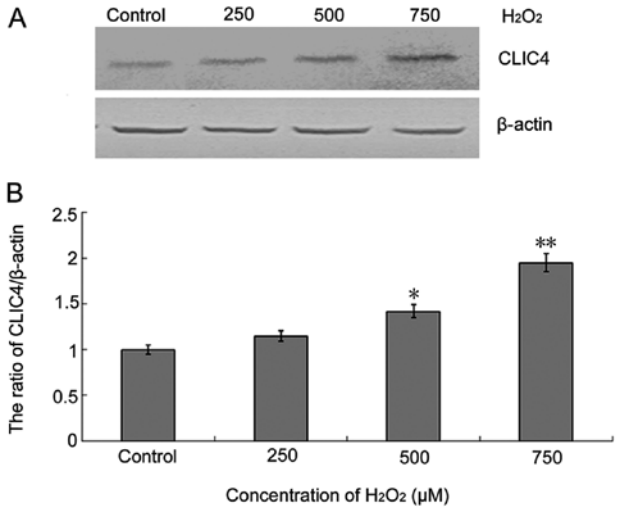

Figure 2. CLIC4 protein expression is upregulated during H_2O_2-induced C6 cell injury. (A) C6 cells were treated with H_2O_2 (0, 250, 500 and 750 μM) for 24 h, and the levels of CLIC4 protein were assessed by western blot analysis. (B) Densitometry was applied to quantify CLIC4 density in western blot analysis. The relative CLIC4 protein level was normalized with reference to the β-actin protein, and the normalized signal of control was expressed as 1.0. Values are the means ± SD (n=3), *P<0.05, **P<0.01 vs. the control group.

examined during H_2O_2-induced C6 cell injury. Western blot analysis demonstrated that Bax, cytosol cytochrome *c* and cleaved caspase-3 protein expression was upregulated, whereas Bcl-2 protein expression was downregulated in a dose-dependent manner (Fig. 3A). The ratio of Bax/Bcl-2 was therefore increased (Fig. 3B). These results indicated that mitochondrial apoptosis pathways were involved in H_2O_2-induced C6 cell apoptosis.

CLIC4-RNAi promotes apoptosis during H_2O_2-induced C6 cell injury. To evaluate the function of CLIC4 in H_2O_2-induced C6 cell injury, C6 cells were transfected with three different pSH1Si-CLIC4 plasmids. The transfection efficiency was estimated by RT-PCR and western blot analysis. Compared with the mock group or pSH1Si-scramble plasmid group, the CLIC4 mRNA and protein expression in the pSH1Si-CLIC4 plasmid-2 group was significantly decreased (Fig. 4). Therefore, the pSH1Si-CLIC4 plasmid-2 was chosen

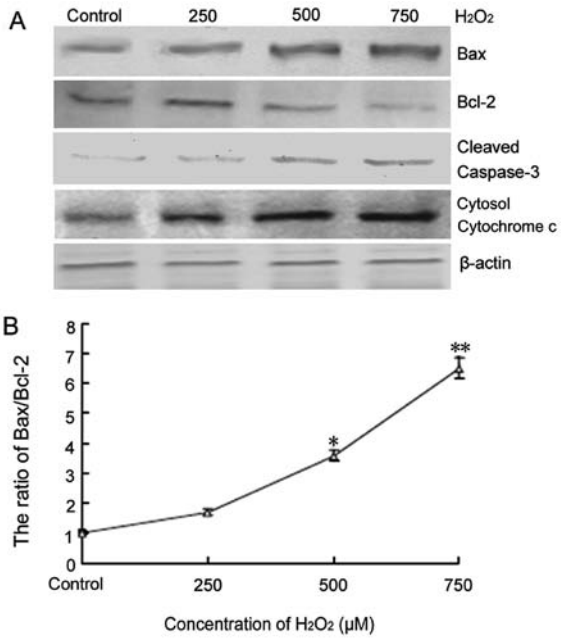

Figure 3. The mitochondrial apoptosis pathway is involved in H_2O_2-induced C6 cell injury. (A) C6 cells were exposed to 0, 250, 500 and 750 μM H_2O_2 for 24 h, and the levels of Bax, Bcl-2, cytochrome *c* and cleaved caspase-3 protein were assessed by western blot analysis. (B) The ratio of normalized Bax to Bcl-2. Densitometry was applied to quantify the Bax and Bcl-2 density in western blot analysis. The Bax and Bcl-2 protein levels were normalized to β-actin protein, and the normalized signal of control was expressed as 1.0. The ratio was caculated and plotted against various concentrations of H_2O_2. Values are the means ± SD (n=3), *P<0.05, **P<0.01 vs. the control group.

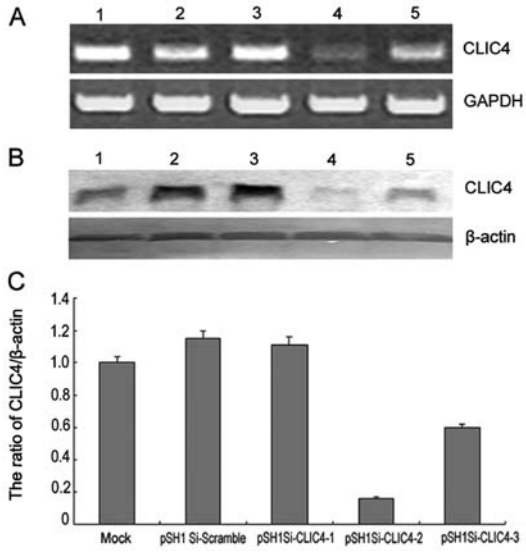

Figure 4. Small interfering RNA constructs specifically reduce CLIC4 mRNA and protein expression in C6 cells. The DNA fragments encoding CLIC4-targeted siRNA (pSH1Si-CLIC4 plasmid-1, pSH1Si-CLIC4 plasmid-2, pSH1Si-CLIC4 plasmid-3) were subcloned into the pSilencer 3.1-H1 plasmids and were confirmed by restrictive enzyme digestion and DNA sequencing. The plasmids were then transfected using Lipofectamine 2000. The transfection efficiency was estimated by RT-PCR and western blot analysis. (A) The CLIC4 mRNA expression in C6 cells transfected with the pSH1Si-CLIC4 plasmids. (B) CLIC4 protein expression in C6 cells transfected with the pSH1Si-CLIC4 plasmids. Lane 1, mock group; lane 2, pSH1Si-scramble plasmid; lane 3, pSH1Si-CLIC4 plasmid-1; lane 4, pSH1Si-CLIC4 plasmid-2; lane 5, pSH1Si-CLIC4 plasmid-3. (C) Densitometry was applied to quantify CLIC4 density in western blot analysis. The relative CLIC4 protein level was normalized with reference to the β-actin protein, and the normalized signal of control was expressed as 1.0.

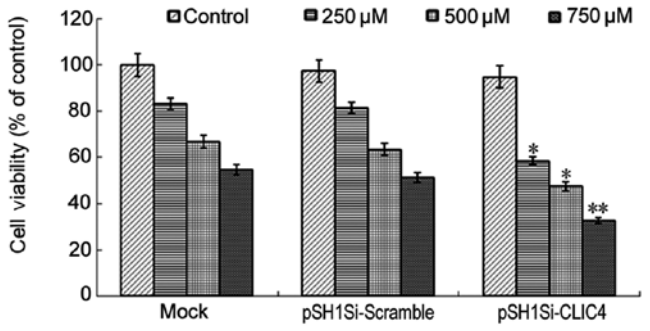

Figure 5. Effect of CLIC4-RNAi on the cell viability of C6 cells treated with H_2O_2. C6 cells were transfected with pSH1Si-scramble plasmid or pSH1Si-CLIC4 plasmid-2 and then treated with 250, 500 and 750 μM H_2O_2. The viability of the C6 cells was determined by MTT assay. Values are the means ± SD (n=3), *P<0.05, **P<0.01 vs. corresponding mock or pSH1Si-scramble group.

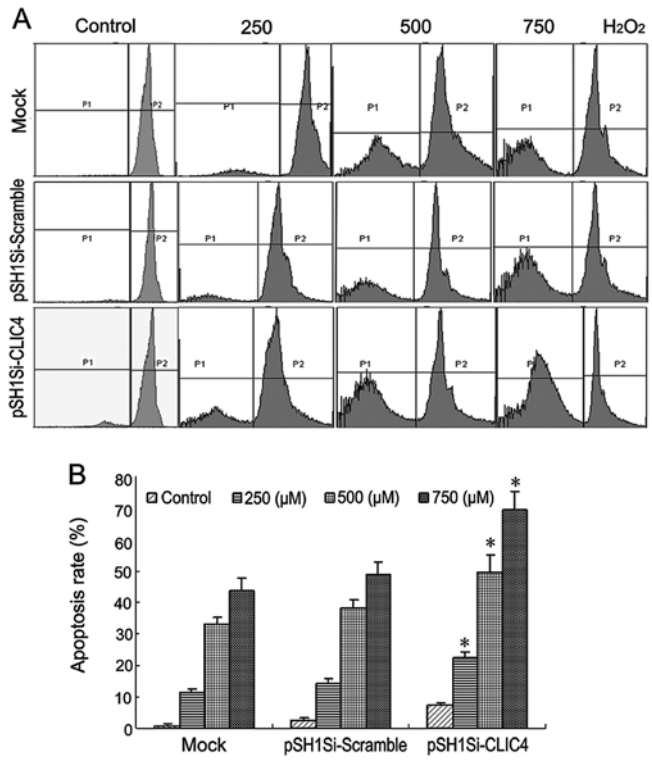

Figure 6. CLIC4-RNAi enhances C6 cell apoptosis induced by H_2O_2. (A) C6 cells were transfected with pSH1Si-scramble plasmid or pSH1Si-CLIC4 plasmid-2 and then treated with 250, 500 and 750 μM H_2O_2 for 24 h. The apoptosis rates were analyzed by flow cytometry. (B) Quantitative analysis of the apoptosis rate. Values are the means ± SD (n=3), *P<0.05 vs. corresponding mock or pSH1Si-scramble group.

as a candidate for further experiments. First, the effect of CLIC4-RNAi on cell viability in C6 cells treated with different concentrations of H_2O_2 was measured by MTT assay. The cell viability was decreased in the mock group, pSH1Si-scramble group and pSH1Si-CLIC4 group in a dose-dependent manner after 24 h (Fig. 5). Compared with the mock group or pSH1Si-scramble plasmid group, the cell viability in the pSH1Si-CLIC4 plasmid group was significantly decreased (P<0.05), indicating that CLIC4-RNAi promotes C6 cell death induced by H_2O_2.

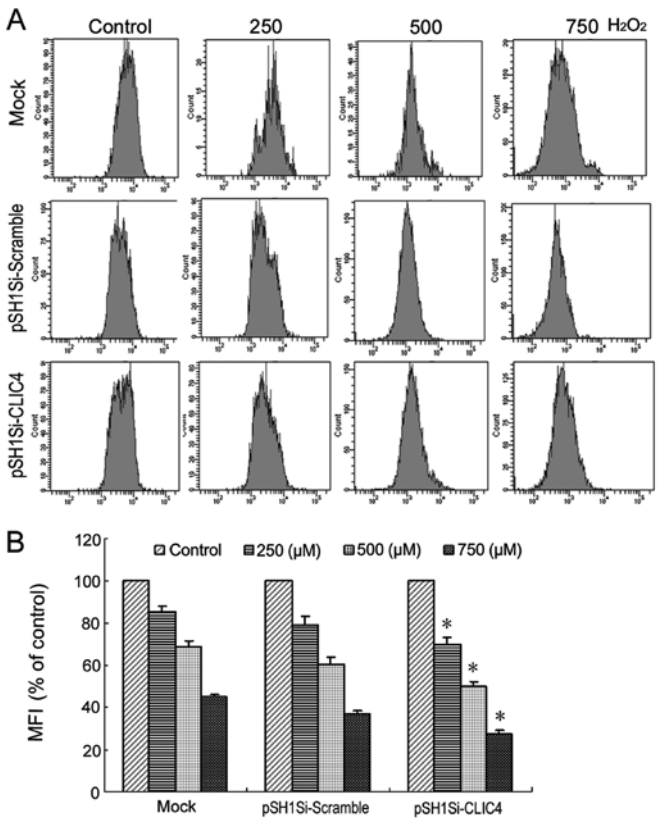

Figure 7. Effect of CLIC4-RNAi on mitochondrial membrane potential in C6 cells treated with different concentration of H_2O_2. (A) C6 cells were transfected with pSH1Si-scramble plasmid or pSH1Si-CLIC4 plasmid-2 and then treated with 250, 500 and 750 μM H_2O_2 for 24 h. The mean mitochondrial potential was measured by flow cytometric analysis using Rhodamine 123 probe. (B) Quantitative analysis of the mean fluorescence intensity (MFI) of Rhodamine 123. Values are the means ± SD (n=3), *P<0.05 vs. corresponding mock or pSH1Si-scramble group.

Next, we evaluated the effects of CLIC4-RNAi on the apoptosis rate in C6 cells treated with different concentrations of H_2O_2. As shown in Fig. 6, the apoptosis rate was increased in the mock group, pSH1Si-scramble group and pSH1Si-CLIC4 group in a dose-dependent manner. Compared with the mock group or pSH1Si-scramble plasmid group, the apoptosis rate in the pSH1Si-CLIC4 plasmid group was significantly increased (P<0.05). The C6 cells transfected with the pSH1Si-CLIC4 plasmid exposed to 750 μM H_2O_2 exhibited an increase of 69.3% in the apoptosis rate (P<0.05). These results suggest that suppression of CLIC4 expression by RNA interference enhanced cell apoptosis in H_2O_2-treated C6 cells.

Dissipation of MMP is a critical event in the early stages of apoptosis. FACS was used to measure the mean mitochondrial membrane potential as determined by Rhodamine 123. Treatment with 250, 500 and 750 μM H_2O_2 induced decreases in MMP that reached 85.5, 68.9 and 45.1% of the control levels, respectively (P<0.05, P<0.01, P<0.01). There was no difference between the mock group and the pSH1Si-scramble plasmid group in regards to MMP. Compared with the mock group or pSH1Si-scramble plasmid group, a significant decrease in MMP that reached 69.8, 49.9 and 27.7% of control levels was observed in the CLIC4 siRNA-transfected cells after H_2O_2 exposure (P<0.05, P<0.05, P<0.05) (Fig. 7). This data clearly

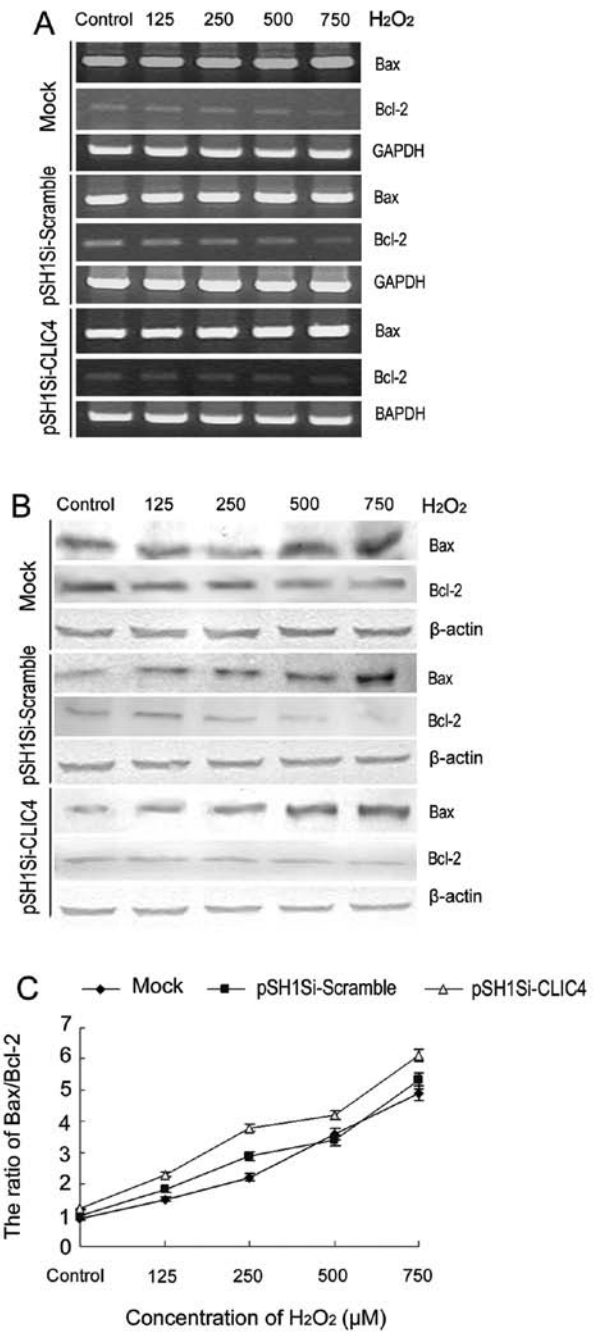

Figure 8. Effect of CLIC4-RNAi on the ratio of Bax/Bcl-2 in C6 cells treated with different concentrations of H_2O_2. C6 cells were transfected with pSH1Si-scramble plasmid or pSH1Si-CLIC4 plasmid-2 and then treated with 125, 250, 500 and 750 μM H_2O_2 for 24 h. The mRNA and protein expression of Bax and Bcl-2 was measured by RT-PCR and western blot analysis. (A) RT-PCR analysis of the mRNA expression of Bax and Bcl-2 in the different C6 cell groups treated with H_2O_2. (B) Western blot analysis for protein expression of Bax and Bcl-2 in the different C6 cell groups treated with H_2O_2. (C) The ratio of normalized Bax to Bcl-2. Densitometry was applied to quantify the Bax and Bcl-2 density in western blot analysis. The Bax and Bcl-2 protein levels were normalized to the β-actin protein, and the normalized signal of control was expressed as 1.0. The ratio was caculated and plotted against various concentrations of H_2O_2. The result of RT-PCR analysis was consistent with the western blot analysis.

showed that H_2O_2 caused mitochondrial dysfunction, characterized by decreased MMP which may subsequently decrease cellular viability leading to cell death.

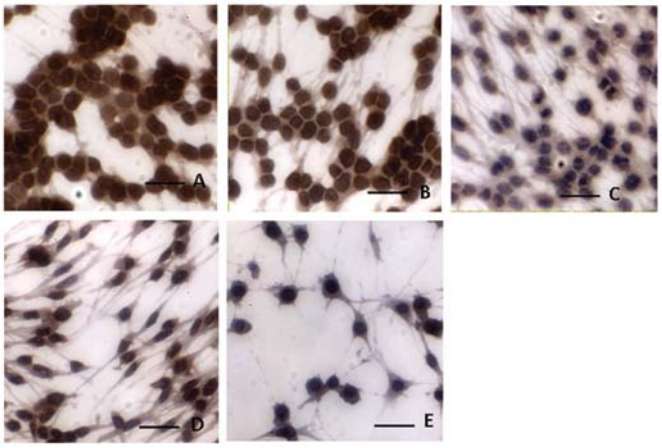

Figure 9. CLIC4 protein expression in C6 cells transfected with the pSH1Si-CLIC4 plasmid as determined by immunocytochemical staining (x400). C6 cells were transfected with pSH1Si-scramble plasmid or pSH1Si-CLIC4 plasmid-2 and then treated with 250, 500 and 750 μM H_2O_2 for 24 h. The CLIC4 expression in C6 cells was evaluated by immunocytochemistry. (A) Mock group; (B) pSH1Si-scramble plasmid group; (C) pSH1Si-CLIC4 plasmid-2 group + 250 μM H_2O_2; (D) pSH1Si-CLIC4 plasmid-2 group + 500 μM H_2O_2; (E) pSH1Si-CLIC4 plasmid-2 group + 500 μM H_2O_2. Scale bar, 25 μm.

Apoptotic cell death is also known to be regulated by Bax and Bcl-2 proteins. The fate of cells is thought to be determined by the balance between pro-apoptotic and anti-apoptotic genes. mRNA and protein expression of pro-apoptotic gene Bax was upregulated and mRNA and protein expression of the anti-apoptotic gene Bcl-2 was downregulated in a dose-dependent manner in the mock group and pSH1Si-scramble plasmid group. Similar results were also found in the pSH1Si-CLIC4 plasmid group (Fig. 8A and B). The Bax/Bcl-2 ratio is the determination of the cell fate. As shown in Fig. 8C, the ratio of Bax/Bcl-2 in each group was increased in a dose-dependent manner, but no differences were noted among the mock, pSH1Si-scramble and pSH1Si-CLIC4 groups. These results suggest that CLIC4-RNAi promotes apoptosis induced by H_2O_2, but Bax/Bcl-2 is not involved in this process.

CLIC4-RNAi enhances CLIC4 nuclear translocation during H_2O_2-induced C6 cell apoptosis. To further investigate the effect of CLIC4-RNAi on apoptosis, we examined the levels of CLIC4 protein by immunocytochemistry. As shown in Fig. 9, the number of C6 cells decreased in the pSH1Si-CLIC4 group in a dose-dependent manner. Compared to the mock group and the pSH1Si-scramble group, CLIC4 translocation to the nucleus in the pSH1Si-CLIC4 group increased in a dose-dependent manner (Fig. 9).

The levels of CLIC4 protein in the nucleus after H_2O_2 treatment were examined in the mock cells and cells transfected with the pSH1Si-scramble or pSH1Si-CLIC4 plasmids. The results showed that the nuclear CLIC4 protein expression was upregulated in both the mock group and the pSH1Si-scramble group in a dose-dependent manner (Fig. 10). Although the CLIC4 protein expression was inhibited by RNAi in the pSH1Si-CLIC4 group, nuclear CLIC4 protein was still increased in a dose-dependent manner in response to increased concentration of H_2O_2 (Fig. 10). Compared to the baseline, the

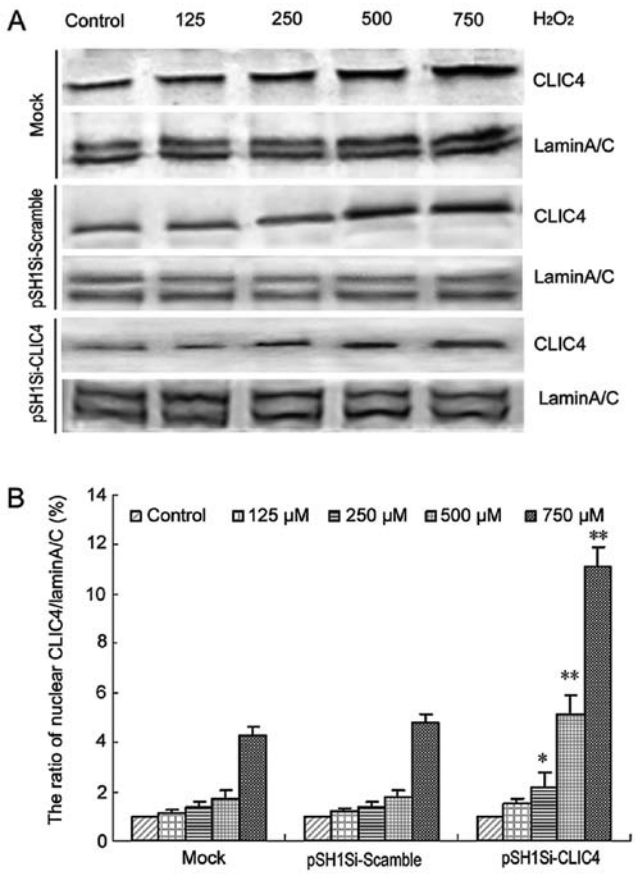

Figure 10. Effect of CLIC4-RNAi on CLIC4 nuclear translocation in C6 cells treated with different concentrations of H_2O_2. (A) C6 cells were transfected with the pSH1Si-scramble plasmid or pSH1Si-CLIC4 plasmid-2 and then treated with 125, 250, 500 and 750 μM H_2O_2 for 24 h. The nuclear protein was then extracted and the level of CLIC4 protein in the nucleus was assessed by western blotting. (B) Densitometry was applied to quantify the CLIC4 density in western blotting. The relative CLIC4 protein level was normalized with reference to the LaminA/C protein and the normalized signal of the control was expressed as 1.0. Values are the means ± SD (n=3), *P<0.05, **P<0.01 vs. corresponding mock or pSH1Si-scramble group.

relative fold increases in nuclear CLIC4 protein levels in the pSH1Si-CLIC4 group were much more than those in the mock or the pSH1Si-scramble groups. These results indicated that CLIC4 nuclear translocation plays an important role during H_2O_2-induced C6 cell apoptosis.

Discussion

Apoptosis is a tightly regulated form of cell death, also known as programmed cell death (PCD), which is an inherent cellular response for effective cellular disposal against developmental and environmental insults. It is an important process during normal development and is also involved in various diseases such as cancer, viral infections and nerve degeneration. Apoptosis can be induced by diverse stimuli. Common signaling mediators, including reactive oxygen species (ROS), may damage DNA, lipids, proteins and other macromolecules (20,21). As a type of ROS, hydrogen peroxide (H_2O_2) produces cytotoxicity (22). Thus, H_2O_2 has been extensively used to duplicate the cell model of oxidative damage and apoptosis (23).

CLIC4/mtCLIC (referred to here as CLIC4) is one of the seven-member family of chloride intracellular channels and its biological functions have been thoroughly studied. c-Myc and p53 binding sites have been found in the CLIC4 promoter and CLIC4 is a direct response gene for both c-Myc and p53 (12,13). The expression of CLIC4 transcripts is also regulated by the tumor necrosis factor (TNF)α (11). CLIC4 is upregulated in response to p53-, TNFα- or c-Myc-mediated apoptosis which is characterized by changes in the intrinsic mitochondrial apoptotic pathway (11-13).

In the present study, we demonstrated that H_2O_2 induces C6 cell apoptosis in a dose-dependent manner and this apoptosis is associated with upregulation of cleaved caspase-3 protein expression and cytochrome *c* and an increased ratio of Bax/Bcl-2. We also demonstrated that H_2O_2 induced decreases in MMP along with an increased apoptosis rate. This suggests activation of the mitochondrial apoptosis pathway in H_2O_2-induced C6 cell apoptosis, consistent with previous reports (3,4). Notably, the CLIC4 protein expression was also upregulated during H_2O_2-induced apoptosis in C6 cells, indicating CLIC4 was involved in H_2O_2-induced C6 cell apoptosis.

In the present study, we identified the cDNA nucleotide and deduced amino acid sequences of CLIC4 in SD rat glioma cells (GenBank accession nunber EF397567). For the first time according to the best of our knowledge, we successfully constructed a recombinant siRNA expression plasmid which significantly inhibits CLIC4 gene expression. Since there was an upregulation of CLIC4 detected after H_2O_2 treatment in C6 cells, we considered that downregulation of CLIC4 would protect C6 cells against H_2O_2-induced apoptosis. In contrast, suppression of CLIC4 expression by RNA interference enhanced H_2O_2-induced C6 cell apoptosis. The enhancement of cell apoptosis by suppression of CLIC4 expression suggests the importance of CLIC4 in H_2O_2-induced cell apoptosis, while the result that CLIC4 protein expression was upregulated in cells treated with H_2O_2 further confirmed the importance of CLIC4 in cell apoptosis induced by H_2O_2. The same results were also found to occur in TNFα-mediated apoptosis (11,12).

The mitochondrion is a sensitive target for oxidative damage under certain pathological conditions, e.g exposure to H_2O_2. Accumulating evidence suggests a central role of the mitochondrion in cellular apoptosis (24,25). Dissipation of MMP is a critical event in the early stages of apoptosis (26). The localization to the mitochondrial inner membrane and the putative pore-forming and ion transport activities of CLIC4 are involved in mitochondrial dysfunction and apoptosis (8). It was reported that CLIC4 levels were elevated in mitochondria isolated from mtDNA-depleted cells, and specific siRNA for CLIC4 reduced MMP measured in mtDNA-depleted L929 cells (27). Another report showed that overexpression of CLIC4 reduced MMP, resulting in the release of cytochrome *c* into the cytoplasm, activation of caspases, and induction of apoptosis (12). In the present study, we showed that suppression of CLIC4 expression by RNA interference promoted a decreases in MMP along with an increased apoptosis rate. This suggests that the MMP can be regulated by the level of CLIC4 expression and CLIC4 is involved in H_2O_2-induced C6 cell apoptosis.

The Bcl-2 family proteins play critical roles in the control of apoptosis. The stoichiometry of pro-apoptotic and anti-apoptotic Bcl-2 family members decides the fate of the cell. Pro-apoptotic proteins such as Bax by translocation from the cytosol to the mitochondria induce cytochrome *c* release, whereas Bcl-2 exerts its anti-apoptotic activity, at least in part by preventing Bax redistribution to mitochondria (28,29). Our experiments demonstrated that H_2O_2 exposure caused an increase in the Bax/Bcl-2 ratio and cleaved caspase-3 expression in a dose-dependent manner and a simultaneous increase in CLIC4 expression in C6 cells. It was reported that overexpression of CLIC4 induces apoptosis, and CLIC4 cooperates with Bax in the induction of cell death (12). Thus, it appears that CLIC4 is important in cell apoptosis induced by H_2O_2. However, suppression of CLIC4 expression by RNA interference did not increase the ratio of Bax/Bcl-2, but did increased the apoptosis rate during H_2O_2-induced C6 cell apoptosis. These results suggest that CLIC4-RNAi promotes apoptosis induced by H_2O_2, but Bax/Bcl-2 is not involved in this process. It was also reported that antisense CLIC4 did not prevent apoptosis induced by Bax, suggesting that CLIC4 and Bax function through independent pathways (12). Other molecules in addition to the Bcl-2 family, which are associated with CLIC4, may be involved in H_2O_2-induced C6 cell apoptosis.

It was reported that nuclear translocation of CLIC4 was detected in cells undergoing p53-mediated apoptosis. When CLIC4 is targeted directly to the nucleus, apoptosis is accelerated and proceeds in the absence of mitochondrial-dependent caspase activation (15). To further investigate the effect of CLIC4-RNAi on apoptosis, we examined the levels of CLIC4 in the nucleus after H_2O_2 treatment. We demonstrated that H_2O_2 caused nuclear translocation of CLIC4 in a dose-dependent manner. Suppression of CLIC4 expression by RNA interference enhanced the upregulation of CLIC4 in the nucleus and increased the apoptosis in H_2O_2-treated C6 cells. This, therefore, suggests that the translocation of CLIC4 to the nucleus occurs in H_2O_2-induced C6 cell apoptosis. The nuclear localization signal (NLS) on the C terminus of CLIC4 appears to play a crucial role in nuclear translocation (15). CLIC4 translocation to the nucleus may participate in the alteration of pH and chloride ion content that could be involved in apoptotic events in the nucleus, such as activation of nucleases and DNA fragmentation (30). The translocation of CLIC4 to the cortical actin cytoskeleton and its association with AKAP350 at the centrosome and midbody may be important for regulating the cell cycle (31). Inhibiting the expression of CLIC4 was found to trigger both mitochondrial apoptosis involved in Bax/Bcl-2 and cytochrome *c* release under starvation and endoplasmic reticulum stress-induced apoptosis (32). These results suggest a role for CLIC4 in apoptosis independent of ion channel regulation. Together, this data show that H_2O_2-induced C6 cell apoptosis is associated with the redistribution of CLIC4 in several cellular compartments.

In summary, we demonstrated that suppression of CLIC4 expression by RNA interference enhanced the apoptotic activity of H_2O_2. Dissipation of MMP and nuclear translocation of CLIC4 were involved in the activation of apoptosis induced by H_2O_2. These data suggest that the CLIC4 protein plays an important role in the regulation of oxidative stress and apoptosis, yet further studies are needed to confirm these findings.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (nos. 30570687 and 81272876), the Jilin Provincial Research Foundation for Basic Research, China (nos. 201015200, 200705373 and 200505139) and the '211 Project' of Jilin University. We greatly appreciate Professor Stuart H. Yuspa of the National Institutes of Health in Bethesda, MD for his generous contribution of the antibodies against CLIC4.

References

1. Castillo SS, Levy M, Thaikoottathil JV and Goldkorn T: Reactive nitrogen and oxygen species activate different sphingomyelinases to induce apoptosis in airway epithelial cells. Exp Cell Res 313: 2680-2686, 2007.
2. Son JH, Yoo HH and Kim DH: Activation of de novo synthetic pathway of ceramides is responsible for the initiation of hydrogen peroxide-induced apoptosis in HL-60 cells. J Toxicol Environ Health A 70: 1310-1318, 2007.
3. Singh M, Sharma H and Singh N: Hydrogen peroxide induces apoptosis in HeLa cells through mitochondrial pathway. Mitochondrion 7: 367-373, 2007.
4. Singh M and Singh N: Induction of apoptosis by hydrogen peroxide in HPV 16-positive human cervical cancer cells: involvement of mitochondrial pathway. Mol Cell Biochem 310: 57-65, 2008.
5. Qian Y, Du YH, Tang YB, Lv XF, Liu J, Zhou JG and Guan YY: ClC-3 chloride channel prevents apoptosis induced by hydrogen peroxide in basilar artery smooth muscle cells through mitochondria dependent pathway. Apoptosis 16: 468-477, 2011.
6. Ryu SY, Peixoto PM, Teijido O, Dejean LM and Kinnally KW: Role of mitochondrial ion channels in cell death. Biofactors 36: 255-263, 2010.
7. Jentsch TJ, Friedrich T, Schriever A and Yamada H: The CLC chloride channel family. Pflugers Arch 437: 783-795, 1999.
8. Ashley RH: Challenging accepted ion channel biology: p64 and the CLIC family of putative intracellular anion channel proteins. Mol Membr Biol 20: 1-11, 2003.
9. Harrop SJ, DeMaere MZ, Fairlie WD, *et al*: Crystal structure of a soluble form of the intracellular chloride ion channel CLIC1 (NCC27) at 1.4-A resolution. J Biol Chem 276: 44993-5000, 2001.
10. Littler DR, Harrop SJ, Brown LJ, *et al*: Comparison of vertebrate and invertebrate CLIC proteins: the crystal structures of *Caenorhabditis elegans* EXC-4 and *Drosophila melanogaster* DmCLIC. Proteins 71: 364-378, 2008.
11. Fernández-Salas E, Sagar M, Cheng C, Yuspa SH and Weinberg WC: p53 and tumor necrosis factor alpha regulate the expression of a mitochondrial chloride channel protein. J Biol Chem 274: 36488-36497, 1999.
12. Fernández-Salas E, Suh KS, Speransky VV, *et al*: mtCLIC/CLIC4 an organellular chloride channel protein is increased by DNA damage and participates in the apoptotic response to p53. Mol Cell Biol 22: 3610-3620, 2002.
13. Shiio Y, Suh KS, Lee H, Yuspa SH, Eisenman RN and Aebersold R: Quantitative proteomic analysis of myc-induced apoptosis: a direct role for Myc induction of the mitochondrial chloride ion channel mtCLIC/CLIC4. J Biol Chem 281: 2750-2756, 2006.
14. Suh KS, Mutoh M, Gerdes M, *et al*: Antisense suppression of the chloride intracellular channel family induces apoptosis enhances tumor necrosis factor α-induced apoptosis and inhibits tumor growth. Cancer Res 65: 562-571, 2005.
15. Suh KS, Mutoh M, Nagashima K, *et al*: The organellular chloride channel protein CLIC4/mtCLIC translocates to the nucleus in response to cellular stress and accelerates apoptosis. J Biol Chem 279: 4632-4641, 2004.
16. Konturek PC, Burnat G, Rau T, Hahn EG and Konturek S: Effect of adiponectin and ghrelin on apoptosis of Barrett adenocarcinoma cell line. Digest Dis Sci 53: 597-605, 2008.
17. Elingold I, Isollabella MP, Casanova MB, *et al*: Mitochondrial toxicity and antioxidant activity of a prenylated flavonoid isolated from *Dalea elegans*. Chem Biol Interact 171: 294-305, 2008.

18. Buchet K and Godinot C: Functional F1-ATPase essential in maintaining growth and membrane potential of human mitochondrial DNA-depleted rho degrees cells. J Biol Chem 273: 22983-22989, 1998.
19. Park KS, Nam KJ, Kim JW, *et al*: Depletion of mitochondrial DNA alters glucose metabolism in SK-Hepl cells. Am J Physiol Endocrinol Metab 280: E1007-E1014, 2001.
20. Huang SX, Partridge MA, Ghandhi SA, Davidson MM, Amundson SA and Hei TK: Mitochondria-derived reactive intermediate species mediate asbestos-induced genotoxicity and oxidative stress-responsive signaling pathways. Environ Health Perspect 120: 840-847, 2012.
21. Peng X and Gandhi V: ROS-activated anticancer prodrugs: a new strategy for tumor-specific damage. Ther Deliv 3: 823-833, 2012.
22. Kitamura Y, Ota T, Matsuoka Y, *et al*: Hydrogen peroxide-induced apoptosis mediated by p53 protein in glial cells. Glia 25: 154-164, 1999.
23. Zhang H, Kong X, Kang J, Su J, Li Y, Zhong J and Sun L: Oxidative stress induces parallel autophagy and mitochondria dysfunction in human glioma U251 cells. Toxicol Sci 110: 376-388, 2009.
24. Green DR and Kroemer G: The pathophysiology of mitochondrial cell death. Science 305: 626-629, 2004.
25. Shih CM, Ko WC, Wu JS, *et al*: Mediating of caspase-independent apoptosis by cadmium through the mitochondria-ROS pathway in MRC-5 fibroblasts. J Cell Biochem 91: 384-397, 2004.
26. Kakkar P and Singh BK: Mitochondria: a hub of redox activities and cellular distress control. Mol Cell Biol 305: 235-253, 2007.
27. Arnould T, Mercy L, Houbion A, *et al*: mtCLIC is up-regulated and maintains a mitochondrial membrane potential in mtDNA-depleted L929 cells. FASEB J 17: 2145-2147, 2003.
28. Wong WW and Puthalakath H: Bcl-2 family proteins: the sentinels of the mitochondrial apoptosis pathway. IUBMB Life 60: 390-397, 2008.
29. Murphy KM, Ranganathan V, Farnsworth ML, Kavallaris M and Lock RB: Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. Cell Death Differ 7: 102-111, 2000.
30. Pervaiz S and Clément MV: A permissive apoptotic environment: function of a decrease in intracellular superoxide anion and cytosolic acidification. Biochem Biophys Res Commun 290: 1145-1150, 2002.
31. Berryman MA and Goldenring JR: CLIC4 is enriched at cell-cell junctions and colocalizes with AKAP350 at the centrosome and midbody of cultured mammalian cells. Cell Motil Cytoskeleton 56: 159-172, 2003.
32. Zhong J, Kong X, Zhang H, *et al*: Inhibition of CLIC4 enhances autophagy and triggers mitochondrial and ER stress-induced apoptosis in human glioma U251 cells under starvation. PLoS One 7: e39378, 2012.