Sirt3 attenuates hydrogen peroxide-induced oxidative stress through the preservation of mitochondrial function in HT22 cells

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Received May 24, 2014; Accepted July 14, 2014

DOI: 10.3892/ijmm.2014.1876

Abstract. Sirtuins (Sirt) are a family of phylogenetically conserved nicotinamide adenine nucleotide (NAD^+)-dependent protein deacetylases, among which Sirt3 resides primarily in the mitochondria and serves as a stress responsive deacetylase, playing a role in protecting cells from damage under stress conditions. The present study aimed to investigate the role of Sirt3 in hydrogen peroxide (H_2O_2)-induced oxidative neuronal injury in HT22 mouse hippocampal cells. Treatment with H_2O_2 increased the expression of Sirt3 in a dose- and time-dependent manner, and the knockdown of Sirt3 using specific small interfering RNA (siRNA) exacerbated the H_2O_2-induced neuronal injury. The overexpression of Sirt3 induced by lentiviral transfection significantly reduced the generation of reactive oxygen species (ROS) and lipid peroxidation following injury, whereas the activities of endogenous antioxidant enzymes were not affected. Further experiments revealed that the H_2O_2-induced inhibition of mitochondrial complex activity and adenosine triphosphate (ATP) synthesis, the decrease in mitochondrial Ca^{2+} buffering capacity and mitochondrial swelling were all partly reversed by Sirt3. Furthermore, the overexpression of Sirt3 attenuated the release of cytochrome *c*, the increase in the Bax/Bcl-2 ratio, as well as caspase-9/caspase-3 activity induced by H_2O_2, and eventually inhibited apoptotic neuronal cell death. These results suggest that Sirt3 acts as a prosurvival factor, playing an essential role in protecting HT22 cells under H_2O_2-induced oxidative stress, possibly by inhibiting ROS accumulation and the activation of the mitochondrial apoptotic pathway.

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Key words: Sirt3, reactive oxygen species, oxidative stress, mitochondria

Introduction

Reactive oxygen species (ROS) are a group of molecules generated in the process of oxygen metabolism, among which the endogenous stable oxidant, hydrogen peroxide (H_2O_2), is considered as the principal ROS member and has been the main focus of studies on ROS biology in recent years (1). For many years after its discovery, H_2O_2 was viewed as a non-specific agent of destruction to human tissues (2); however, growing evidence over the past few years suggests that H_2O_2 may act as a 'Jekyll and Hyde' signaling molecule in cell proliferation, migration, survival and death (1). At low concentrations, H_2O_2 can act as a classical second messenger with a pro-survival role by regulating kinase-driven pathways in several physiological processes. At high concentrations, H_2O_2 induces cellular injury by damaging key cellular molecules, such as DNA and lipids, and by inducing apoptosis, necrosis or autophagy. Several pharmacological agents targeting H_2O_2 metabolism have been demonstrated to have therapeutic potential in the treatment of neurological disorders, ranging from acute insults, such as ischemic and traumatic brain injury to chronic neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease (3,4).

Mitochondria are usually described as 'cellular power plants' due to their ability to generate most of the chemical energy of cells, namely adenosine triphosphate (ATP), and they can also receive cellular signals and propagate a targeted response to mediate several basic cellular functions (5). Due to their important role in regulating cell metabolism and ROS generation, the damage and ensuing dysfunction of the mitochondria in neurons has been demonstrated to be a key factor in various types of oxidative stress related to neurological diseases (6). Following oxidative stress, the majority of the mitochondria develop varying degrees of swelling, and several pro-apoptotic molecules are released or activated, such as cytochrome *c*, caspase-9 and the pro-apoptotic Bcl-2 family of proteins (which includes Bax). A number of studies have demonstrated that many pharmacological agents and mitochondria-associated

molecules exert protective effects against neuronal injury through the preservation of mitochondrial function, and this may be an ideal neuroprotective strategy (6,7).

The sirtuins (or Sir2-like proteins) are a conserved family of nicotinamide adenine nucleotide (NAD^+)-dependent protein deacetylases, and have been reported to be involved in transcriptional silencing, the genetic control of aging and the longevity of organisms ranging from yeast to humans (8). Among these sirtuins, Sirt3 resides primarily in the mitochondria and serves as a primary regulator of mitochondrial function and metabolism by binding and deacetylating several metabolic and respiratory enzymes (9). The increased expression of Sirt3 protects cardiomyocytes against genotoxic and oxidative stress-mediated cell death by hindering the translocation of Bax to the mitochondria (10). A recent study demonstrated that the Sirt3-mediated deacetylation of forkhead box O3 (FOXO3) attenuates oxidative stress-induced mitochondrial dysfunction through the coordination of mitochondrial biogenesis, fission/fusion and mitophagy (11). However, the exact role of Sirt3 in oxidative stress-induced neuronal cell injury has not yet been fully elucidated. Therefore, the aim of the present study was to investigate the effects of Sirt3 knockdown and its overexpression in H_2O_2-induced neuronal injury in HT22 cells, as well as the potential mechanisms involved with focus on mitochondrial oxidative phosphorylation, calcium metabolism and the intrinsic apoptotic pathway.

Materials and methods

Cell culture. HT22 mouse hippocampal cells were obtained from the Institute of Biochemistry and Cell Biology (IBCB), Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (CAS), Shanghai, China. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum and 1% antibiotics (penicillin/streptomycin) in a humidified incubator with 5% CO_2 and 95% air. The growth medium was removed and replaced by medium containing H_2O_2 for the induction of apoptosis.

Cell viability assay. Cell viability assay was performed using the Cell Proliferation Reagent WST-1 following the manufacture's instructions (Roche, Basel, Switzerland). Briefly, the HT22 cells were cultured at a concentration of 0.5-5x10^4 in microplates in a final volume of 100 μl/well culture medium. Following the various treatments, 10 μl of the cell proliferation reagent, WST-1, were added to each well followed by incubation for 4 h at 37˚C. Subsequently, 100 μl/well culture medium and 10 μl WST-1 were added to one well in the absence of HT22 cells, and its absorbance was used as a blank position for the ELISA reader. The cells were shaken thoroughly for 1 min on a shaker and the absorbance of the samples was measured using a microplate (ELISA) reader (Bio-Rad Laboratories, Cambridge, MA, USA).

Lactate dehydrogenase (LDH) release assay. Cytotoxicity was determined by the release of LDH, a cytoplasmic enzyme released from cells, and a marker of membrane integrity. The LDH release into the culture medium was detected using a diagnostic kit according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Briefly, 50 μl of supernatant from each well were collected to measure the release of LDH. The samples were incubated with a reduced form of nicotinamide adenine dinucleotide (NADH) and pyruvate for 15 min at 37˚C and the reaction was terminated by the addition of 0.4 mol/l NaOH. The activity of LDH was calculated from the absorbance at 440 nm and the background absorbance from the culture medium that was not used for any cell cultures was subtracted from all the absorbance measurements. The results were normalized to the maximal LDH release, which was determined by treating the control wells for 60 min with 1% Triton X-100 to lyse all cells.

Small interfering RNA (siRNA) and transfection. Specific siRNA targeting Sirt3 (Si-Sirt3, sc-61556) and control siRNA (Si-Control, sc-37007), which should not knock down any known proteins, were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The above-mentioned siRNA molecules were transfected into the cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) in 6-well plates for 48 h. Following transfection, the HT22 cells were treated with H_2O_2 (500 μM) for 24 h and subjected to various measurements.

Lentivirus construction and transfection. The coding sequence of Sirt3 was amplified by RT-PCR. The primer sequences were as follows: forward, 5'-TACTTCCTTCGGCTGCTTCA-3' and reverse, 5'-AAGGCGAAATCAGCCACA-3'. The PCR fragments and the pGC-FU plasmid (Shanghai Genechem Co., Ltd., Shanghai, China) were digested with *Age*I and then ligated with T4 DNA ligase to produce pGC-FU-Sirt3. To generate the recombinant lentivirus, LV-Sirt3, 293T cells were co-transfected with the pGC-FU plasmid (20 μg) with a cDNA encoding Sirt3, pHelper1.0 plasmid (15 μg) and pHelper 2.0 plasmid (10 μg) using Lipofectamine 2000 (100 μl). The supernatant was harvested and the viral titer was calculated by transducing 293T cells. As a control, we also generated a control lentiviral vector that expresses GFP alone (LV-Control). The HT22 cells were transfected with the lentiviral vectors for 72 h and subjected to various treatments.

Measurement of the NAD^+/NADH ratio. To investigate whether Sirt3 is enzymatically active under our experimental conditions, measurement of the NAD^+/NADH ratio was performed using the NAD^+/NADH Quantification kit (BioVision, Milpitas, CA, USA) according to the manufacturer's instructions. Briefly, 5x10^5 HT22 cells seeded in 6-well plates were washed with cold PBS, collected and centrifuged at 1,500 rpm for 5 min. The cells were then lysed by 2 freeze/thaw cycles in NADH/NAD extraction buffer and subsequently vortexed for 10 sec. The cellular extracts were transferred into a 96-well plate in duplicate, and incubated with NAD Cycling Mix for 5 min. NAD^+_{total} was quantified by the addition of NADH developer to each sample and by reading the plate at OD450 nm for 30 min. To detect NADH, the extracted samples were incubated at 60˚C for 30 min in order to decompose NAD^+. Subsequently, the samples were incubated with NAD Cycling Mix for 5 min and, after the addition of NADH developer, were read as previously described (12). The NAD^+/NADH ratio was calculated as follows: (NAD^+_{total} - NADH)/NADH.

Measurement of ROS generation. Briefly, the HT22 cells were incubated with 2,7-dichlorodihydrofluorescein diace-

tate (DCF-DA) (Sigma, St. Louis, MO, USA) (10 μM) for 1 h at 37˚C in the dark and then resuspended in phosphate-buffered saline (PBS). Intracellular ROS production was detected using the fluorescence intensity of the oxidant-sensitive probe, H_2DCF-DA, in a microscope and fluorescence was read using an excitation wavelength of 480 nm and an emission wavelength of 530 nm.

Measurement of lipid peroxidation. Malonyldialdehyde (MDA) and 4-hydroxynonenal (4-HNE), 2 indexes of lipid peroxidation, were determined using assay kits from Cell Biolabs (San Diego, CA, USA) strictly following the manufacturer's instructions. The absorbance of the samples was measured using a microplate (ELISA) reader.

Detection of antioxidant enzyme activity. The enzyme activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST) was determined using commercially available assay kits following the manufacturer's instructions (Cayman Chemical Co., Mountain View, CA, USA). The protein concentration was determined using the BCA protein kit (Nanjing Jiancheng Bioengineering Institute). The enzyme activities were then normalized to the corresponding protein concentration for each sample.

Determination of mitochondrial respiratory chain complex activity. The mitochondria were purified by Percoll density gradient centrifugation in extraction buffer (50 mM Tris HCl, pH 7.5, 500 mM NaCl, 0.03% reduced Triton X-100, 1 mM EDTA, 1 mM PMSF, 0.5 mM benzamidine, and 1 mg/ml each of pepstatin-A, leupeptin and aprotinin). All the samples were subjected to 3 freeze-thaw cycles to disrupt the membranes and expose the enzymes before analysis. The enzymatic activity was measured at 37˚C spectrophotometrically using the following methods: complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (ubiquinol cytochrome *c* reductase) and complex IV (cytochrome *c* oxidase), as previously described (13-15). The data were expressed as a percentage of the control.

Measurement of ATP synthesis. Isolated mitochondria were utilized to measure ATP synthesis with a luciferase/luciferin-based system as previously described (16). Mitochondria-enriched pellets (30 μg) were resuspended in 100 μl of buffer A (150 mM KCl, 25 mM Tris-HCl, 2 mM potassium phosphate, 0.1 mM $MgCl_2$, pH 7.4) with 0.1% BSA, 1 mM malate, 1 mM glutamate and buffer B (containing 0.8 mM luciferin and 20 mg/ml luciferase in 0.5 M Tris-acetate pH 7.75). The reaction was initiated by the addition of 0.1 mM adenosine diphosphate (ADP) and monitored for 5 min using a microplate reader (Bio-Rad Laboratories).

Measurement of mitochondrial swelling. Mitochondrial swelling was measured following a previously published protocol (17). Briefly, the isolated mitochondria were suspended in fresh swelling buffer (0.2 M sucrose, 10 mM Tris-MOPS, pH 7.4, 5 mM succinate, 1 mM phosphate, 2 μM rotenone and 1 μM EGTA-Tris, pH 7.4) at 0.5 mg/ml, and the swelling of the mitochondria was monitored by a decrease in absorbance at 540 nm in the presence of calcium chloride ($CaCl_2$; 200 μM).

Measurement of mitochondrial calcium buffering capacity. Mitochondrial calcium buffering capacity was estimated using the Ca^{2+} sensitive Calcium Green 5N fluorescent dye. The incubation medium was composed of 125 mM KCl, 20 mM HEPES (pH 7.2), 2 mM KH_2PO_4, 2 mM $MgCl_2$, 5 mM succinate, 1 μM rotenone and 0.2 mM ADP, with 1 μg/ml oligomycin and 1 μM Calcium Green 5N. Bolus additions of $CaCl_2$ were made to the 60 μg of mitochondria in suspension in 30 nM increments and changes in Calcium Green 5N fluorescence were recorded at an emission of 532 nm.

Flow cytometry. The HT22 cells were harvested 24 h following exposure to H_2O_2, washed with ice-cold Ca^{2+} free PBS, and re-suspended in binding buffer. Cell suspension was transferred into a tube and double-stained for 15 min with Alexa Fluor 488-conjugated Annexin V (AV) and propidium iodide (PI) at room temperature in the dark. After the addition of 400 μl binding buffer, the stained cells were analyzed using an FC500 flow cytometer (Beckman-Coulter, Brea, CA USA) with the fluorescence emission at 530 nm and >575 nm. CXP cell quest software (Beckman-Coulter) was used to count the number of apoptotic cells (AV^+/PI^+, late phase apoptotic cells and AV^+/PI^-, early phase apoptotic cells) and analyzed the results.

Quantification of cytochrome c release. Cytochrome *c* release into the cytoplasm was assessed following subcellular fraction preparation. The HT22 cells were washed with ice-cold PBS 3 times and lysed with lysis buffer containing protease inhibitors. The cell lysate was centrifuged for 10 min at 750 x g at 4˚C, and the pellets containing the nuclei and unbroken cells were discarded. The supernatant was then centrifuged at 15,000 x g for 15 min. The resulting supernatant was removed and used as the cytosolic fraction. The pellet fraction containing the mitochondria was further incubated with PBS containing 0.5% Trition X-100 for 10 min at 4˚C. Following centrifugation at 16,000 x g for 10 min, the supernatant was collected as the mitochondrial fraction. The levels of cytochrome *c* in the cytosolic and mitochondrial fractions were measured using the Quantikine M Cytochrome C Immunoassay kit obtained from R&D Systems (Minneapolis, MN, USA). Data were expressed as ng/mg protein.

Measurement of caspase-3 activity. The activity of caspase-3 was measured using the colorimetric assay kit according to the manufacturer's instructions (Cell Signaling Technology, Danvers, MA, USA). Briefly, after being harvested and lysed 10^6 cells were mixed with 32 μl of assay buffer and 2 μl of 10 mM Ac-DEVD-pNA substrate. Absorbance at 405 nm was measured following incubation at 37˚C for 4 h. The absorbance of each sample was determined by subtraction of the mean absorbance of the blank and corrected by the protein concentration of the cell lysate. The results were described as the relative activity to that of the control group.

Western blot analysis. Equivalent amounts of protein (40 μg per lane) were loaded and separated by 10% SDS-PAGE gels, and transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% non-fat milk solution in Tris-buffered saline with 0.1% Triton X-100 (TBST) for 1 h,

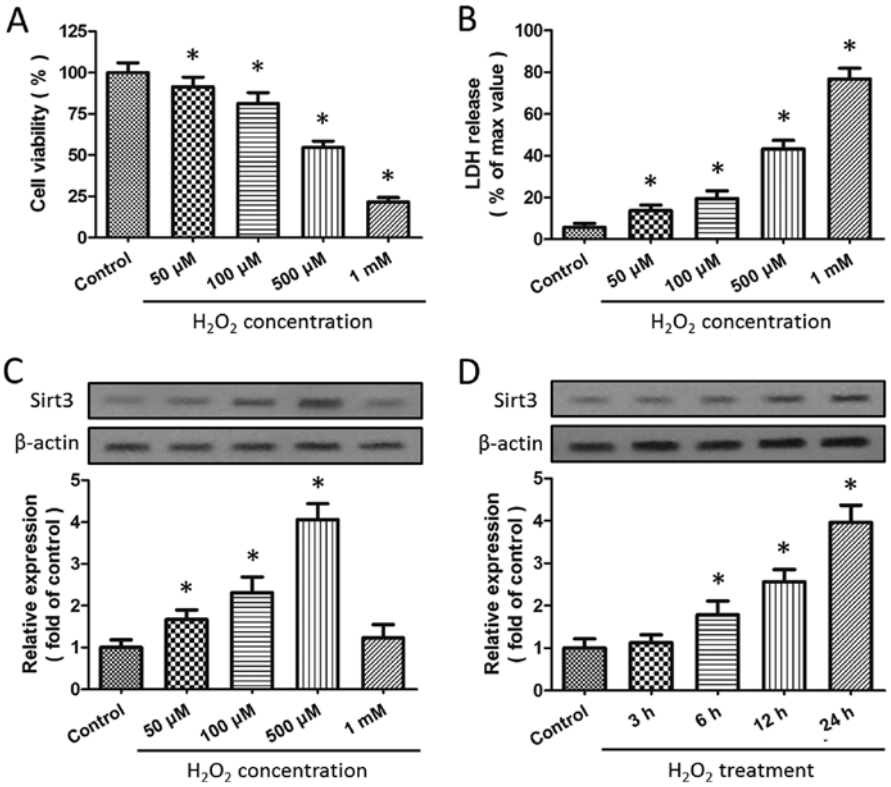

Figure 1. Expression of Sirt3 following H_2O_2-induced injury in HT22 cells. HT22 cells were treated with H_2O_2 at various concentrations for 24 h. Cell viability was measured by (A) WST-1 assay and (B) cytotoxicity was measured by lactate dehydrogenase (LDH) assay. (C) The expression of Sirt3 was measured by western blot analysis. (D) HT22 cells were treated with 500 μM H_2O_2, and the expression of Sirt3 was measured by western blot analysis. Data are shown as the means ± SD of 5 experiments. *P<0.05 vs. control.

and then incubated overnight at 4˚C with primary Sirt3 antibody (1:1,000), Bax (1:800), Bcl-2 (1:800), cleaved caspase-9 (1:500) or caspase-9 (1:600) antibody dilutions in TBST. Subsequently, the membranes were washed and incubated with secondary antibody (anti-rabbit and anti-goat IgG; Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactivity was detected with Super Signal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL, USA). The band densities were corrected for the β-actin signals. ImageJ analysis software (Scion Corp.) was used to quantify the optical density of each band.

Statistical analysis. Statistical analysis was performed using the SPSS 16.0, a statistical software package. Statistical evaluation of the data was performed by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of Sirt3 following H_2O_2-induced injury in HT22 cells. The HT22 cells were incubated in the presence of H_2O_2 at various concentrations (50, 100, 500 μM or 1 mM) for 24 h, and the cytotoxicity was determined by WST-1 assay and LDH release assay. The results revealed that incubation with H_2O_2 significantly decreased cell viability (Fig. 1A) and increased LDH release (Fig. 1B) (both P<0.05) in a dose-dependent manner. As the exposure of the cells to 500 μM H_2O_2 caused almost half of the cells to die, it was used in the subsequent experiments. Western blot analysis was used to investigate the effects of H_2O_2 insults on Sirt3 expression, and the results revealed that incubation with H_2O_2 significantly increased the expression of Sirt3 in a dose-dependent manner (P<0.05; Fig. 1C). As shown in Fig. 1D, a time-dependent increase in Sirt3 expression was also observed following exposure to 500 μM H_2O_2.

H_2O_2-induced Sirt3 expression promotes HT22 cell survival. To investigate the biological functions of Sirt3 in H_2O_2-induced neurotoxicity, the HT22 cells were transfected with Sirt3-specific siRNA (Si-Sirt3) or control siRNA (Si-Control). Western blot analysis indicated that Sirt3 expression was significantly reduced in the cells following transfection with Si-Sirt3 (P<0.05; Fig. 2A). Following treatment with 500 μM H_2O_2 for 24 h, the viability of the cells transfected with Si-Sirt3 was lower than that of the cells transfected with Si-Control (Fig. 2B). By contrast, the knockdown of Sirt3 further increased the release of LDH induced by H_2O_2 treatment in the HT22 cells (Fig. 2C). These data suggest that the knockdown of Sirt3 aggravates H_2O_2-induced neuronal injury and that H_2O_2-induced Sirt3 expression may be an endogenous protective mechanism.

Overexpression of Sirt3 reduces ROS generation and lipid peroxidation. To determine whether Sirt3 affects the generation of intracellular ROS, the HT22 cells were transfected with lentivirus expressing Sirt3 (LV-Sirt3) or a control lentivirus (LV-Control). The results of western blot analysis indicated that the expression of Sirt3 was significantly increased by transfection with LV-Sirt3 as compared to transfection with the

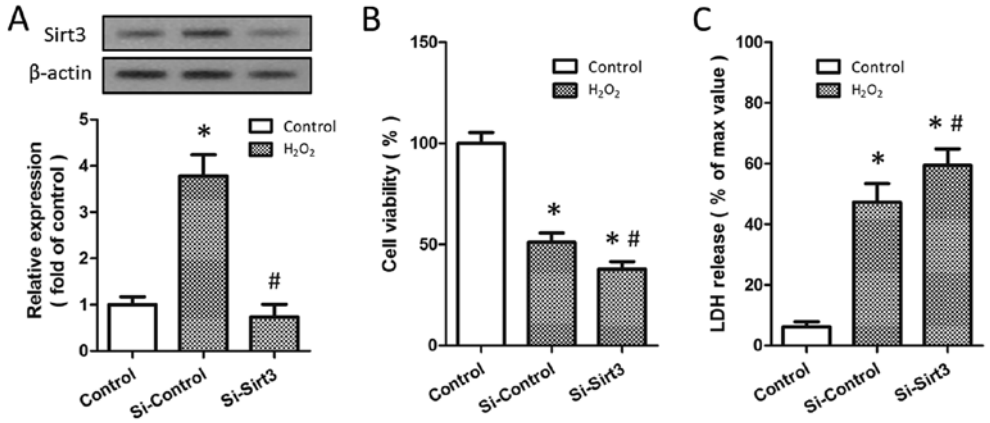

Figure 2. Downregulation of Sirt3 aggravates H_2O_2-induced cytotoxicity. HT22 cells were transfected with Sirt3-specific siRNA (Si-Sirt3) or control siRNA (Si-Control) for 72 h, and then treated with or without 500 μM H_2O_2. (A) The expression of Sirt3 was measured by western blot analysis. Cell viability was measured by (B) WST-1 assay, and (C) cytotoxicity was measured by lactate dehydrogenase (LDH) assay. Data are shown as the means ± SD of 5 experiments. *P<0.05 vs. control. #P<0.05 vs. Si-Control.

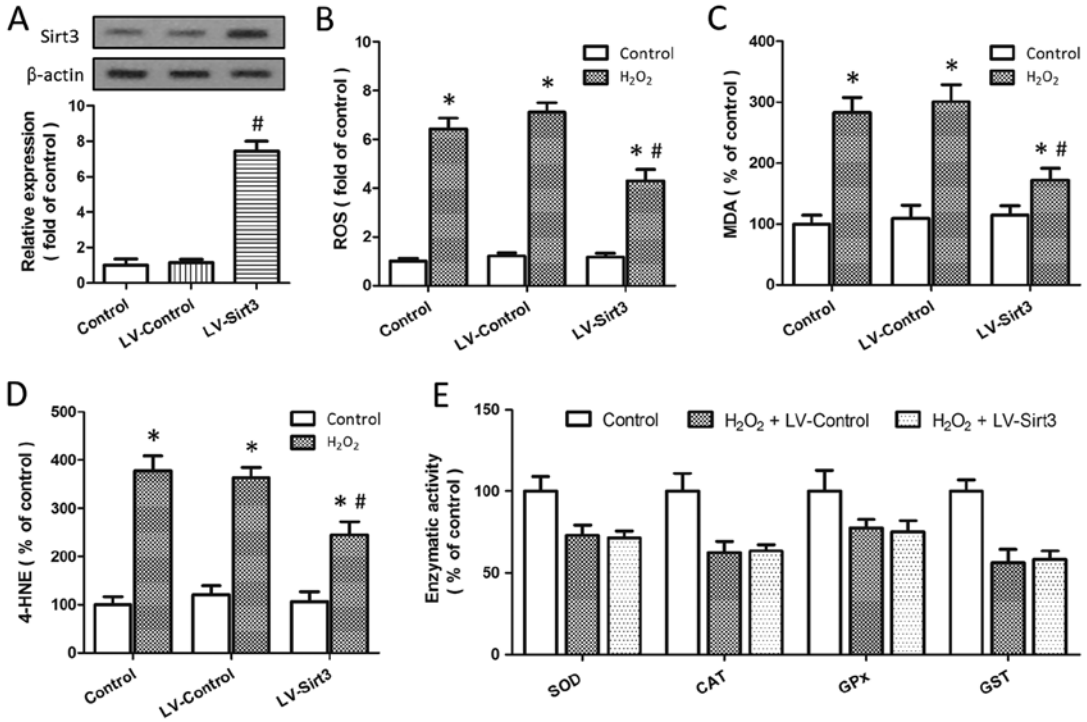

Figure 3. Overexpression of Sirt3 reduces reactive oxygen species (ROS) generation and lipid peroxidation. (A) HT22 cells were transfected with lentivirus expressing Sirt3 (LV-Sirt3) or control lentivirus (LV-Control) for 72 h, and the expression of Sirt3 was measured by western blot analysis. (B) Following transfection, the HT22 cells were treated with or without 500 μM H_2O_2, and ROS production was measured by H_2DCFDA. The expression of (C) malonyldialdehyde (MDA) and (D) 4-hydroxynonenal (4-HNE), and (E) the enzymatic activity of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione S-transferase (GST) was assayed. Data are shown as the means ± SD of 5 experiments. *P<0.05 vs. control. #P<0.05 vs. LV-Control.

LV-Control (P<0.05; Fig. 3A). ROS generation induced by H_2O_2 treatment was reduced by transfection with LV-Sirt3, but not by transfection with LV-Control (Fig. 3B). We also measured the expression levels of MDA and 4-HNE, 2 bioactive markers of lipid peroxidation, following H_2O_2-induced injury, and the results revealed that the knockdown of Sirt3 significantly decreased the levels of MDA (Fig. 3C) and 4-HNE (Fig. 3D) (both P<0.05). As shown in Fig. 3E, treatment with H_2O_2 significantly inhibited the enzymatic activity of SOD, CAT, GPx and GST; however, neither LV-Sirt3 nor LV-Control had any effect on the enzymatic activity of these endogenous antioxidant enzymes, indicating the presence of an endogenous antioxidant system independent of the neuroprotective mechanisms.

To assess whether Sirt3 is enzymatically active under our experimental conditions, we detected the NAD^+/NADH ratio following transfection and/or H_2O_2 treatment (Fig. 4). The results revealed that treatment with H_2O_2 significantly decreased the NAD^+/NADH ratio compared with the control group, indicating that the enzymatic activity of Sirt3 was inhibited by oxidative stress in our *in vitro* model. The knock-

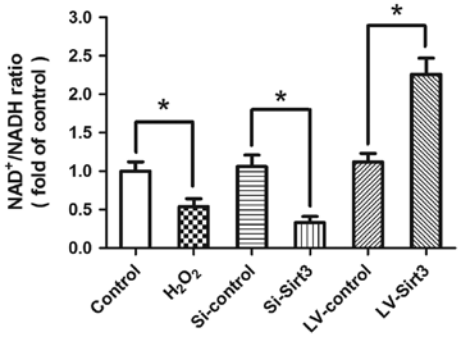

Figure 4. Effects of H_2O_2 treatment, Sirt3 knockdown and Sirt3 overexpression on the NAD^+/NADH ratio. Following treatment with H_2O_2, and transfection with Sirt3-specific siRNA (Si-Sirt3), control siRNA (Si-Control), lentivirus expressing Sirt3 (LV-Sirt3) or control lentivirus (LV-Control) for 72 h, the NAD^+/NADH ratio was measured. Data are shown as the means ± SD of 5 experiments. *P<0.05.

down of Sirt3 decreased the ratio of NAD^+/NADH, whereas the overexpression of Sirt3 by LV-Sirt3 transfection significantly increased the NAD^+/NADH ratio.

Overexpression of Sirt3 preserves mitochondrial respiration and ATP production. The activity assays of electron transfer complexes I-IV in the cell homogenates were performed to investigate the effects of Sirt3 overexpression on mitochondrial respiration. The results revealed that the activity of complexes I-IV was markedly inhibited by H_2O_2 treatment (Fig. 5A). The overexpression of Sirt3 by LV-Sirt3 transfection significantly increased the activity of complexes I, III and IV following H_2O_2 insult, whereas the activity of complex II was not affected by Si-Sirt3 as compared to Si-Control (P>0.05). A dominant role for the mitochondria is the production of ATP through the electron transport chain. Thus, we measured ATP synthesis with a luciferase/luciferin-based system following mitochondrial isolation and purification. As shown in Fig. 5B, compared to the LV-Control-transfected cells, the overexpression of Sirt3 in the HT22 cells reversed the decrease in mitochondrial ATP production which was observed following treatment with H_2O_2.

Overexpression of Sirt3 blocks H_2O_2-induced mitochondrial dysfunction. To characterize the effects of Sirt3 on mitochondrial calcium homeostasis, we examined the calcium buffering capacity in isolated mitochondria following transfection and treatment with H_2O_2. As shown in Fig. 6A, the peaks corresponded to sequential bolus additions of 30 nM of Ca^{2+}, and the downward deflections reflected mitochondrial Ca^{2+} uptake. Treatment with H_2O_2 resulted in a ~50% reduction in Ca^{2+} buffering capacity in the isolated mitochondria, whereas the overexpression of Sirt3 significantly preserved the Ca^{2+} buffering capacity compared to that in the LV-Control-transfected cells (Fig. 6B). Mitochondrial swelling under oxidative stress resulted in damage to the organelle, and is a signature of mitochondrial dysfunction. Thus, we also examined the effects of Sirt3 on mitochondrial swelling, which was induced by the addition of 200 μM Ca^{2+} into isolated mitochondria (Fig. 6C). The results revealed that the decreased absorbance at 540 nm induced by H_2O_2 treatment was partly prevented by LV-Sirt3 transfection in

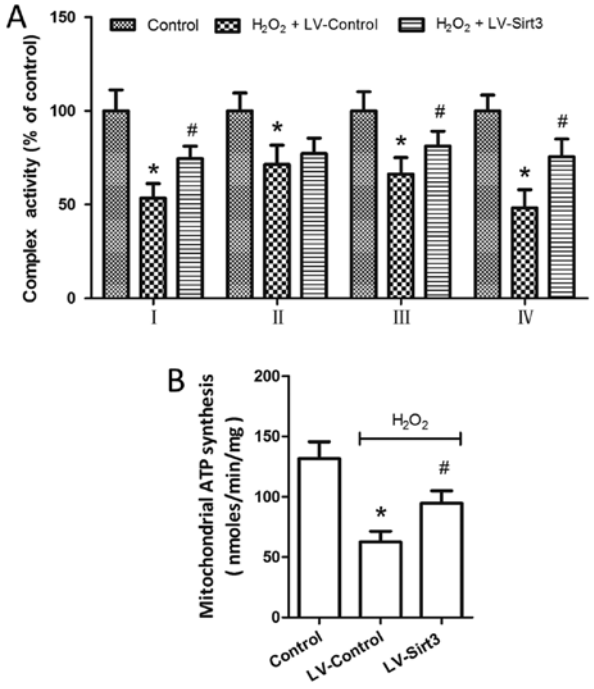

Figure 5. Overexpression of Sirt3 preserves mitochondrial respiration and adenosine triphosphate (ATP) synthesis. Following transfection with lentivirus expressing Sirt3 (LV-Sirt3) or control lentivirus (LV-Control) for 72 h, the HT22 cells were treated with or without 500 μM H_2O_2, and the activity of mitochondrial respiratory chain complex I, complex II, complex III and complex IV was detected (A). (B) The levels of ATP synthesis in the mitochondria isolated from each group were measured. Data are shown as the means ± SD of 5 experiments. *P<0.05 vs. control. #P<0.05 vs. LV-Control.

the HT22 cells, indicating that Sirt3 overexpression attenuated mitochondrial swelling following neuronal oxidative damage.

Overexpression of Sirt3 inhibits mitochondrial-associated apoptosis. To determine the protective effects of Sirt3 against apoptotic neuronal death induced by oxidative stress, flow cytometric analysis was performed at 24 h following treatment with H_2O_2. As shown in Fig. 7A, treatment with H_2O_2 resulted in apparent apoptotic cell death (evidenced by AV^+/PI^+ and AV^+/PI^- cells) in the HT22 cells, and SIRT3 overexpression markedly decreased the number of early apoptotic cells and late apoptotic cells. We also measured the release of cytochrome *c* into the cytoplasm by an immunoassay kit following subcellular fraction preparation. The results revealed that treatment with H_2O_2 induced a significant decrease in mitochondrial cytochrome *c* and a marked increase in cytosolic cytochrome *c*; these effects were partly reversed by transfection with LV-Sirt3 (Fig. 7B and C). To further confirm the anti-apoptotic activity of Sirt3, we examined the expression of Bax, Bcl-2 and caspase-9 by western blot analysis (Fig. 7D). The Bax/Bcl-2 ratio and cleaved-caspase-9/caspase-9 ratio significantly increased following treatment with H_2O_2 (Fig. 7E and F), whereas Sirt3 overexpression exerted a significant inhibitory effect on the H_2O_2-induced increase in the Bax/ Bcl-2 ratio and cleaved-caspase-9/caspase-9 ratio. The results of caspase-3 activity assay indicated that Sirt3 overexpression also attenuated the activation of caspase-3 induced by treatment with H_2O_2 (Fig. 7G).

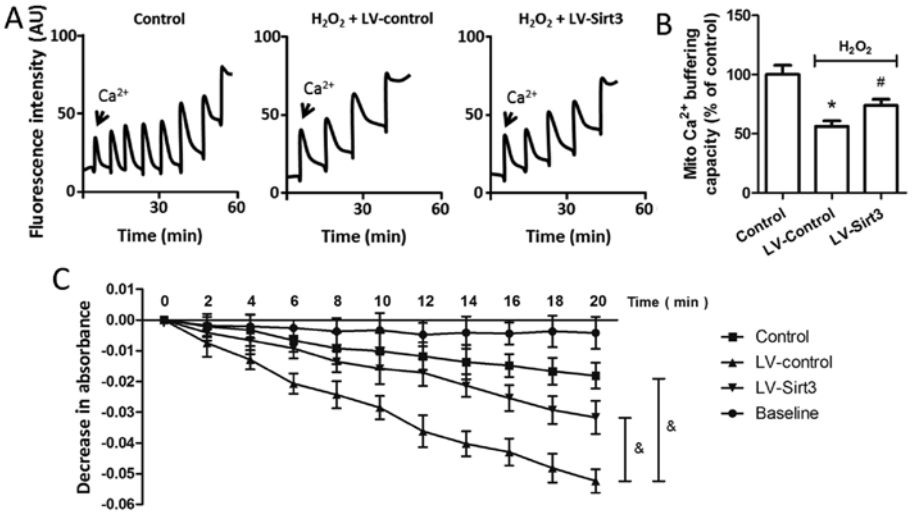

Figure 6. Overexpression of Sirt3 blocks H_2O_2-induced mitochondrial dysfunction. Following transfection with lentivirus expressing Sirt3 (LV-Sirt3) or control lentivirus (LV-Control) for 72 h, the HT22 cells were treated with or without 500 μM H_2O_2, and the mitochondria in each group were isolated and purified. Relative Ca^{2+} uptake capacity of isolated mitochondria was (A) determined and (B) calculated. Mitochondrial swelling was examined by monitoring the absorbance at 540 nm induced by 200 μM Ca^{2+} (C), and the baseline absorbance was measured without Ca^{2+}. Data are shown as the means ± SD of 5 experiments. *P<0.05 vs. control. #P<0.05 vs. LV-Control. &P<0.05. AU, arbitrary units.

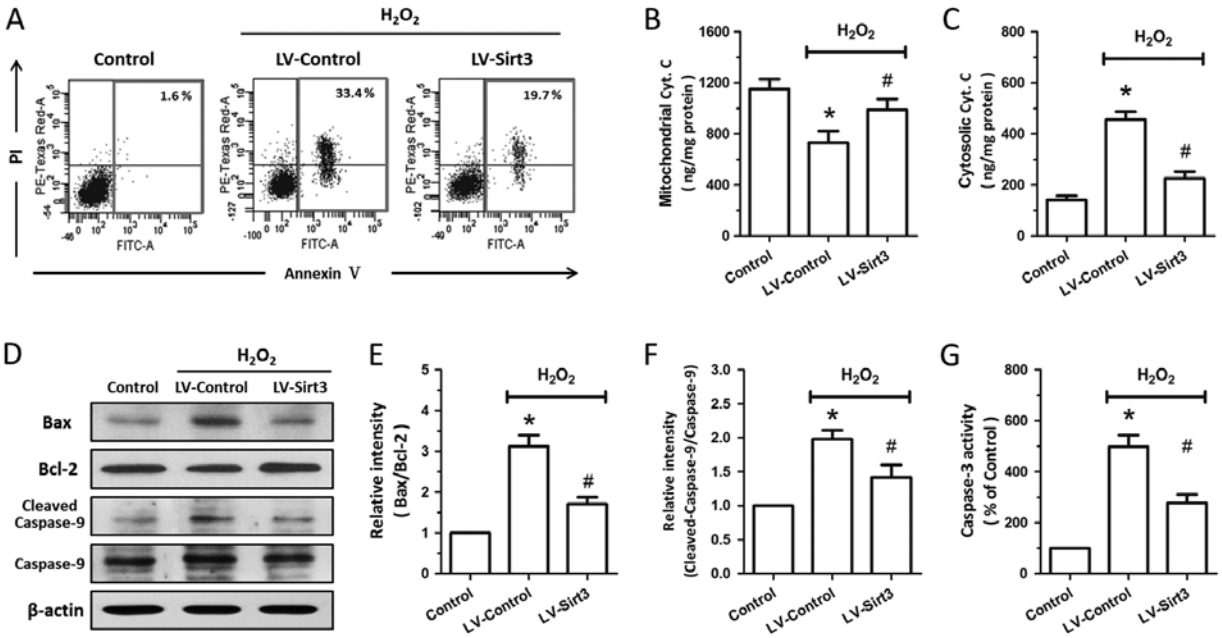

Figure 7. Overexpression of Sirt3 inhibits mitochondrial-associated apoptosis. Following transfection with lentivirus expressing Sirt3 (LV-Sirt3) or control lentivirus (LV-Control) for 72 h, the HT22 cells were treated with or without 500 μM H_2O_2, and (A) apoptotic cell death was detected by flow cytometry. (B) Mitochondrial cytochrome *c* and (C) cytosolic cytochrome *c* were measured. (D) The expression levels of Bax, Bcl-2, cleaved caspase-9 and caspase-9 were determined by western blot analysis, and (E) the Bax/Bcl-2 ratio and (F) activity of caspase-9 were calculated. (G) The activity of caspase-3 was measured by an immunoassay kit. Data are shown as the means ± SD of 5 experiments. *P<0.05 vs. control. #P<0.05 vs. LV-Control.

Discussion

Mitochondrial proteins are acetylated at a high frequency (approximately 20%), and acetylation is an important mechanism for the regulation of mitochondrial function through the modulation of protein-protein interactions, altering complex stability or affecting enzyme activities (18). Among the 3 members of the sirtuin family localized in the mitochondria (Sirt3, Sirt4 and Sirt5), which are referred to as mitochondrial stress sensors (19), only Sirt3 has been demonstrated to robustly deacetylate mitochondrial proteins (20). The increased expression of Sirt3 has been shown to be associated with the extended lifespan of humans, and the Sirt3-mediated reprogramming of cellular metabolism may be of particular importance under oxidative stress conditions (21,22). As previously demonstrated, cells in organs with high ATP demands (such as the heart, liver and brain) present higher levels of Sirt3 expression (23), which was also found in our *in vitro* neuronal

model. The increased expression of Sirt3 has also been observed in the heart under stress conditions, whereas Sirt3 levels are reduced in hypertrophied or failing hearts (24,25). In the present study, we found that the expression of Sirt3 was increased following treatment with H_2O_2 in a dose- and time-dependent manner, and the knockdown of Sirt3 by transfection with specific siRNA aggravated the H_2O_2-induced neuronal injury. These data indicate that Sirt3 may be an endogenous protective mechanism under oxidative stress conditions, and that the overexpression of Sirt3 may provide an incremental protective effect against H_2O_2 injury.

H_2O_2 is often considered to be a toxic molecule since it was discovered by Thénard in 1818. It has been implicated in several neuropathological conditions, such as brain trauma, cerebral ischemia and neurodegenerative diseases (3,4). In previous studies, a marked and rapid increase in H_2O_2 levels was recorded in the reperfusion phase following transient brain ischemia (26), and the concentrations of H_2O_2 in rat vascular smooth muscle cells following ischemia and reperfusion injury were higher than 1 mM (27). H_2O_2-induced oxidative stress and ROS generation contributes to cell death by the oxidation of several important lipids, proteins and nucleic acids, which further damage mitochondrial membrane integrity and inhibit energy production. In the present study, we found that the overexpression of Sirt3 attenuated the H_2O_2-induced ROS generation and lipid peroxidation, suggesting that Sirt3 activates the endogenous antioxidant system through the recruitment of antioxidants, such as SOD, CAT, GPx and GST, thereby attenuating H_2O_2-induced neuronal injury. To confirm this assumption, we also measured the enzymatic activity of SOD, CAT, GPx and GST following H_2O_2-induced injury in cells with or without Sirt3 overexpression. SOD, in its 3 isoforms (cytosolic Cu, Zn-SOD, extracellular Cu, Zn-SOD and mitochondrial Mg-SOD), is responsible for H_2O_2 production from $^{\cdot}O2^{-}$ (28), and H_2O_2 is subsequently converted to H_2O by GPx or decomposed in peroxisomes to H_2O and O_2 by CAT (29,30). Accumulating evidence has suggested that the elevation of these endogenous antioxidants plays a protective role against H_2O_2-induced oxidative stress. Intriguingly, our results demonstrated that the overexpression of Sirt3 had no effect on the decreased enzymatic activity of SOD, CAT, GPx and GST induced by H_2O_2 insults, indicating the presence of an endogenous antioxidant system which is independent of the protective mechanisms of Sirt3 overexpression; this requires further investigation.

The electron transport chain (ETC) plays important roles in oxidative stress, not only due to its function in creating a transmembrane proton gradient, which is required for the generation of ATP through protons, but also as the dysregulated ECT function results in electron leak and increased ROS production through a series of protein complexes (I-IV) (9). Sirt3 physically interacts with the 39-kDa protein, NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 9 (NDUFA9), one of the known subunits of complex I, and regulates the complex I acetylation level and activity (23). Both the increased expression of Sirt3 and complex II activity have been observed in K562 cell lines following treatment with kaempferol (31). Recently, a 56-kDa core I subunit of complex III was identified as a potential target of Sirt3 by immunoprecipitation with anti-acetyl-lysine antibody from mitochondrial lysates (32). Our results revealed that the overexpression of Sirt3 preserved the decreased activity of mitochondrial complex I, II and IV, and increased mitochondrial ATP synthesis following H_2O_2-induced injury. It is conceivable that the upregulation of Sirt3 preserves ATP generation in injured mitochondria to meet the cellular energy requirements, and this action of Sirt3 on mitochondrial energy metabolism may contribute to its neuroprotective effects against H_2O_2-induced oxidative injury.

Even though Ca^{2+} is well known as a crucial second messenger regulating cellular physiological functions, there are two opposite sides to the effects of Ca^{2+} on mitochondrial functions: it may either be beneficial for mitochondrial function in the processes of oxidative phosphorylation and ATP synthesis or it may be detrimental in instigating subsequent pathological cascades (33). Although the endoplasmic reticulum (ER) is considered to be the main site of intracellular Ca^{2+} storage, the mitochondria also serve as an important intracellular calcium buffer shaping Ca^{2+} signaling (34). Recent evidence has validated that mitochondrial Ca^{2+} loading plays crucial roles in apoptotic cell death under oxidative stress, possibly through nitric oxide production, cytochrome *c* dissociation, mitochondrial permeability transition pore (mPTP) opening with the release of cytochrome *c* and Ca^{2+}-calmodulin dependent protein kinase activation (35). A previous study demonstrated that when exposed to Ca^{2+} under normoxic conditions, mitochondria isolated from rat cerebral cortex generate reactive hydroxyl ($^{\cdot}OH$), even in the absence of respiratory chain inhibitors (36). In the present study, we found that treatment with H_2O_2 resulted in a ~50% reduction in Ca^{2+} buffering capacity in the isolated mitochondria, whereas the overexpression of Sirt3 significantly preserved the Ca^{2+} buffering capacity compared to that in the LV-Control-transfected cells. Considering the present data that the Ca^{2+} buffering capacity in the mitochondria decreases with the process of mitochondrial Ca^{2+} loading and the subcellular localization of Sirt3 in the mitochondria (37,38), a hypothetical molecular mechanism explaining the relation between Sirt3 and the mitochondrial Ca^{2+} flux is that Sirt3 is involved in the Ca^{2+} transfer from the cytoplasm to the mitochondria through the regulation of mitochondrial calcium uniporters (mCU), such as uncoupling proteins (UCPs) and voltage-dependent anion channels (VDAC) (39). These data suggest that Sirt3 acts as a mitochondrial Ca^{2+} regulator through its interactions with other mitochondrial proteins; this requires confirmation in *in vitro* neuronal models.

The mitochondrion plays a prominent role in the induction of apoptotic cell death following oxidative stress. The mitochondria-associated intrinsic apoptotic pathway is mediated by the interplay between anti-apoptotic and pro-apoptotic Bcl-2 family proteins, which is initiated by the translocation of the pro-apoptotic protein, Bax, from the cytoplasm to the mitochondria (40). The disruption of mitochondrial membrane integrity and the opening of mPTP result in the release of cytochrome *c*, which further activates caspase-9 and in turn causes the cleavage of caspase-3 (40,41). Previous studies have indicated that under caloric restriction (CR) conditions, the upregulated expression of Sirt3 directly deacetylates cyclophilin D (CypD), preventing its association with the adenine nucleotide translocator (ANT) and therefore blocking mPTP formation (42,43). SIRT3 is required for the regulation of

cytochrome *c* superoxide-scavenging capacity through deacetylation and the activation of complex IV (44). However, the exact role of Sirt3 in apoptosis is contradictory, largely depending upon cell types. Previous studies have demonstrated the pro-apoptotic effects of Sirt3 in leucocythemia and colorectal cancer cells (45,46), whereas the anti-apoptotic activity of Sirt3 and the Sirt3-mediated anti-apoptotic mechanisms have been reported by several other studies (10,47,48). Recent observations also hint at additional neuroprotective effects of SIRT3, involving the regulation of mitochondrial dynamics (9,49,50). In the present study, we found that the overexpression of Sirt3 significantly inhibited cytochrome *c* release, the increase in the Bax/Bcl-2 ratio, as well as caspase-3 and caspase-9 activation, and attenuated neuronal apoptosis following treatment with H_2O_2. These data indicate that the protective effects of Sirt3 in oxidative stress may partly be mediated by mitochondrial-associated apoptosis, and the anti-apoptotic activity of Sirt3 following treatment with H_2O_2 was confirmed in our *in vitro* neuronal injury model.

In conclusion, our results demonstrate that Sirt3 not only reduces H_2O_2-induced ROS overgeneration and lipid peroxidation, but also attenuates the mitochondrial dysfunction and subsequent activation of apoptosis. The increased expression of Sirt3 induced by oxidative stress may be an endogenous protective mechanism, which is partly dependent on the preservation of mitochondrial calcium homeostasis. Thus, metabolic rescue observed upon the overexpression of Sirt3 may represent an appropriate strategy to avoid neuronal death in a broad range of neuronal disorders, where H_2O_2-related oxidative stress may play a major role, such as in cerebral ischemia-reperfusion injury and neurodegenerative diseases.

Acknowledgements

The present study was supported by grants from the National Natural Science Foundation of China (nos. 81071034, 81371447, 81301037, 81200949 and 30930093), research funds from the government (nos. AWS11J008 and 2012BAI11B02) and the Program for Changjiang Scholars and Innovative Research Team in University (no. IRT1053).

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