Lycopene protects human SH-SY5Y neuroblastoma cells against hydrogen peroxide-induced death via inhibition of oxidative stress and mitochondria-associated apoptotic pathways

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Abstract. Oxidative stress, which is characterized by excessive production of reactive oxygen species (ROS), is a common pathway that results in neuronal injury or death due to various types of pathological stress. Although lycopene has been identified as a potent antioxidant, its effect on hydrogen peroxide (H_2O_2)-induced neuronal damage remains unclear. In the present study, pretreatment with lycopene was observed to protect SH-SY5Y neuroblastoma cells against H_2O_2-induced death via inhibition of apoptosis resulting from activation of caspase-3 and translocation of apoptosis inducing factor (AIF) to the nucleus. Furthermore, the over-produced ROS, as well as the reduced activities of anti-oxidative enzymes, superoxide dismutase and catalase, were demonstrated to be alleviated by lycopene. Additionally, lycopene counteracted H_2O_2-induced mitochondrial dysfunction, which was evidenced by suppression of mitochondrial permeability transition pore opening, attenuation of the decline of the mitochondrial membrane potential, and inhibition of the increase of Bax and decrease of Bcl-2 levels within the mitochondria. The release of cytochrome *c* and AIF from the mitochondria was also reduced. These results indicate that lycopene is a potent neuroprotectant against apoptosis, oxidative stress and mitochondrial dysfunction, and could be administered to prevent neuronal injury or death.

Introduction

Oxidative stress is important in mediating brain injury caused by different pathological conditions, such as cerebral ischemia/reperfusion, head trauma, epilepsy and neurodegenerative diseases (1-4). A previous study demonstrated that neurons, glial cells, and cerebral microvasculature could be damaged during the process of oxidative stress (5). The crucial feature of oxidative stress is excessive production of reactive oxygen species (ROS), such as superoxide anions (O_2^-), hydroxyl radicals and hydrogen peroxide (H_2O_2), which are regarded as being the result of disrupted equilibrium between their formation and clearance (5). ROS may attack intracellular biological macromolecules, including nuclear acids, proteins and lipids, induce dysfunction of mitochondria, and activate signaling pathways leading to apoptosis (6). Among the various ROS, H_2O_2 easily diffuses in and out of cells and tissues (6), and is readily converted into highly reactive hydroxyl radicals by the Fenton reaction (7). Thus, H_2O_2 has been considered to be an appropriate molecule to induce oxidative stress and is extensively used to investigate the protective effect of certain agents on cellular injury (8).

Lycopene, a lipid-soluble carotenoid compound, is often present in tomatoes and red fruits, including watermelon, pink grapefruit and guava (9). Although it has multiple biological functions, such as inhibition of inflammation, and suppression of cellular carcinogenesis and tumor growth (10,11), accumulating evidence has indicated that lycopene exerts a strong protective effect against brain damage. A population-based follow-up study demonstrated that males in the highest quartile of serum lycopene concentrations exhibited 59 and 55% lower risks of ischemic stroke when compared with males in the lowest quartile (12). Furthermore, animal experiments using rat models demonstrated that lycopene prevents brain injury caused by focal or global ischemia and reperfusion (13,14), and alleviates cognition dysfunction induced by colchicine and rotenone (15,16). *In vitro* research indicated that lycopene protects against neuronal apoptosis induced by different neurotoxic compounds, including 1-methyl-4-phenylpyridinium (MPP^+), methylmercury, amyloid β, trimethyltin and 6-hydroxydopamine (17-21). Despite previous data, which revealed that lycopene possesses a potent antioxidant capacity (22) and inhibition of oxidative stress is the common mechanism responsible for its neuroprotection, the effect of lycopene on activation of neuronal apoptosis pathways remains unclear.

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SH-SY5Y cells are human neuroblastoma cells, which are comparable to neurons with regards to their morphological, neurochemical and electrophysiological properties and have been extensively applied to evaluate neuronal injury or death in neurodegenerative disease, cerebral ischemia/reperfusion and epilepsy (23-25). Therefore, the present study used H_2O_2-induced apoptosis in SH-SY5Y cells as a model to investigate the effect of lycopene on the activation of apoptotic pathways induced by oxidative stress.

Materials and methods

Drugs and chemicals. Lycopene was purchased from Sigma-Aldrich (St. Louis, MO, USA) and was dissolved in tetrahydrofuran (Sigma-Aldrich) prior to each experiment. H_2O_2 was purchased from Wuhan Boster Biological Technology, Ltd. (Wuhan, China). Superoxide Dismutase (SOD) activity and Catalase assay kits were obtained from BioVision Inc. (Milpitas, CA, USA). A lactate dehydrogenase (LDH) assay was purchased from Beyotime Institute of Biotechnology (Haimen, China). Rabbit anti-caspase-3 polyclonal antibody (ab44976), rabbit anti-apoptosis inducing factor (AIF) polyclonal antibody ab1998) and mouse anti-β-actin monoclonal antibody (ab8226) were purchased from Abcam (Cambridge, MA, USA). Rabbit anti-Bcl-2-like protein 4 (Bax) polyclonal antibody (cat. no. 2774) and rabbit anti-B-cell lymphoma 2 (Bcl-2; cat. no. 4223), rabbit anti-lamin B1 (cat. no. 13435), rabbit anti-cytochrome *c* (Cyt *c;* cat. no. 4280) and mouse anti-cytochrome *c* oxidase IV (COX IV; cat. no. 11967) monoclonal antibodies were from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig) G and horse anti-mouse IgG were from Cell Signaling Technology, Inc. (cat. nos. 7074 and 7076, respectively). Enhanced chemiluminescence (ECL) western blotting detection reagents were purchased from GE Healthcare Life Sciences, and polyvinylidene difluoride (PVDF) membranes were from EMD Millipore (Billerica, MA, USA).

Cell culture and groups. Human SH-SY5Y neuroblastoma cells were obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum, 2 mmol/l glutamine (Sigma-Aldrich), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich), and maintained in a humid environment at 37˚C and 5% CO_2 atmosphere. The medium was replaced twice per week.

The cells were randomized to the following groups: Control (normal SH-SYSY cells), lycopene (the cells were treated only with lycopene at the indicated concentrations), H_2O_2 (the cells were treated alone with H_2O_2 at indicated concentration), and H_2O_2 + lycopene (the cells were treated for 2 h with lycopene followed by a 24-h incubation with H_2O_2).

Cellular viability assay. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Sigma-Aldrich) was performed to determine cell viability. SH-SY5Y cells were seeded at a density of $4x10^4$ cells per well on collagen-coated 96-well plates for 24 h. After being treated as described in each experimental group, the absorbance value at 570 nm was read using an automatic multi-well spectrophotometer (xMark™; Bio-Rad Laboratories, Inc., Richmond, CA, USA). The results are expressed as the percentage of MTT reduction relative to the absorbance of control cells.

LDH release assay. SH-SY5Y cells were seeded into 96-well culture plates at a density of $4x10^4$ cells/well. The cells, with or without lycopene pretreatment, were incubated with 400 μmol/l H_2O_2 for 24 h. According to the manufacturer's instructions, the supernatant from each sample was used in the LDH assay. The LDH activity was measured by monitoring the reduction of pyruvic acid. The absorbance of each sample was determined at 490 nm with a microplate reader (xMark™). LDH release was expressed as the percentage (%) of the total LDH activity (LDH in the medium + LDH in the cell), according to the following equation: % LDH release = (LDH activity in the medium / total LDH activity) x100.

Detection of apoptosis by flow cytometry. Apoptosis was examined by analysis of DNA fragmentation using flow cytometry. After a 24-h incubation with 400 μmol/l H_2O_2 following a 2-h treatment with or without lycopene, SH-SY5Y cells were collected by 0.25% trypsin (Sigma-Aldrich) and washed once with phosphate-buffered saline (PBS). The cells were then fixed in 70% ethanol at 4˚C overnight, treated with 100 mg/l RNase (Sigma-Aldrich) at 37˚C for 30 min and stained with 50 mg/l propidium iodide (Sigma-Aldrich) for 30 min. Finally, the cells were analyzed using flow cytometry (FACScan; BD Biosciences, San Jose, CA, USA). The rate of apoptosis was analyzed using CellQuest software, version 5.1 (BD Biosciences). Data acquisition was conducted by collecting 20,000 cells per tube, and the numbers of viable and apoptotic cells were determined for each experimental condition.

Transmission electron microscopy. SH-SY5Y cells from the control group and 400 μmol/l H_2O_2 group were harvested using 0.25% trypsin, washed with PBS, collected by centrifugation for 10 min at 1,500 x g and treated as previously described by Watkins and Cullen (26). Briefly, the cells were fixed in ice-cold 2.5% glutaraldehyde (Sigma-Aldrich) in PBS (pH 7.3), rinsed with PBS, post-fixed in 1% osmium tetroxide (Sigma-Aldrich) with 0.1% potassium ferricyanide (Sigma-Aldrich), dehydrated through a graded series of ethanol (30-90%) and embedded in Epon (Energy Beam Sciences, Agawam, MA, USA). Semi-thin (300 nm) sections were sliced using a Reichart Ultracut E Ultra-Microtome (Leica Microsystems, Inc., Buffalo Grove, IL, USA), stained with 0.5% Toluidine Blue (Sigma-Aldrich) and examined under a light microscope (Olympus IX71; Olympus Corporation, Tokyo, Japan). Ultra-thin sections (65 nm) were stained with 1% uranyl acetate and 0.1% lead citrate (both Sigma-Aldrich), and examined on a JEM-2000EX transmission electron microscope (JEOL USA, Inc., Pleasanton, CA, USA).

Hoechst 33342 staining. SH-SY5Y cells were seeded into 6-well plates at a density of $2x10^5$ cells/well. After pretreatment with or without lycopene, the cells were exposed to 400 μmol/l H_2O_2 for 24 h, washed with PBS solution and

loaded with 10 μg/ml Hoechst 33342 dye (Sigma-Aldrich) for 15 min. The cells were subsequently visualized under a fluorescence microscope (Olympus IX71). Individual nuclei were visualized at a magnification of x400 to distinguish the normal uniform nuclear pattern from the characteristic, condensed coalesced chromatin pattern of apoptotic cells. To quantify apoptosis, 400 nuclei from six random microscopic fields were analyzed by an observer blinded to the treatment groups. The total number of apoptotic cells in each section was summed and expressed as the percentage of the total cell number. A minimum of 10 individual sections were evaluated per slide.

Measurement of intracellular ROS levels. The average density of intracellular ROS was evaluated in cells loaded with the redox-sensitive dye, dichloro-dihydro-fluorescein diacetate (Molecular Probes DCFH-DA; Thermo Fisher Scientific, Inc.). SH-SY5Y cells, with or without lycopene pretreatment, were seeded into 96-well culture plates at a density of $4x10^4$ cells/well, exposed for 24 h to 400 μmol/l H_2O_2, washed twice with PBS, stained with 20 μmol/l DCFH-DA in the dark for 30 min and harvested. The cells were then dissolved with 1% Triton X-100 (Sigma-Aldrich). Fluorescence was observed under a fluorescent light microscope, and measured at excitation and emission wavelengths of 485 and 530 nm, respectively using a fluorescence spectrometer (HTS 7000; PerkinElmer, Inc., Waltham, MA, USA). The ROS levels were expressed as arbitrary unit/mg protein and as a percentage of the control.

Measurement of mitochondrial membrane potential. Mitochondrial membrane potential was determined by the retention of the dye, rhodamine 123 (Sigma-Aldrich). SH-SY5Y cells, with or without lycopene pretreatment, were exposed for 24 h to 400 μmol/l H_2O_2. The cells were collected using 0.25% trypsin and washed twice with PBS, followed by incubation with 10 μg/ml rhodamine 123 at 37˚C for 30 min. The cells were washed again with PBS, and fluorescence intensities of rhodamine 123 in cells were analyzed by flow cytometry (FACScan).

Determination of mitochondrial permeability transition pore (MPTP) opening. The opening of MPTP was determined using a calcein-cobalt assay kit (Genmed Scientifics, Inc., Wilmington, DE, USA) as described previously (20). Briefly, SH-SY5Y cells were seeded into 24-well plates at a density of $5x10^4$ cells/well. After pretreatment with or without lycopene, the cells were exposed to 400 μmol/l H_2O_2 for 24 h, washed twice with 1 ml Reagent A, incubated for 20 min at 37˚C with 250 μl each of Reagents B (calcein AM) and C (cobalt dichloride; 1:50; 500 μl per well). The cells were subsequently washed twice with pre-warmed Reagent A. The fluorescence intensity was measured on a microplate reader with excitation and emission wavelengths of 488 and 505 nm, respectively. After the fluorescence intensity was determined, the protein concentration for each well was measured using a Bradford protein assay kit (Bio-Rad Laboratories, Inc.). The fluorescent signals were normalized to total protein content in the corresponding cell extract and data were presented as normalized relative fluorescence units.

Measurement of cellular anti-oxidative enzymes. The activity of antioxidant enzymes, SOD and catalase were measured according to the manufacturer's instructions. After incubation for 24 h with 400 μmol/l H_2O_2 following pretreatment with or without lycopene, SH-SY5Y cells were collected from the culture dishes using a scraper, centrifuged at 1,000 x g for 10 min at 4˚C, and the cell pellets were washed with PBS. For the SOD activity assay, the cells were suspended in ice-cold lysis buffer [0.1 M Tris/HCl (pH 7.4), 0.25 mol/l sucrose, 5 mmol/l β-mercaptoethanol and 0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF); Sigma-Aldrich], homogenized with a glass Pyrex microhomogenizer (20 strokes; Beyotime Institute of Biotechnology), and centrifuged at 1,500 x g for 5 min at 4˚C. The supernatant was then collected for assaying. For the catalase activity assay, the cells were suspended in ice-cold assay buffer (Sigma-Aldrich), homogenized with a glass Pyrex microhomogenizer (20 strokes), centrifuged at 10,000 x g for 15 min at 4˚C. The supernatant was then collected for assaying. The catalase was spectrophotometrically determined by measuring decreased absorbance at 570 nm, using the catalase assay kit, and SOD was measured spectrophotometrically by monitoring the absorbance at 450 nm using the SOD assay kit. Catalase and SOD activities were expressed as U/mg protein.

Immunocytochemistry. The collected SH-SY5Y cells were washed with PBS and fixed in 4.0% paraformaldehyde (Sigma-Aldrich) for 30 min at 4˚C. After being washed with PBS three times for 5 min each time, cells were incubated with 1% Triton X-100 for 10 min, and blocked at nonspecific antibody binding sites by incubating with 5% bovine serum albumin (BSA; Sigma-Aldrich) in PBS containing 0.3% Triton X-100 for 1 h at room temperature. The cells were then incubated with a polyclonal antibody against AIF (1:100) at 4˚C overnight followed by incubation in cy3-conjugated goat anti-rabbit IgG (1:200) for 50 min at room temperature. After washing with PBS, cells were incubated with Hoechst 33342 for 30 min. After three washes, the cells were mounted on slides and visualized under a fluorescence microscope.

Differential centrifugation and cellular fraction. The collected cells were washed with PBS, and centrifuged for 10 min at 1,000 x g, after which the cell pellets were suspended in ice-cold lysis buffer containing 15 mmol/l Tris, pH 7.6, 250 mmol/l sucrose, 1 mmol/l $MgCl_2$, 2.5 mmol/l EDTA, 1 mmol/l ethylene glycol-bis (β-aminoethyl ether) tetraacetic acid, 1 mmol/l dithiothreitol, 1.25 mg/ml pepstatin A, 10 mg/ml leupeptin, 2.5 mg/ml aprotinin, 1.0 mmol/l PMSF, 0.1 mmol/l Na_3VO_4, 50 mmol/l NaF, and 2 mmol/l $Na4P_2O_7$ (Sigma-Aldrich) and homogenized with a glass Pyrex microhomogenizer (20 strokes). The homogenates were centrifuged at 800 x g at 4˚C for 10 min to obtain the pellet containing the nuclear fraction. The supernatants were then centrifuged at 15,000 x g at 4˚C for 10 min to obtain the pellet containing the mitochondria and the supernatant containing the cytosolic fraction. All the pellets were resuspended in lysis buffer. The protein content of each cellular fraction was determined using the Bradford protein assay kit.

Gel electrophoresis and western blotting. Equal protein quantities were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to PVDF

membranes. The membranes were blocked with 3% BSA in Tris-buffered saline for 30 min and then incubated overnight at 4˚C with primary antibodies against AIF (1:1,000), Bax (1:1,000), Bcl-2 (1:1,000), caspase-3 (1:1,000), Cyt *c* (1:1,000), COX IV (1:1,000), β-actin (1:3,000) and lamin B1 (1:1,000). After being incubated with HRP-conjugated goat anti-rabbit IgG (1:2,000) or horse anti-mouse IgG (1:2,000), blots were washed and immunoreactive proteins were visualized on a Kodak X-omat LS film (Eastman Kodak Co., New Haven, CT, USA) with ECL. Densitometry was performed using Kodak ID Image Analysis Software, version 3.4.5 (Eastman Kodak Co.).

Statistical analysis. All data represent at least four independent experiments and are expressed as means ± standard deviation. Statistical comparisons were conducted using one-way analysis of variance and P<0.05 was considered to indicate a statistically significant difference.

Results

Lycopene inhibited H_2O_2-induced cell death in SH-SY5Y cells. H_2O_2-induced changes in the viability of SH-SY5Y cells were firstly examined by MTT assay. When compared with the control group, the viability of the cells exposed for 24 h to H_2O_2 at the indicated concentrations decreased significantly (Fig. 1A; P<0.01). Furthermore, the H_2O_2-induced reduction in cellular viability was concentration-dependent. Given that the viability of the cells treated with 400 μmol/l H_2O_2 reduced to 52.1±2.3% in comparison with that in the control group, the 400-μmol/l concentration was used in subsequent experiments to examine the effect of lycopene on H_2O_2-induced changes in SH-SY5Y cells.

As shown in Fig. 1B, no obvious differences were identified in the cellular viability between the cells in the control group and the cells treated lycopene alone at the indicated concentrations. By contrast, decreased cellular viability caused by 400 umol/l H_2O_2 was improved significantly by pretreatment with lycopene at the concentration of 2.0, 4.0 and 8.0 μmol/l lycopene (P<0.01), despite no significant difference being observed in the protective effect of lycopene between the 4.0 and 8.0 μmol/l groups.

Additionally, the effect of lycopene on H_2O_2-induced toxicity was examined by measuring the quantity of LDH in the culture medium. As Fig. 1C shows, the leakage of LDH significantly increased to 1,250.6±85.8% in the group exposed to 400 μmol/l H_2O_2 when compared with that in the control group (P<0.01). However, the elevated LDH leakage caused by H_2O_2 was suppressed to 962.5±79.8 and 378.9±36.7% by pretreatment with 2.0 and 4.0 μmol/l lycopene, respectively (Fig. 1C; P<0.01). These results indicate that lycopene exerts a protective effect on cells exposed to a lethal concentration of H_2O_2.

Lycopene inhibited H_2O_2-induced apoptosis in SH-SY5Y cells. Apoptosis is a form of programmed neuronal death caused by H_2O_2 (27); therefore, the present study examined the effect of lycopene on H_2O_2-induced apoptosis in SH-SY5Y cells. Fluorescence microscopy in combination with Hoechst 33342 staining showed that the nuclei in the H_2O_2 group were polygonal, condensed and bright blue in color

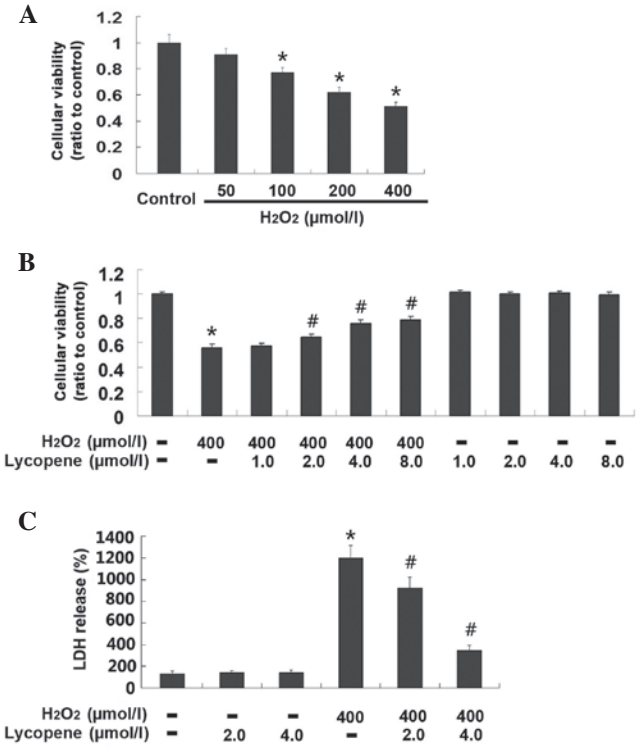

Figure 1. Lycopene inhibited H_2O_2-induced cell death in SH-SY5Y cells. (A) MTT assay of the viability of SH-SY5Y cells exposed to H_2O_2 for 24 h at the indicated concentrations. (B) MTT assay of the changes in the viability of SH-SY5Y cells exposed to H_2O_2 as a result of pretreatment with lycopene. (C) LDH release assay of H_2O_2-induced toxicity in SH-SY5Y cells pretreated with or without lycopene. *P<0.01 vs. control group; #P<0.01 vs. H_2O_2 group. H_2O_2, hydrogen peroxide; LDH, lactate dehydrogenase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

when compared with those in the control group (Fig. 2A). Cell counting under a florescence microscope demonstrated that the percentage of cells with apoptotic features was 32.6±4.8% in the H_2O_2 group, which was significantly higher than in the control group (5.2±0.7%; P<0.01). However, this reduced to 25.1±1.9 and 15.2±1.7% in the cells pretreated with 2.0 and 4.0 μmol/l lycopene, respectively (Fig. 2B). Fig. 2C demonstrates the transmission electronic microscopy images of the cells exposed to H_2O_2, demonstrating membrane blebbing, chromatin accumulation beneath the nucleus membrane and nucleus condensation, which are consistent with the morphological features of apoptotic cells (Fig. 2C). Furthermore, flow cytometry indicated that the apoptosis rate in the control and H_2O_2 groups was 3.21 and 42.04%, respectively. By contrast, 2.0 and 4.0 μmol/l lycopene decreased the apoptosis rate to 26.55 and 17.87%, respectively (Fig. 2D). These results indicate that lycopene protects SH-SY5Y cells against H_2O_2-induced death via inhibition of apoptosis.

Lycopene mitigated oxidative stress caused by H_2O_2. ROS is an important indicator of oxidative stress, which is also a trigger of apoptosis. Therefore, ROS production caused by H_2O_2 was examined in cells pretreated with or without lycopene. The green fluorescence in the group exposed to 400 μmol/l H_2O_2 was observed to be markedly stronger than that in the control group, however, was attenuated by pretreatment with either 2.0 or 4.0 μmol/l lycopene

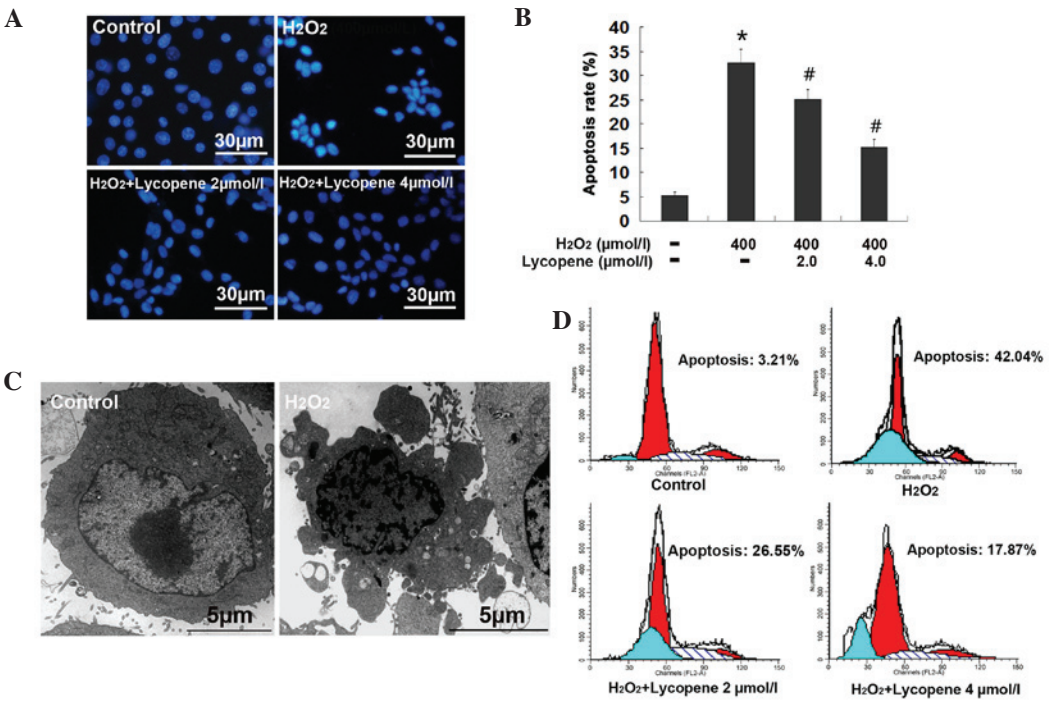

Figure 2. Lycopene inhibited H_2O_2-induced apoptosis in SH-SY5Y cells. (A) Representative images of nuclei stained with Hoechst 33342. (B) Quantification of the nuclei demonstrating apoptotic features observed under a fluorescence microscope. (C) Representative images of the control and apoptotic cells observed under a transmission electronic microscope. The cells treated with H_2O_2 had membrane blebbing, chromatin accumulation beneath the nuclear membrane and nuclear condensation. (D) Flow cytometric analysis of the effect of lycopene on H_2O_2-induced apoptosis in SH-SY5Y cells. *P<0.01 vs. control group; #P<0.01 vs. H_2O_2 group. H_2O_2, hydrogen peroxide.

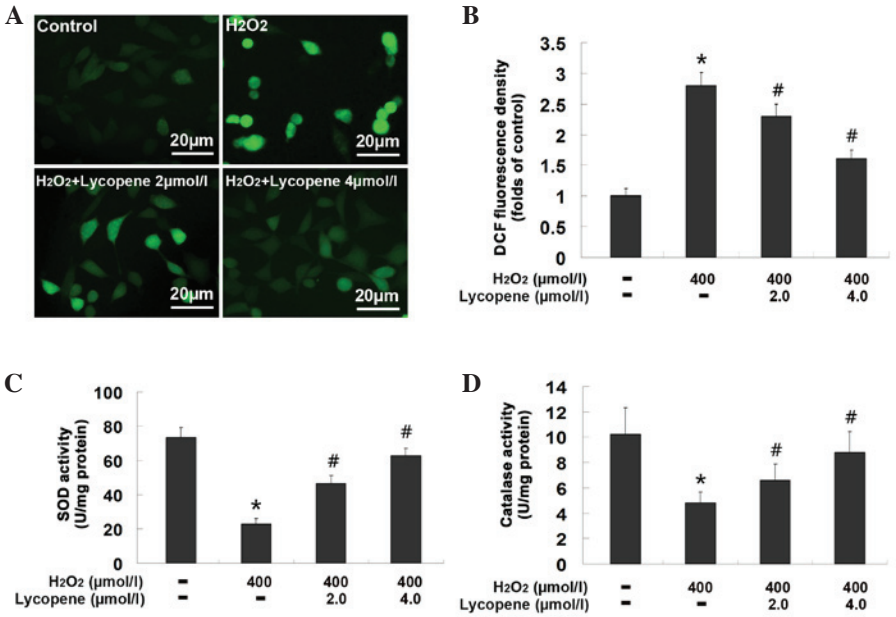

Figure 3. Lycopene mitigated H_2O_2-induced oxidative stress in SH-SY5Y cells. (A) Representative fluorescence microscopy images of the cells stained with DCFH-DA. (B) Quantification of fluorescence density of the cells stained with DCFH-DA. (C) Activity assay of SOD. (D) Activity assay of catalase. *P<0.01 vs. control group. #P<0.01 vs. H_2O_2 group. DCFH-DA, dichloro-dihydro-fluorescein diacetate; DCF, 2',7'-dichlorofluorescein; SOD, superoxide dismutase; H_2O_2, hydrogen peroxide.

(Fig. 3A). Additionally, the spectrometric assay showed that the fluorescence intensity in the 400 μmol/l H_2O_2 group was 2.83±0.21 times higher than that in the control group (P<0.01), but reduced to 2.34±0.19 times and 1.67±0.15 times in the cells pretreated with 2.0 and 4.0 μmol/l lycopene, respectively (Fig. 3B; P<0.01 vs. the H_2O_2 group). These results indicate that pretreatment with lycopene inhibits H_2O_2-induced production of ROS in SH-SY5Y cells.

As the antioxidant enzymes, SOD and catalase may inhibit the ROS level within cells, the effect of lycopene on the enzy-

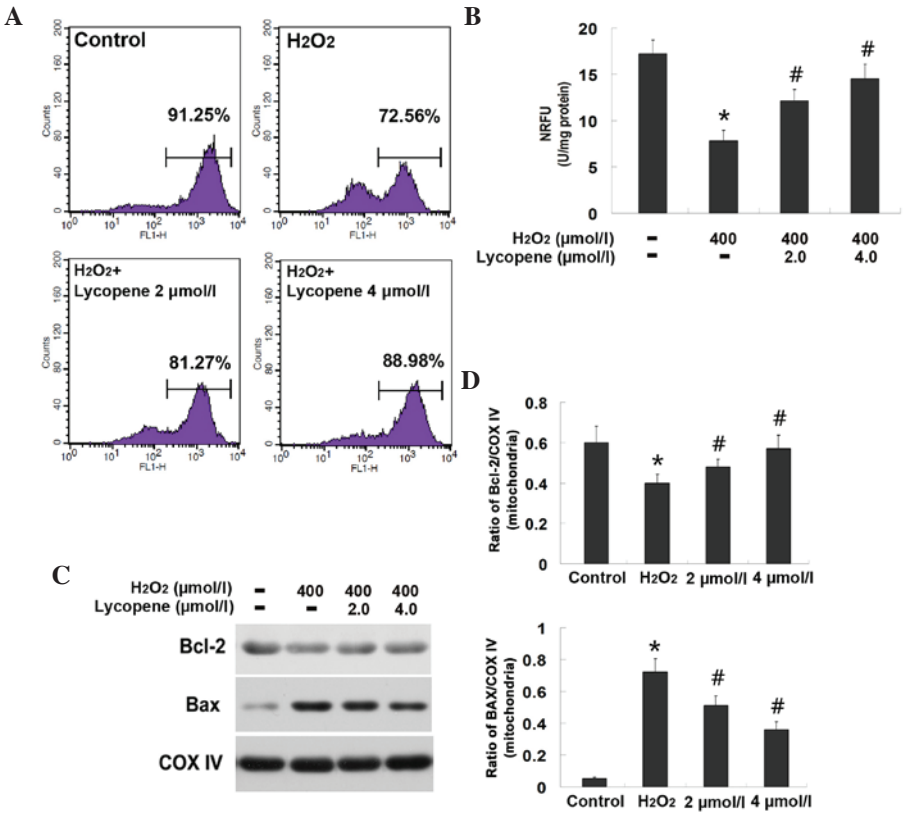

Figure 4. Lycopene counteracted mitochondrial dysfunction caused by H_2O_2. (A) Measurement of mitochondrial permeability transition pore opening. (B) Analysis of mitochondrial membrane potentials by flow cytometry. (C) Western blot analysis of Bax and Bcl-2 protein levels within mitochondria. (D) Quantification of Bax and Bcl-2 protein levels. *P<0.01 vs. control group. #P<0.01 vs. H_2O_2 group. NRFU, normalized relative fluorescence units; Bcl-2, B-cell lymphoma 2; Bax, Bcl-2-like protein 4; COX IV, cytochrome *c* oxidase subunit IV; H_2O_2, hydrogen peroxide.

matic activities of SOD and catalase were examined in the present study. As shown in Fig. 3C and D, the activity of SOD in the cells exposed to 400 μmol/l H_2O_2 was 22.8±3.23 U/mg protein, significantly lower than 73.66±5.39 U/mg protein in the control group (P<0.01). By contrast, it was improved to 46.72±4.53 and 62.77±5.36 U/mg protein in the cells pretreated with 2.0 and 4.0 μmol/l lycopene, respectively (P<0.01 vs. the H_2O_2 group). Similarly, the catalase activity reduced from 10.22±2.11 to 4.81±0.88 U/mg protein when SH-SY5Y cells were exposed to 400 μmol/l H_2O_2 (P<0.01), whereas pretreatment with 2.0 and 4.0 μmol/l lycopene restored its activity to 6.58±1.28 and 8.76±1.69 U/mg protein, respectively (P<0.01 vs. the H_2O_2 group). These results indicate that lycopene facilitates the maintenance of SOD and catalase activities in the cells exposed to H_2O_2.

Lycopene protected against H_2O_2-induced mitochondrial dysfunction. Mitochondrial dysfunction due to ROS attack is proposed to participate in cellular apoptosis. Therefore, depolarization of the mitochondrial membrane and opening of the MPTP, which are sensitive indicators of mitochondrial function, were assayed in the present study. As shown in Fig. 4A, the mitochondrial membrane potentials reduced from 91.25 to 72.56% after the SH-SY5Y cells were exposed to H_2O_2. However, the mitochondrial membrane potentials were restored to 81.27 and 88.98% in the groups pretreated with 2.0 and 4.0 μmol/l lycopene, respectively. This indicates that pretreatment with lycopene inhibits H_2O_2-induced depolarization of the mitochondrial membrane in SH-SY5Y cells.

Similar changes were observed in the MPTP opening (Fig. 4B). Compared with the control group, the fluorescence intensity of cells in the H_2O_2 group was significantly weakened (from 17.22±1.51 to 7.83±1.12; P<0.01), which was indicative of the opening of the MPTP. When the cells were pretreated with lycopene at concentrations of 2.0 or 4.0 μmol/l, the fluorescence intensity was significantly strengthened to 12.18±1.33 (P<0.01) and 14.49±1.47 (P<0.01), respectively, indicating closure of the MPTP.

Since it has been reported that Bax and Bcl-2 regulate the opening of MPTP (28), the mitochondrial fraction was isolated via differential centrifugation, and the levels of Bax and Bcl-2 were examined by western blotting (Fig. 4C). The protein expression level of Bax was significantly increased from 0.05±0.01 to 0.72±0.08 (P<0.01), and the expression level of Bcl-2 was significantly decreased from 0.61±0.07 to 0.38±0.04 (P<0.01), in the mitochondria of the cells exposed to H_2O_2 when compared with those in the control group (Fig. 4D). By contrast, the changes in the expression levels of Bax and Bcl-2 within the mitochondria were reversed by pretreatment with 2.0 and 4.0 μmol/l lycopene. Following pretreatment with 2.0 μmol/l lycopene, the expression levels of Bax were decreased to 0.51±0.06 and the expression levels of Bcl-2 were increased to 0.48±0.04. Notably, 4.0 μmol/l demonstrated stronger effects compared with 2.0 μmol/l lycopene; the expression levels of Bax were decreased to 0.35±0.04 and those of Bcl-2 were increased to 0.57±0.07 following pretreatment with 4.0 μmol/l lycopene (Fig. 4D). These results indicate

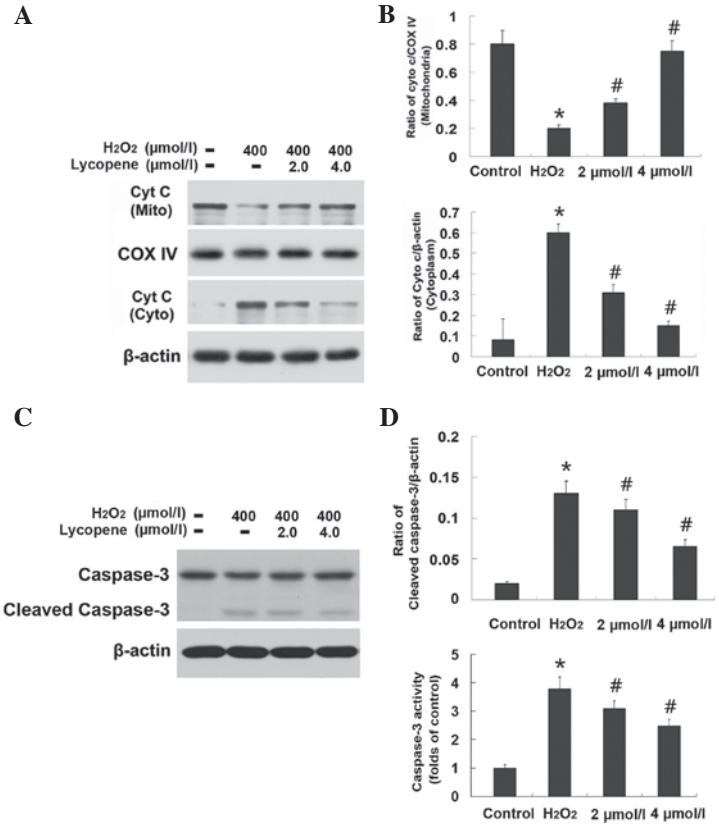

Figure 5. Lycopene attenuated the release of Cyt *c* from the mitochondria to the cytoplasm and inhibited the activation of caspase-3. (A) Western blot analysis of translocation of Cyt *c* from the mitochondria (Mito) to the cytoplasm (Cyto). (B) Quantification of the Cyt *c* protein levels in the mitochondria and cytoplasm. (C) Western blot analysis of the expression level of cleaved caspase-3. (D) Quantification of the cleaved caspase-3 protein level. *P<0.01 vs. control group. #P<0.01 vs. H_2O_2 group. Cyt *c*, cytochrhome *c*; COX IV, cytochrome *c* oxidase subunit IV; H_2O_2, hydrogen peroxide.

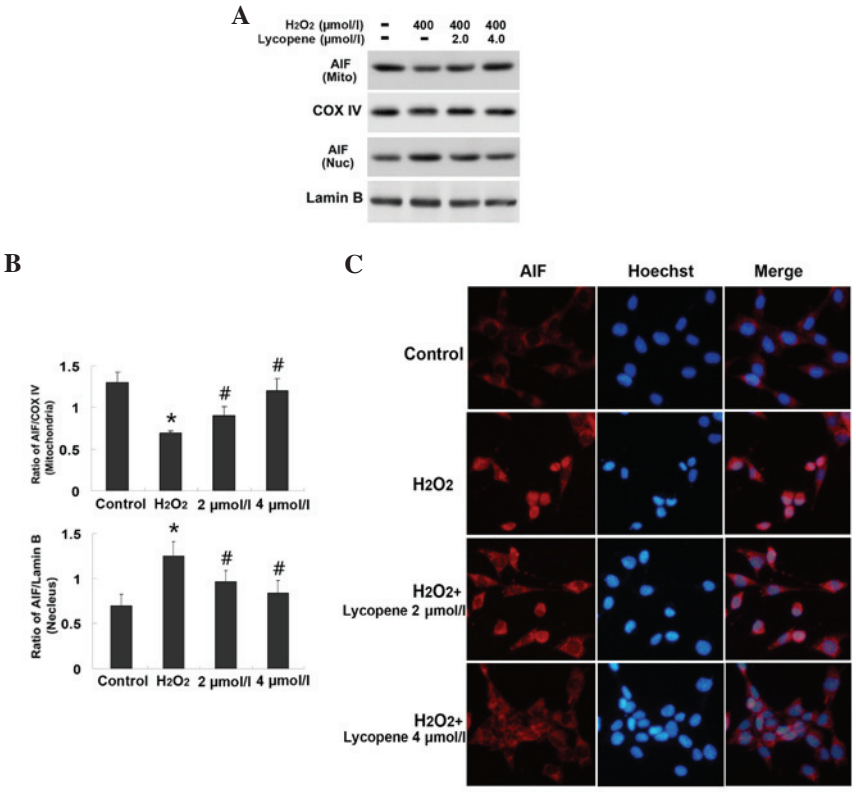

Figure 6. Lycopene suppressed the translocation of AIF from the mitochondria to the nuclei. (A) Western blot analysis of the AIF levels within the mitochondria (Mito) and nucleus (Nuc). (B) Quantification of the AIF levels. (C) Representative fluorescence microscopy images of the distribution of AIF. *P<0.01 vs. control group. #P<0.01 vs. H_2O_2 group. H_2O_2, hydrogen peroxide; AIF, apoptosis inducing factor; COX IV, cytochrome *c* oxidase subunit IV.

that lycopene is able to modulate the level of mitochondrial Bax and Bcl-2, which may clarify the molecular mechanism underlying the inhibitory effect of lycopene on H_2O_2-induced MPTP opening.

Lycopene inhibited the mitochondrial-associated apoptosis pathway. Mitochondrial proteins, including Cyt *c* and AIF, are critical in the initiation of intrinsic apoptosis signaling pathways. Thus, the sub-cellular mitochondrial, nuclear and cytoplasm fractions were isolated, and the distribution of Cyt *c* (Fig. 5) and AIF (Fig. 6) were examined by western blotting. The protein levels of Cyt *c* and AIF within the mitochondria were significantly decreased from 0.82±0.13 to 0.23±0.02 ($P<0.01$) and 1.33±0.15 to 0.73±0.03 ($P<0.01$), respectively, in the H_2O_2 group. Conversely, the level of Cyt *c* was elevated from 0.08±0.07 to 0.62±0.04 ($P<0.01$) in the cytoplasm, and the level of AIF was increased from 0.71±0.07 to 1.25±0.16 ($P<0.01$) in the nucleus (Figs. 5 and 6). Cleaved caspase-3 is a downstream apoptosis executor of the Cyt *c* signaling pathway. Notably, the levels of cleaved caspase-3 were increased from 0.02±0.0018 to 0.13±0.015 ($P<0.01$), and the activity of caspase-3 was elevated from 1.02±0.11 to 3.83±0.42 ($P<0.01$), in the cells exposed to H_2O_2 (Fig. 5). In addition, immunocytochemistry demonstrated that a proportion of mitochondrial AIF translocated into the nuclei of the cells treated with H_2O_2 (Fig. 6). By contrast, pretreatment with lycopene at either 2.0 or 4.0 μmol/l suppressed these alterations in levels of Cyt *c*, cleaved caspase-3 and AIF that were caused by H_2O_2 (Figs. 5 and 6). These results indicate that lycopene inhibits H_2O_2-induced apoptosis in SH-SY5Y cells via inhibition of caspase and AIF apoptotic pathways.

Discussion

The present study demonstrated that pretreatment with lycopene significantly inhibited H_2O_2-induced apoptosis in SH-SY5Y cells via attenuation of ROS production, protection against mitochondrial dysfunction and mitigation of the activation of mitochondria-associated apoptotic pathways. The underlying mechanisms of the inhibitory effects of lycopene on H_2O_2-induced apoptosis in SH-SY5Y cells are the inhibition of oxidative stress and suppression of mitochondria-associated apoptosis pathways.

Apoptosis is an important form of programmed neuronal death, which has been identified under various pathological conditions, such as cerebral ischemia, neurotrauma, and Parkinson's disease (29-31). H_2O_2-induced cell death in human SH-SY5Y cells is often used as an *in vitro* model to investigate neuronal apoptosis caused by oxidative stress (27,32,33). In the current study, transmission electronic and fluorescence microscopy revealed that SH-SY5Y cells treated with H_2O_2 presented morphological features of apoptosis, including membrane blebbing, chromatin accumulation beneath the nucleus membrane and nucleus condensation. By contrast, pretreatment with lycopene significantly attenuated H_2O_2-induced apoptosis in SH-SY5Y cells, which was demonstrated by Hoechst 33342 staining and flow cytometric analysis. As previous reports demonstrated that lycopene prevented apoptosis (induced by cerebral ischemia and reperfusion) in gerbil hippocampal neurons and in cardiomyocytes (where apoptosis was induced *in vitro* by hypoxia and re-oxygenation) (14,34), it was hypothesized that lycopene inhibits H_2O_2-induced death in SH-SY5Y cells via suppression of apoptosis.

Oxidative stress, characterized by accumulation of ROS, is proposed to be an initiator of neuronal apoptosis (35). The brain is an organ that is prone to producing reactive oxidative species (such as O_2^-, H_2O_2 and hydroxyl radicals) as it requires a large quantity of oxygen to maintain its normal function, but is deficient in anti-oxidants (36). Furthermore, neuronal cells are more sensitive to oxidative stress than the cells in other types of tissue (37). Therefore, inhibition of oxidative stress is considered to be a strategy to prevent neuronal apoptosis. Previous studies have shown that lycopene is a potent agent that inhibits oxidative stress, which occurs within human cells. Tang *et al* (38) found that lycopene protected human endothelial cell against H_2O_2-induced oxidative injury. Palozza *et al* (39) reported that lycopene prevented 7-ketocholesterol-induced oxidative stress in human macrophages. Consistent with these previous findings, the present study found that lycopene significantly inhibited H_2O_2-induced accumulation of ROS in SH-SY5Y cells. Lycopene is a chemical found to inhibit oxidative stress by quenching singlet oxygen and trapping peroxyl radicals (10), which may elucidate why pretreatment with lycopene in the present study inhibited the ROS levels in the SH-SY5Y cells that were exposed to H_2O_2. Furthermore, it was demonstrated that lycopene mitigated the H_2O_2-induced reduction in SOD and catalase activities. Consistently, Srinivasan *et al* (40) reported that lycopene was able to recover the damaged activities of SOD and catalase in human lymphocytes caused by gamma-radiation. SOD and catalase are the predominant members of the intracellular enzymes that have the ability to clear ROS. SOD converts O_2^- into H_2O_2, which is subsequently transformed into water and oxygen via catalase. Therefore, maintaining the activities of the anti-oxidative enzymes, SOD and catalase is another method by which lycopene clears H_2O_2-indcued excessive ROS.

The mitochondrion is an organelle that may activate apoptosis when its function is damaged. ROS, which are produced during the process of oxidative stress, may target mitochondria, resulting in the opening of the MPTP and the decline in mitochondrial membrane potentials (41). Although it was observed that lycopene is an effective agent that could prevent mitochondrial dysfunction caused by MPP^+ and hypoxia/reoxygenation (17,34), its underlying molecular mechanism remains unclear. Previously, Sandhir *et al* (42) reported that lycopene maintained mitochondrial function by suppression of mitochondrial oxidative stress that was caused by 3-nitropropionic acid in the rat brain. By contrast, the present study demonstrated that the inhibitory effect of lycopene on the H_2O_2-induced decline in mitochondrial membrane potentials and opening of MPTP in SH-SY5Y cells was associated with the fact that lycopene significantly counteracted the H_2O_2-induced increase in pro-apoptotic Bax level and reduction in anti-apoptotic Bcl-2 level within mitochondria. The Bax and Bcl-2 within mitochondria has been found to participate in the regulation of mitochondrial function, where Bax promotes and Bcl-2 blocks the formation and opening of MPTP (43). Furthermore, inhibition of MPTP opening has been identified as a promising therapeutic target for preventing cell damage (44). Therefore, the present study hypothesized

that lycopene mitigated H_2O_2-induced mitochondrial dysfunction in SH-SY5Y cells by counteracting the H_2O_2-induced reduction in Bcl-2 and elevation in Bax expression levels.

It was reported previously that lycopene attenuated apoptosis via inhibition of the nuclear factor-κB signaling pathway (45), alleviation of c-Jun N-terminal kinase activation (46), mitigation of endoplasmic reticulum stress (47) and induction of protective autophagy (48). In the present study, lycopene inhibited mitochondria-associated apoptotic pathways, which were activated in the SH-SY5Y cells that were exposed to H_2O_2. The opening of MPTP results in the release of pro-apoptotic proteins, Cyt *c* and AIF, which are normally located in the space between the mitochondrial inner and outer membranes (45). After being released as a result of apoptotic stress, Cyt *c* translocates into the cytoplasm to activate the caspase-dependent apoptotic pathway by cleaving caspase-3. Thus, cleaved caspase-3 is often regarded as a marker representing the activation of caspase-3. In the current study, pretreatment with lycopene mitigated the release of Cyt *c* from the mitochondria and suppressed the level of cleaved caspase-3 in SH-SY5Y cells that were exposed to H_2O_2. Results from previous studies indicate that inhibition of the activation of caspase-3 is one factor that is responsible for the protection of lycopene against apoptosis in various types of cells (19,38). Qu *et al* (19) reported that lycopene inhibited amyloid β-induced caspase-3 activation in cultured rat cortical neurons and Tang *et al* (38) demonstrated that lycopene attenuated the level of activated caspase-3 caused by H_2O_2 exposure in human endothelial cells. Thus, the inhibitory effect of lycopene on H_2O_2-induced apoptosis in SH-SY5Y cells is associated with its attenuation of caspase-3 apoptosis pathway activation.

AIF, as another protein that is released from the mitochondria of cells stressed by apoptotic stimuli, represents a caspase-independent apoptotic pathway. AIF translocates into the nucleus to initiate chromatin condensation and DNA fragmentation (49) and has been found to be involved in neuronal apoptosis caused by different neuropathological conditions. Ba *et al* (50) demonstrated that AIF nuclear translocation occurred following focal cerebral ischemia-reperfusion injury in rats. Yang *et al* (51) found that AIF translocation into nuclei contributed to epilepsy-induced apoptosis in hippocampal neurons *in vitro*. Thus, *in vitro* and *in vivo* studies have demonstrated that AIF is a crucial factor leading to neuronal injury or apoptosis. By contrast, *in vitro* and *in vivo* studies have demonstrated that inhibition of AIF translocation into nuclei is responsible for the protection of certain chemicals against neuronal apoptosis. Huang *et al* (52) reported that the protection of forsythiaside against H_2O_2-induced apoptosis in PC12 cells was associated with inhibiting the accumulation of AIF within the nuclei. Similarly, an *in vivo* study revealed that blocking the mitochondrio-nuclear translocation of AIF by ginsenoside Rd benefited neuronal survival following focal cerebral ischemia in rats (53). In the present study, the H_2O_2-induced elevation of AIF level within the nuclei of SH-SY5Y cells was observed to be suppressed when cells were pre-treated with lycopene. Although there was no report regarding the effect of lycopene on AIF translocation from the mitochondria into the nuclei, lycopene was demonstrated to inhibit H_2O_2-induced apoptosis in SH-SY5Y cells via mitigation of AIF nuclear translocation.

In conclusion, the present study demonstrated that lycopene inhibits H_2O_2-induced excessive production of ROS in SH-SY5Y cells by maintaining the activity of cellular anti-oxidative enzymes, SOD and catalase. Lycopene protects against mitochondrial dysfunction by counteracting H_2O_2-induced upregulation of Bax expression and downregulation of Bcl-2 expression. Furthermore, lycopene suppressed H_2O_2-induced activation of caspase-3 and AIF apoptotic pathways in SH-SY5Y cells. Thus, lycopene is considered to be a potent agent that exerts a protective effect against oxidative stress, mitochondrial dysfunction and apoptosis, and may serve as a therapeutic strategy for preventing neuronal injury or death.

Acknowledgements

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