Curcumin exerts protective effects against hypoxia-reoxygenation injury via the enhancement of apurinic/apyrimidinic endonuclease 1 in SH-SY5Y cells: Involvement of the PI3K/AKT pathway

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Abstract. Curcumin, a polyphenolic compound extracted from the plant *Curcuma longa*, has been reported to exert neuroprotective effects against cerebral ischemia reperfusion (I/R) injury. However, the mechanisms underlying these effects remain to be fully elucidated. Emerging evidence indicated that apurinic/apyrimidinic endonuclease 1 (APE1), a multifunctional enzyme, participates in neuronal survival against I/R injury. Therefore, the aim of the present study was to investigate whether curcumin alleviates oxygen-glucose deprivation/reperfusion (OGD/R)-induced SH-SY5Y cell injury, which serves as an *in vitro* model of cerebral I/R injury, by regulating APE1. The results revealed that curcumin increased cell viability, decreased LDH activity, reduced apoptosis and caspase-3 activity, downregulated the pro-apoptotic protein Bax expression and upregulated the anti-apoptotic protein Bcl-2 expression in SH-SY5Y cells subjected to OGD/R. Simultaneously, curcumin eliminated the OGD/R-induced decreases in APE1 protein and mRNA expression, as well as 8-hydroxy-2'-deoxyguanosine (8-OHdG) level and AP sites in SH-SY5Y cells. However, APE1 knockdown by siRNA transfection markedly abrogated the protective effects of curcumin against OGD/R-induced cytotoxicity, apoptosis and oxidative stress, as illustrated by the decreases in reactive oxygen species production and NADPH oxidase 2 expression, and the increase in superoxide dismutase activity and glutathione levels in SH-SY5Y cells. Furthermore, curcumin mitigated the OGD/R-induced activation of phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway. Treatment with LY294002, an inhibitor of PI3K/AKT pathway activity, attenuated the protective effects of curcumin on cytotoxicity and apoptosis, and reversed the curcumin-induced upregulation of APE1 protein expression in SH-SY5Y cells subjected to OGD/R. Taken together, these results demonstrated that curcumin protects SH-SY5Y cells against OGD/R injury by inhibiting apoptosis and oxidative stress, and via enhancing the APE1 level and activity, promoting PI3K/AKT pathway activation.

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Introduction

Cerebral ischemia/reperfusion (I/R) injury is a leading cause of mortality and permanent adult disability worldwide, causing a significant clinical and socioeconomic impact (1). Cerebral I/R injury is a complex pathophysiologic process, during which apoptosis, oxidative stress, excitotoxicity, inflammation and mitochondrial dysfunction are all involved (2,3). Among these, oxidative stress caused by the excess production of reactive oxygen species (ROS) can induce protein dysfunction, DNA damage and lipid peroxidation, resulting in neuronal apoptosis and death, playing a pivotal role in the process of cerebral I/R injury (4,5). To date, significant efforts have been made to mitigate neuronal injury following cerebral injury; however, no effective treatment strategies are currently available (6,7). Therefore, the search for suitable therapeutic agents to alleviate oxidative stress may be an effective means of treating cerebral I/R-induced nervous system injury, which continues to be a major research endeavor.

In recent years, the benefits of traditional Chinese medicine conferring neuroprotective effects have been increasingly investigated in cerebral I/R injury (8-10). Curcumin, a natural antioxidant mainly extracted from the root of turmeric

(*Curcuma longa*), exhibits a range of pharmacological effects, including anticancer, anti-apoptosis, ant-inflammatory and antioxidant effects (11-13). Curcumin can cross the blood-brain barrier and exerts neuroprotective effects in central neurological disease (14). Accumulating evidence has indicated that the most vital biological function of curcumin associated with neuroprotection is its antioxidant effect, which can improve the antioxidant ability of neurons in cerebral ischemia (15,16). Although emerging studies have indicated the neuroprotective effects of curcumin against cerebral I/R injury, the exact mechanisms underlying these effects are not yet fully understood.

Apurinic/apyrimidinic endonuclease 1 (APE1) is a multifunctional protein involved in the base-excision repair of oxidative DNA damage and in the redox activation of transcription factors (17). Recently, several studies have demonstrated that APE1 is implicated in the development and progression of cerebral I/R injury, which is conversely associated with the reduction of oxidative DNA damage, while APE1 expression has been reported to decrease following ischemic injury (18,19). In addition, the overexpression of APE1 induced by an APE-mimicking peptide or adenovirus-mediated APE1 following reperfusion increased DNA repair, attenuated apoptosis and reduced the infarct volume (20,21). It has also been demonstrated that APE1 is an attractive target for neuroprotection and is involved in neuroprotection against cerebral I/R injury (22,23). Stetler *et al* (23) revealed that APE1 was required for pituitary adenylate cyclase-activating polypeptide (PACAP)-induced neuroprotection against global cerebral ischemia. However, the direct contribution of APE1 to the neuroprotective effects of curcumin has yet to be established.

The present study was undertaken to determine the roles of APE1 in the protective effects of curcumin against cerebral I/R injury, as well as to identify the molecular mechanisms through which curcumin affects SH-SY5Y neuronal cells subjected to oxygen-glucose deprivation/reperfusion (OGD/R), a commonly used *in vitro* model of cerebral I/R injury (24,25). It was observed that curcumin protected the SY-SH5Y cells against OGD/R injury by upregulating APE1 expression, which is associated with the regulation of the phosphatidylinositol 3-kinase/protein kinase B (PI3K/AKT) signaling pathway. The results indicated that APE1 is essential for curcumin-induced neuroprotection.

Materials and methods

Reagents and antibodies. Curcumin was dissolved in 0.01% dimethyl sulfoxide (both purchased from Sigma-Aldrich; Merck KGaA) to prepare a 10 mmol/l stock solution, which was then stored at -20˚C. Drug stocks were prepared for use to avoid repeated freeze-thaw cycles and diluted to the desired concentration, as needed. High-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco), fetal bovine serum (FBS; Gibco), TRIzol reagent (Invitrogen) and Lipofectamine RNAiMAX (Invitrogen) were obtained from Thermo Fisher Scientific, Inc. The Cell Counting Kit-8 (CCK-8) assay kit was supplied by Dojindo Molecular Technologies, Inc., while the Cytotoxicity Detection kit was purchased from Roche Applied Science. The BCA protein assay kit, RIPA lysis buffer and BeyoECL Star were obtained from Beyotime Institute of Biotechnology. The reverse transcription (RT) kit and ABI Prism 7500 Real-Time PCR system with the SYBR® RT-PCR kit were obtained from Takara Bio, Inc. and Thermo Fisher Scientific, Inc. Antibodies against GAPDH (cat. no. 5174), B-cell lymphoma 2 (Bcl-2; cat. no. 3498), Bcl-2-associated X protein (Bax; cat. no. 14796), p-AKT (cat. no. 13038), AKT (cat. no. 4685), p-PI3K (cat. no. 17366) and PI3K (cat. no. 4249) were provided by Cell Signaling Technology. The antibody against NADPH oxidase 2 (NOX2) was obtained from Abcam (cat. no. ab131083).

Cells and cell culture. SH-SY5Y neuroblastoma cells were obtained from the American Type Culture Collection (cat. no. CRL-2266) and cultured in high-glucose DMEM supplemented with 10% (v/v) FBS, 1% (v/v) penicillin (100 μg/ml) and 100 U/ml streptomycin at 37˚C in a humidified incubator containing an atmosphere of 5% CO_2 and 95% air. The cell culture medium was changed every 2-3 days.

OGD and OGD/R models and treatment. In order to establish an *in vitro* cerebral ischemia model, OGD exposure was performed by replacing the culture medium of the cells with glucose-free DMEM without serum, and placing the cells in a controlled humidified hypoxic glove box (Coy Laboratory Products, Inc.) supplemented with a 0% O_2, 5% CO_2 and 95% N_2 gas mixture for 1 h at 37˚C. At the end of the OGD process, the anoxic glucose-free medium was replaced with normal DMEM, and the cells were incubated at 37˚C in a humidified incubator containing an atmosphere of 5% CO_2 and 95% air for 24 h of reperfusion, establishing an OGD/R model. To illustrate the protection of curcumin against OGD/R injury, SH-SY5Y cells were treated with curcumin (1, 5, 10 and 15 μl) for 24 h immediately after 1 h of OGD exposure during reperfusion. To investigate the role of APE1 in this process, SH-SY5Y cells were transfected with chemically synthesized small interfering RNA (siRNA) for 6 h, followed by treatment with curcumin (10 μl) for 24 h immediately after 1 h of OGD exposure during reperfusion. To demonstrate the function of the PI3K/Akt signaling pathway, SH-SY5Y cells were pretreated with 10 μM LY294002 (a PI3K/Akt inhibitor) for 2 h, and then treated with curcumin (10 μl) for 24 h immediately after 1 h of OGD exposure during reperfusion.

APE1 siRNA transfection. siRNA targeting APE1 (APE1t) and scrambled siRNA (APE1s) were purchased from Bioneer Corporation. Before SH-SY5Y neuronal cells were subjected to OGD injury, they were transfected with 20 nM chemically synthesized APE1t (forward, 5'-GUCUGGUACGACUGG AGUACC-3', and reverse, 5'-UACUCCAGUCGUACCAGA CCU-3') or APE1s (forward, 5'-CCAUGAGGUCAGCAU GGUCUG-3', and reverse, 5'GACCAUGCUGACCUCAUG GAA-3') using Lipofectamine RNAiMAX according to the manufacturer protocol. Following transfection for 6 h, the culture medium was changed with fresh DMEM, and the cells were used in subsequent experiments. The transfection efficiency was determined by western blot analysis.

Cell viability assay. Cell viability was assessed using a CCK-8 assay kit, according to the manufacturer's protocol. Briefly, the SH-SY5Y cells were plated into 96-well plates at a density of $3x10^4$ cells per well and treated as described earlier. At the end of the experiment, CCK-8 regent (10 μl) was added to

each well and co-incubated with the cells for 3 h at 37˚C. The absorbance was measured at a wavelength of 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.). The assays were independently performed in triplicate, and cell viability was expressed as a percentage of the control.

Lactate dehydrogenase (LDH) activity assay. Cytotoxicity was evaluated by determining the LDH activity in the culture supernatant using the Cytotoxicity Detection kit, according to the manufacturer's protocol. Following the indicated treatments, the extracellular medium was collected and centrifuged at 400 x g at room temperature for 5 min. The supernatant (20 μl) was then mixed with 2,4-dinitrophenylhydrazine (20 μl) at 37˚C for 15 min, and NaOH (0.4 M, 250 μl) was then added to the mixture and co-incubated for a further 15 min at 37˚C. The LDH concentration was quantified by measuring the absorbance at 490 nm. The protein concentration from the cell lysates was determined using the BCA protein assay kit, and the cytotoxicity was normalized to the protein concentration.

RNA isolation and RT-quantitative polymerase chain reaction (RT-qPCR) assay. Total RNA was isolated from the cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific) in accordance with the manufacturer's protocol. The RNA quality and purity were determined using the NanoDrop-2000 spectrophotometer at an A260/A280 ratio. Next, total RNA (2 μg) was reverse transcribed into cDNA using an RT kit. The qPCR reactions were performed with the SYBR® RT-PCR kit on the ABI 7500 Real-Time PCR System, according to the manufacturer's protocol. The following qPCR conditions were set: Reverse transcription (1 cycle) at 61˚C for 20 sec, denaturation (1 cycle) at 95˚C for 1 min, and annealing (45 cycles) at 95˚C for 5 sec, followed by extension at 72˚C for 20 sec. The amount of amplified GAPDH was used as an external reference gene. The mRNA levels were calculated by the $2^{-\Delta\Delta Cq}$ method (26) and are presented as fold changes relative to the expression levels of internal control (GAPDH). All reactions were performed in triplicate. The primers used in qPCR were synthesized by Sigma Genosys, and their sequences were as follows: APE1/Ref-1 forward, 5'-CTGCCTGGACTCTCT CATCAATAC-3', and reverse, 5'-GAATGCCGTATCCGC TACTCC-3'; GAPDH forward, 5-ACGGCAAGTTCAACG GCAC-3', and reverse, 5'-CGCCAGTAGACTCCACGAC ATA-3'.

Flow cytometric detection of apoptosis. The apoptosis rate was determined using an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences) according to the manufacturer's protocol, in combination with a flow cytometer (Beckman Coulter, Inc.). Briefly, SH-SY5Y cells were seeded in 6-well plates at a density of $1x10^6$ cells/well. Subsequent to the different treatments discussed earlier, cells were washed twice with cold PBS and re-suspended with 1X binding buffer. Next, the cells were stained with Annexin V-FITC (5 μl) and PI (10 μl), and co-incubated for 20 min at room temperature in the dark. Finally, the flow cytometer was used to detect the apoptotic cells.

8-Hydroxy-2'-deoxyguanosine (8-OHdG) level determination. The 8-OHdG level was determined using an ELISA kit from Oxis Health Products, according to the manufacturer's protocol. Briefly, genomic DNA was isolated from the SH-SY5Y cells using the Genomic DNA Isolation kit (RayBiotech). Standards included in the kit and DNA samples were incubated with biotinylated 8-OHdG in microtitration wells coated with other 8-OHdG molecules with defined and unique epitope specificity. Subsequent to washing, streptavidin labeled with enzyme horseradish peroxidase (HRP) was added to each well, followed by the addition of a TMB substrate solution, and color development in proportion to the concentration of 8-OHdG. Finally, stop solution was added, and the color changed from blue to yellow. The intensity was measured at 450 nm using a microplate reader. Sample DNA assays were performed in duplicate.

DNA damage assays. The abasic sites, also known as apurinic/apyrimidinic (AP) sites, in the DNA extracts from the SH-SY5Y cells were determined with a biotin-labeled aldehyde reactive probe in a colorimetric assay (Dojindo Molecular Technologies, Inc.) according to the manufacturer's protocol.

Caspase-3 activity assessment. The activity of caspase-3 was measured with a caspase-3 activity assay kit (Beyotime Institute of Biotechnology), following the protocol provided by the manufacturer. Acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) is catalyzed by caspase-3 to produce a yellow p-nitroaniline (pNA) compound. For caspase-3 activity assessment, the SH-SY5Y cells were collected, washed twice with PBS and then lysed in lysis buffer on ice for 15 min. Following centrifugation at 16,000 x g for 15 min at 4˚C, the supernatant was incubated with Ac-DEVD-pNA at 37˚C for 2 h. The absorbance at a wavelength of 405 nm was then measured. The activity of caspase-3 is expressed as the relative percentage of the optical density in the control group.

Measurement of intracellular reactive oxygen species. Dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen; Thermo Fisher Scientific, Inc.), a membrane-permeable non-fluorescent dye, was used in the present study to detect the endogenous ROS generation in SH-SY5Y neuronal cells according to the protocol provided by the manufacturer. DCFH-DA can be converted by intracellular esterases into 2',7'-dichlorodihydrofluorescein, which is then oxidized by ROS into fluorescent 2',7'-dichlorofluorescein. In brief, following treatment, the SH-SY5Y cells were washed twice with ice-cold PBS and then co-incubated in DMEM solution containing 10 μM DCFH-DA at 37˚C for 20 min. Subsequent to washing twice with PBS, the fluorescence was observed using a fluorescence microplate reader (H1m; BioTek Instruments, Inc.) at an excitation wavelength of 488 nm and an emission wavelength of 528 nm. In addition, the cells were collected, centrifuged at 800 x g for 5 min at room temperature and washed twice with PBS, and then the fluorescence intensity was be quantitatively analyzed using a flow cytometer (BD Biosciences).

Superoxide dismutase (SOD) activity determination. At the end of the treatment period, the SH-SY5Y cells were harvested, washed with ice-cold PBS, and then incubated

for 30 min with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA and 0.2% sodium dodecyl sulfate (SDS). The cell lysates were centrifuged at 10,000 x g at 4˚C for 10 min, and the protein concentration was detected using a BCA kit. SOD activity in the cell lysates was finally evaluated using a SOD determination kit (Assay Designs) as per the manufacturer's protocol, and is expressed as U/mg of protein.

Glutathione (GSH) content determination. The total GSH content in the cellular lysates was determined using a glutathione measurement kit (Assay Designs; Enzo Life Sciences, Inc.). Following treatment, cell lysates were centrifuged at 10,000 x g for 15 min at 4˚C, and the supernatant obtained was used for measuring the GSH content. The protein concentration was detected using a BCA kit. The absorbance of the samples was determined using a microplate reader (Infinite 200; Tecan) at 405 nm. The amount of GSH was detected by means of a calibration curve and normalized to the protein concentration. The results are presented as a percentage of the control cells.

Western blot analysis. Following the indicated treatments, the SH-SY5Y cells were collected and lysed in RIPA buffer containing 1% phenylmethylsulfonyl fluoride. The lysates were centrifuged at 10,000 x g at 4˚C for 30 min, and the concentration of the protein samples was detected using a BCA protein quantification kit. Subsequently, equal amounts of protein (30 μg) were separated by SDS-PAGE (12% gel) and transferred onto polyvinylidene difluoride membranes (Millipore). Subsequently, the membranes were blocked with 5% non-fat milk at room temperature for 2 h, and then incubated at 4˚C overnight with primary antibodies, including anti-GAPDH (1:1,000), anti-APE1 (1:1,000), anti-Bax (1:1,000), anti-Bcl-2 (1:1,000), anti-NOX2 (1:1,000), anti-p-AKT (1:1,000), anti-AKT (1:1,000), anti-p-PI3K (1:1,000) and anti-PI3K (1:1,000). Following washing three times for 10 min with Tris-buffered saline with Tween-20, the membranes were incubated with a HRP-conjugated secondary antibody (1:5,000; Cell Signaling Technology; cat. no. 7076) at room temperature for 2 h. The blots were then developed with an BeyoECL Star according to the manufacturer's protocol, and visualized using Image Lab™ Software (Bio-Rad Laboratories, Inc.).

Statistical analysis. The results are presented as the mean ± standard deviation values. The statistical significance of differences between the mean values was assessed by analysis of variance and post-hoc Bonferroni/Dunn's tests using SPSS software (version 16.0; SPSS, Inc.). A value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Curcumin mitigates OGD/R-induced SH-SY5Y cell injury. First, the protective effects of curcumin on OGD/R injury in SH-SY5Y cells were investigated in the present study. The results of CCK-8 assay revealed that treatment with curcumin alone at various concentrations (1, 5, 10 and 15 μl) exerted no cytotoxicity (Fig. 1A), while treatment with curcumin (5, 10 and 15 μM) markedly reversed the OGD/R-induced the downregulation of cell viability (Fig. 1B). In addition, the results from LDH activity assay revealed that OGD/R significantly increased the LDH activity, and this effect was also blocked by curcumin (5, 10 and 15 μM) treatment (Fig. 1C). According to the aforementioned results, curcumin treatment at 10 μl significantly increased cell viability and decreased LDH activity; thus, this concentration was selected for use in subsequent experiments.

The study further investigated the effects of curcumin on the apoptosis of OGD/R-injured SY-SH5Y cells. Annexin V-FITC/PI double staining followed by flow cytometry revealed that curcumin significantly reversed the OGD/R-induced increase in the apoptotic rate of the SH-SY5Y cells (Fig. 1D). In addition, OGD/R exposure resulted in marked elevation of caspase-3 activity, which was also blocked by curcumin (Fig. 1E). Consistently, the results of western blot analysis (Fig. 1F) demonstrated that OGD/R promoted the pro-apoptotic protein Bax expression (Fig. 1G) and inhibited the anti-apoptotic protein Bcl-2 expression (Fig. 1H) in the SY-SH5Y cells; however, treatment with curcumin reversed these effects. These results thus indicated that curcumin protected the SH-SY5Y cells against OGD/R-induced injury by inhibiting apoptosis.

Curcumin increases the APE1 level and activity in OGD/R-injured SH-SY5Y cells. To determine whether APE1 is involved in the curcumin-induced neuroprotective effects, the effects of curcumin on the APE1 level and activity under OGD/R conditions were examined. As presented in Fig. 2, the levels of APE1 protein (Fig. 2A) and mRNA (Fig. 2B) in cells subjected to OGD/R were significantly lower as compared with those in the control cells. However, post-treatment of the cells with curcumin (10 μM) immediately after OGD exposure markedly blocked these effects. Treatment with curcumin alone had no effect on the APE1 protein and mRNA levels.

APE1 is a multifunctional protein involved in DNA repair and redox regulation, serving an essential role in repairing oxidative DNA damage involving single-strand breaks, and formation of 8-OHdG and AP sites (27,28). The present study further observed that OGD/R significantly increased the 8-OHdG (Fig. 2C) and AP site (Fig. 2D) levels compared with those in the control group. However, these effects were abolished by curcumin post-treatment. Curcumin alone did not affect the 8-OHdG and AP site levels. Taken together, these results suggested that curcumin enhanced the level and DNA repair capacity of APE1 under OGD/R conditions in the SH-SY5Y cells.

APE1 knockdown abrogates the neuroprotective effects of curcumin against OGD/R-induced SH-SY5Y cell injury. To further address the role of APE1 in curcumin-induced neuroprotection, the SH-SY5Y cells were transfected with chemically synthesized APE1t or APE1s siRNAs. The results of western blot analysis (Fig. 3A) revealed that APE1 expression in the APE1t transfection group was significantly reduced to ~35% of that in the APE1s transfection group (Fig. 3B), indicating that APE1 knockdown was successfully induced by APE1t transfection. Subsequently, it was observed that APE1 knockdown further reduced the viability of the OGD/R-injured SH-SY5Y cells. However, APE1t transfection reversed the curcumin-induced upregulation of

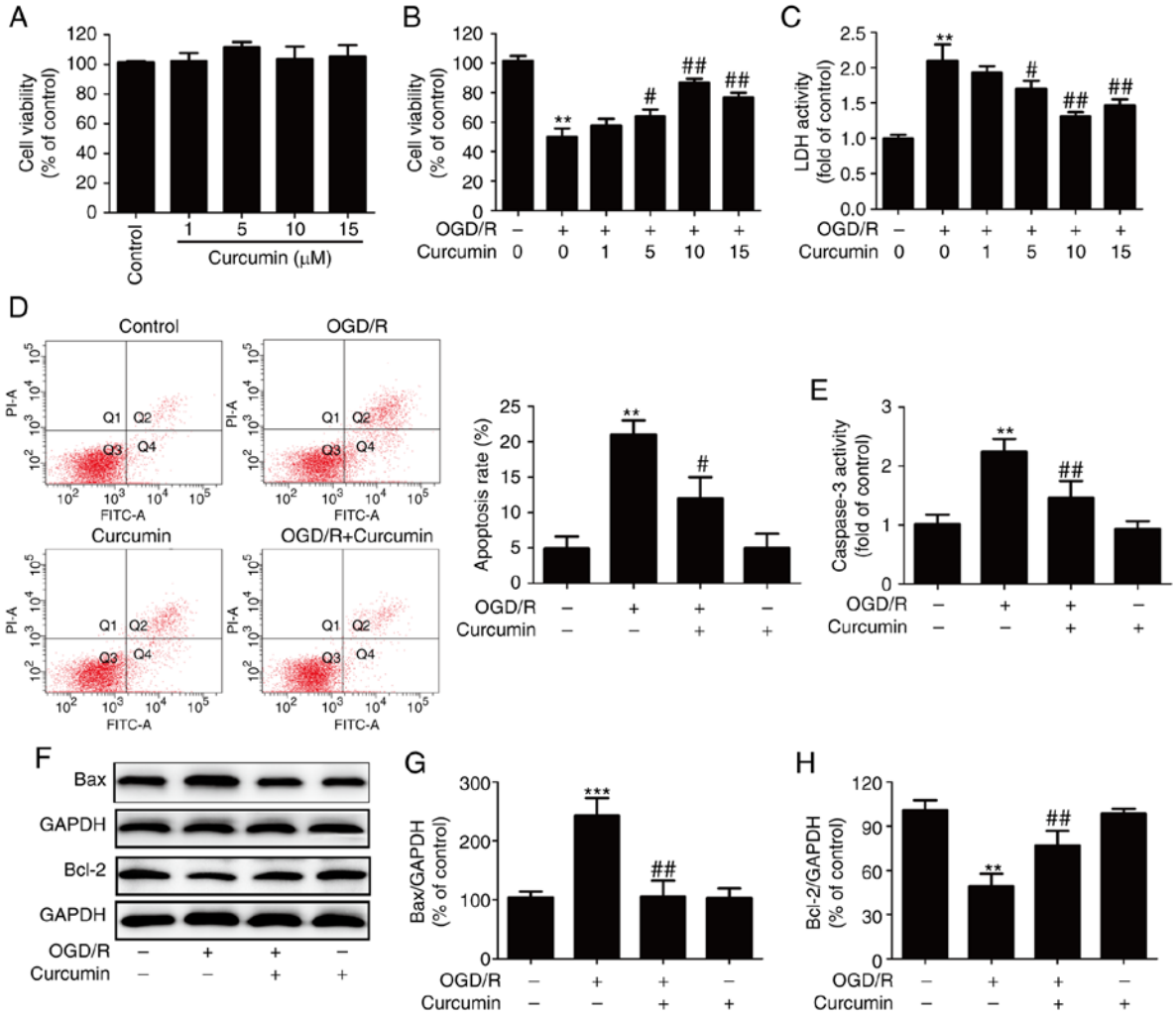

Figure 1. Effects of curcumin on cytotoxicity and the apoptosis of OGD/R-injured SH-SY5Y cells. (A) SH-SY5Y cells were treated with various concentrations of curcumin (1, 5, 10 and 15 μl) for 24 h, and the cell viability was measured by CCK-8 assay. Data were normalized to the control as 100%. (B) Cell viability examined by CCK-8 assay and (C) LDH activity in the culture supernatant were tested in SH-SY5Y cells with curcumin (1, 5, 10 and 15 μl) for 24 h immediately after 1 h of OGD exposure. LDH activity data were normalized to the control, which was set as 1. Subsequently, SH-SY5Y cells were treated with curcumin (10 μl) for 24 h immediately after 1 h of exposure to OGD and then assessed for apoptosis. (D) Apoptosis was measured by Annexin V-FITC/PI double staining, followed by flow cytometry. (E) Caspase-3 activity was measured with an assay kit. (F) Protein expression was detected by western blot analysis. (G) Bax and (H) Bcl-2 protein levels were quantitatively analyzed. Data are expressed as the mean ± standard deviation of at least three independent experiments. **P<0.01 and ***P<0.001, vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01, vs. OGD/R exposure alone. OGD/R, oxygen-glucose deprivation/reperfusion; CCK-8, Cell Counting Kit-8; LDH, lactate dehydrogenase; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2.

cell viability (Fig. 3C) and downregulation of LDH activity (Fig. 3D), as compared with the effect of APE1s transfection in the curcumin + OGD/R-treated SH-SY5Y cells. In addition, the effects of APE1 on cell apoptosis in the presence or absence of curcumin under OGD/R conditions were further investigated. The results revealed that APE1t transfection exacerbated the effect of OGD/R on apoptosis, resulting in the upregulation of caspase-3 activity (Fig. 3E) and Bax protein expression (Fig. 3F and G), as well as in the downregulation of Bcl-2 protein expression (Fig. 3F and H), as compared with those in APE1s-transfected cells. Notably, APE1t transfection attenuated the curcumin-induced inhibition on these apoptosis-associated indexes. In the control group, APE1t transfection did not affect the cell viability, LDH activity, and Bax and Bcl-2 expression levels as compared with the APE1s transfection group. These results indicated that APE1 contributed to the neuroprotective effects of curcumin against cerebral I/R injury *in vitro*.

APE1 knockdown eliminates the inhibitory effects of curcumin on OGD/R-induced oxidative stress in SH-SY5Y cells. Next, the effects of curcumin on oxidative stress in OGD/R-injured SH-SY5Y cells and the role of APE1 in this process were further examined. As illustrated in Fig. 4, in the APE1s-transfected SH-SY5Y cells, OGD/R significantly increased ROS production (Fig. 4A and B) and NADPH oxidase 2 (NOX2) expression (Fig. 4C), which is one of the most important sources of ROS production in the brain, compared with the control group; these effects were then reversed by curcumin post-treatment. Notably, APE1t transfection further increased ROS generation and NOX2 expression compared with those observed in the APE1s-transfeced OGD/R-injured SH-SY5Y cells. Upon curcumin treatment, APE1t transfection blocked the curcumin-induced decrease in ROS production and NOX2 expression in the OGD/R-injured SH-SY5Y cells.

The imbalance of antioxidant endogenous enzymatic activity is known to result in oxidative stress. Thus, the levels

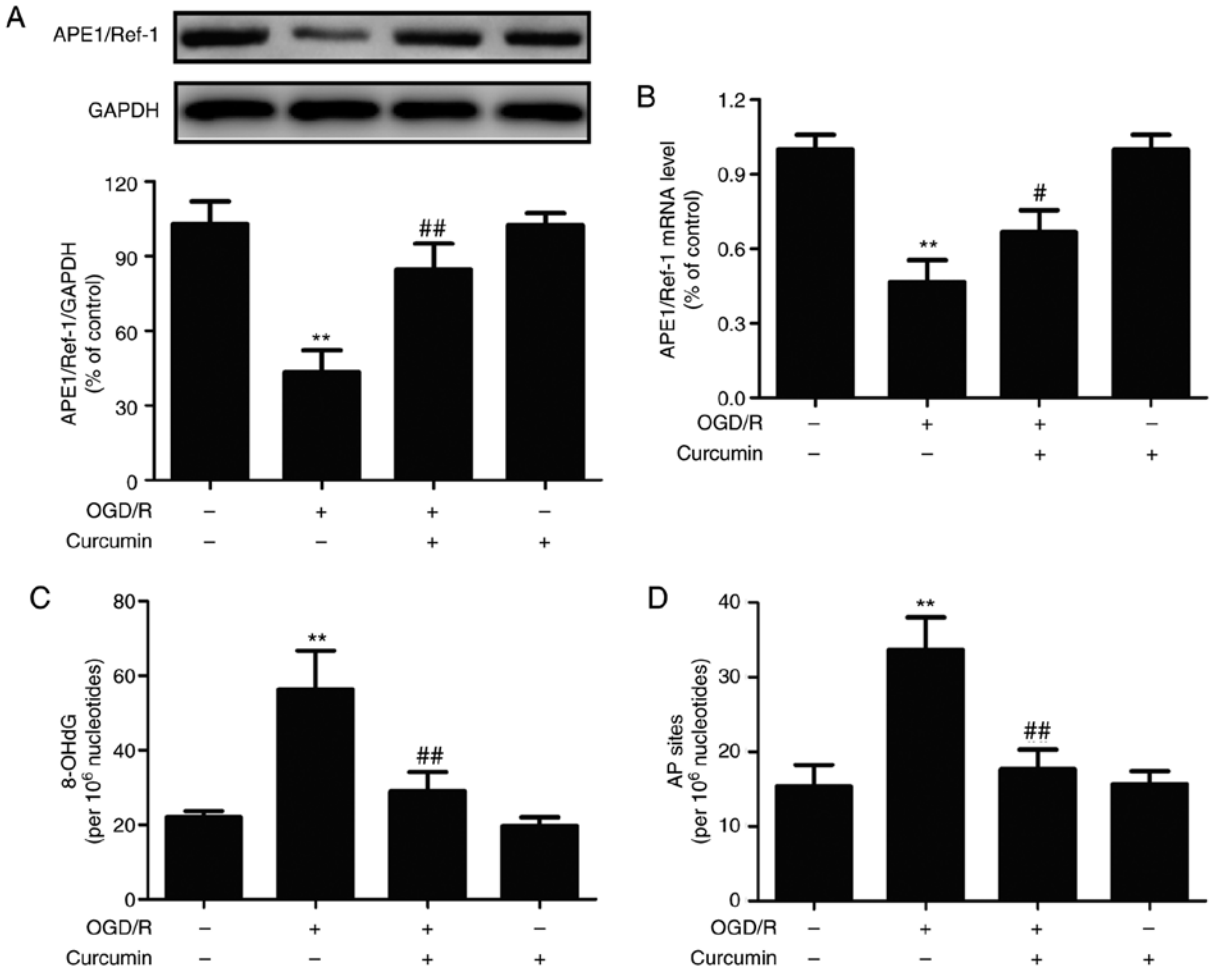

Figure 2. Effects of curcumin on the APE1 level and oxidative DNA damage in OGD/R-injured SH-SY5Y cells. SH-SY5Y cells were treated with curcumin (10 μl) for 24 h immediately after 1 h of exposure to OGD. (A) APE1 protein expression was measured by western blot analysis, and data were normalized to the control, which was set as 100%. (B) APE1 mRNA expression was detected by reverse transcription-quantitative polymerase chain reaction. The levels of the oxidative DNA damage markers, (C) 8-OHdG and (D) AP sites, were measured with a ELISA kit from Oxis Health Products and biotin-labeled aldehyde reactive probe with a colorimetric assay, respectively. Data are expressed as the mean ± standard deviation of at least three independent experiments. **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01, vs. OGD/R exposure alone. OGD/R, oxygen-glucose deprivation/reperfusion; APE1, apurinic/apyrimidinic endonuclease 1; 8-OHdG, 8-hydroxy-2'-deoxyguanosine.

of endogenous enzymes, including SOD and GSH, were measured, and it was observed that OGD/R led to a decrease in SOD activity (Fig. 4D) and in GSH levels (Fig. 4E). The OGD/R-induced decrease in SOD and GSH levels was exacerbated by APE1t transfection, while it was attenuated by curcumin treatment; however, the effect of curcumin was eliminated by APE1t transfection. APE1t transfection alone did not affect these oxidative stress and antioxidant stress indexes compared with the APE1s transfection group. Taken together, these results indicated that APE1 mediated the protective effects of curcumin against oxidative stress in OGD/R-injured SH-SY5Y cells.

Curcumin activates the PI3K/AKT signaling pathway in OGD/R-injured SH-SY5Y cells. The PI3K/AKT pathway is known to be involved in neuronal death associated with cerebral ischemia (29). To further evaluate the mechanisms underlying the neuroprotective effects of curcumin under OGD/R conditions, the effects of curcumin on the PI3K/AKT signaling pathway were then examined. It was observed that the ratios of p-PI3K/PI3K (Fig. 5A) and p-AKT/AKT (Fig. 5B) were significantly decreased in SH-SY5Y cells exposed to OGD/R, as compared with those in cells exposed to normal conditions. However, these effects were reversed by curcumin post-treatment. Curcumin treatment alone had no effect on the expression levels of p-PI3K and p-AKT proteins. These results suggested that curcumin promotes the activation of the PI3K/AKT pathway in OGD/R-injured SH-SY5Y cells.

Inhibition of the PI3K/AKT signaling pathway attenuates the curcumin-induced protective effects against OGD/R injury in SH-SY5Y cells. In order to further determine the roles of the PI3K/AKT pathway in the neuroprotective effects of curcumin, the cells were pre-treated with LY294002, an inhibitor of the PI3K/AKT pathway. The results of CCK-8 assay revealed that LY294002 abolished the curcumin-induced increase in cell viability (Fig. 6A) and the decrease in LDH release (Fig. 6B) in the OGD/R-injured SH-SY5Y cells. In addition, it was observed that LY294002 reversed the curcumin-induced inhibition of apoptosis, as evidenced by the increase in caspase-3 activity (Fig. 6C) and Bax expression (Fig. 6D), as well as by the decrease in Bcl-2

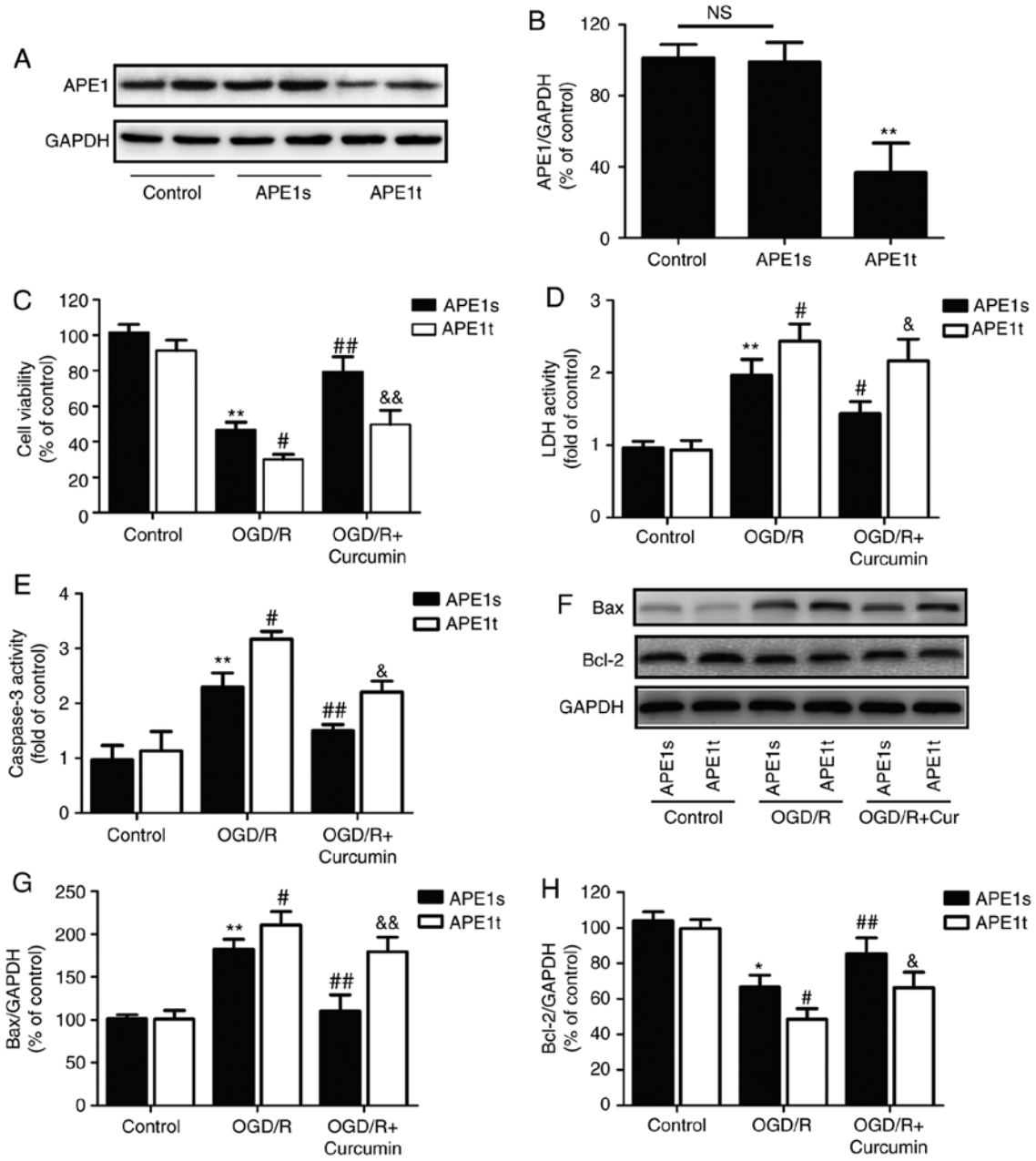

Figure 3. Effects of APE1 knockdown on cytotoxicity and apoptosis in the presence or absence of curcumin in SH-SY5Y cells exposed to OGD/R. SH-SY5Y cells were transfected with APE1t or APE1s, followed by treatment with curcumin (10 μl) for 24 h immediately after 1 h of OGD exposure. (A) Western blot and (B) quantified protein expression of APE1. **P<0.01 vs. control. (C) Cell viability was measured by Cell Counting Kit-8 assay. (D) LDH activity was detected with a cytotoxicity detection kit. (E) Caspase-3 activity was measured using an assay kit. (F) The protein expression levels of Bax and Bcl-2 were determined by western blot analysis. Quantitative analysis of the (G) Bax and (H) Bcl-2 protein bands. Data are expressed as the mean ± standard deviation of at least three independent experiments. *P<0.05 and **P<0.01, vs. APE1s + control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01, vs. APE1s + OGD/R exposure alone; $^{\&}$P<0.05 and $^{\&\&}$P<0.01, vs. APE1s + OGD/R + curcumin treatment. OGD/R, oxygen-glucose deprivation/reperfusion; APE1, apurinic/apyrimidinic endonuclease 1; APE1t, siRNA targeting APE1; APE1s, scrambled siRNA; LDH, lactate dehydrogenase; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2; NS, no significant difference.

expression (Fig. 6E), compared with the curcumin + OGD/R co-treatment group. These results indicated that the PI3K/AKT pathway contributes to the protective effects of curcumin against OGD/R-induced injury in SH-SY5Y cells. Notably, the data revealed that LY294002 also blocked the curcumin-induced upregulation of APE1 in the OGD/R-injured SH-SY5Y cells (Fig. 6F). Taken together, the aforementioned results suggested that curcumin activates the PI3K/AKT pathway and further increases APE1 expression, resulting in neuroprotective effects against OGD/R-induced SH-SY5Y cell injury.

Discussion

The present study revealed that curcumin protected the SH-SY5Y neuronal cells against apoptosis and oxidative stress induced by OGD/R injury by increasing the APE1 levels. In addition, the results demonstrated that curcumin activated the PI3K/AKT signaling pathway, exerting protective effects against OGD/R-induced SH-SY5Y cell injury. Notably, the PI3K/AKT pathway mediated the curcumin-induced upregulation of APE1. To the best of our knowledge, these results

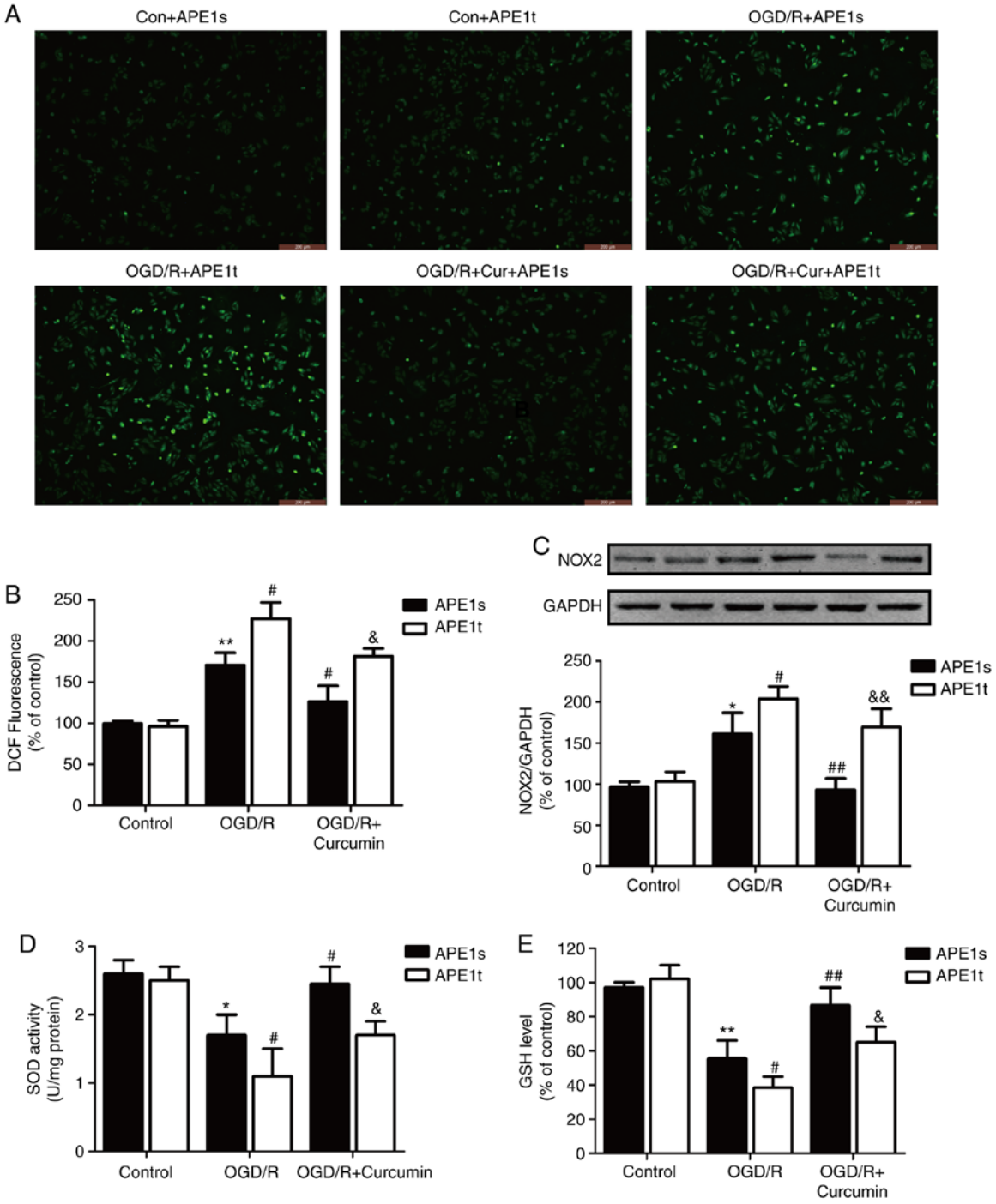

Figure 4. Effects of APE1 knockdown on oxidative stress in the presence or absence of curcumin in SH-SY5Y cells exposed to OGD/R. SH-SY5Y cells were transfected with APE1t or APE1s followed by treatment with curcumin (10 μl) for 24 h immediately after 1 h of OGD exposure. (A) Intracellular ROS generation determined by the DCFH-DA method (magnification, x200). (B) The fluorescence intensity was analyzed by flow cytometry. (C) NOX2 expression was determined by western blot analysis. (D) SOD activity and (E) GSH levels were measured using commercial assay kits. Data are expressed as the mean ± standard deviation of at least three independent experiments. *P<0.05 and **P<0.01, vs. APE1s + control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. APE1s + OGD/R exposure alone; $^{\&}$P<0.05 and $^{\&\&}$P<0.01, vs. APE1s + OGD/R + curcumin treatment. OGD/R, oxygen-glucose deprivation/reperfusion; APE1, apurinic/apyrimidinic endonuclease 1; APE1t, siRNA targeting APE1; APE1s, scrambled siRNA; NOX2, NADPH oxidase 2; SOD, superoxide dismutase; GSH, glutathione.

confirm for the first time that curcumin exerts neuroprotective effects by enhancing the expression and activity of APE1, which is involved in the activation of the PI3K/AKT pathway following OGD/R injury in SH-SY5Y neuronal cells.

Owing to the high mortality and severe neurological disorder of cerebral I/R injury, the development of effective therapeutic drugs for the prevention of ischemic injury is of utmost importance (30,31). Curcumin, a naturally occurring polyphenolic compound isolated from the root of the *Curcuma longa* Linn., can pass through the blood-brain barrier, and has been recommended for the prevention and treatment of cerebrovascular disease due to its anti-apoptotic, antioxidant and anti-inflammatory effects, its limited toxicity and minimal side-effects (32-34). The results of the

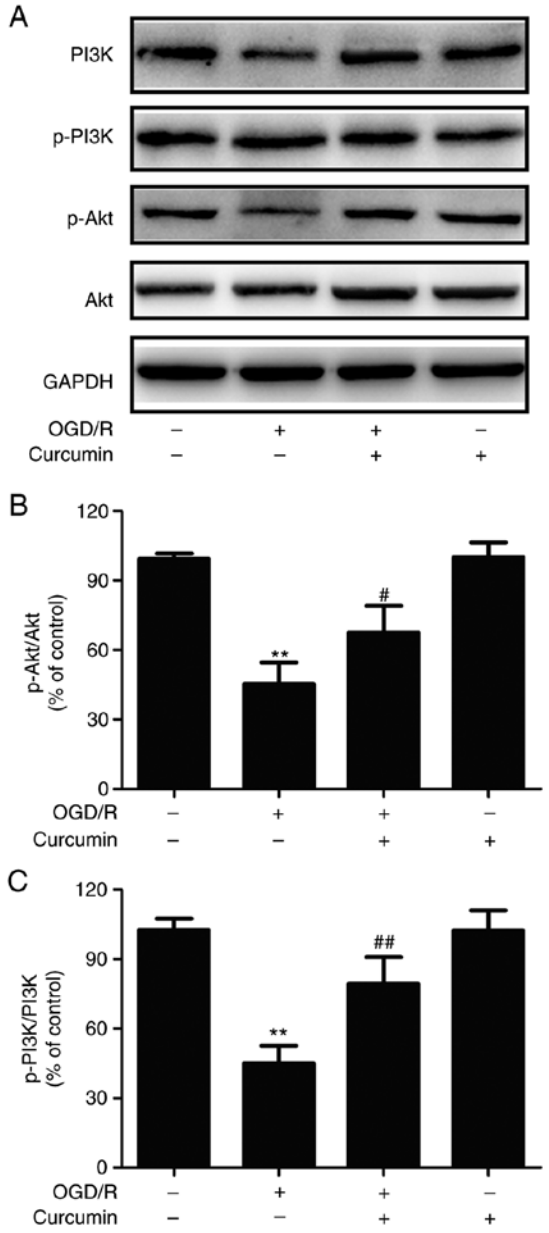

Figure 5. Effects of curcumin on the PI3K/AKT pathway in OGD/R-injured SH-SY5Y cells. SH-SY5Y cells were treated with curcumin (10 μl) for 24 h immediately after OGD exposure for 1 h. (A) Protein expression levels were measured by western blot analysis. Quantitative analysis of the (B) p-PI3K/PI3K and (C) p-AKT/AKT ratio is shown. Data are expressed as the mean ± standard deviation of at least three independent experiments. **P<0.01 vs. control; $^{\#}$P<0.05 and $^{\#\#}$P<0.01, vs. OGD/R exposure alone. OGD/R, oxygen-glucose deprivation/reperfusion; PI3k, phosphatidylinositol 3-kinase; AKT, protein kinase B.

present study revealed that curcumin markedly increased the viability and decreased LDH activity in OGD/R-injured SH-SY5Y cells, which is in accordance with the findings of studies by Xie *et al* (35) and Zhang *et al* (36). These results suggested that curcumin exerts protective effects against OGD/R-induced injury in SH-SY5Y cells. While a number of studies have attempted to elucidate the possible mechanisms underlying the neuroprotective effects of curcumin, the complete mechanisms through which curcumin protects cells against cerebral I/R injury have not yet been elucidated.

A growing number of studies have suggested that oxidative stress refers to the elevated production of intracellular ROS, which may lead to damage in tissue, lipids, proteins and DNA, involved in the pathophysiological processes of cerebral ischemia (37,38). It is well known that the antioxidant activity of curcumin is critical for its neuroprotective effects (39). APE1 is a master regulator of the cellular response to oxidative stress, and is involved in gene transcriptional regulation during the adaptive cellular response to oxidative stress, as well as in the base excision repair pathway of oxidative DNA lesions, which consist of DNA-protein crosslinks, AP sites, 8-OHdG formation and single-strand breaks (40,41). Several studies have examined APE1 in the context of cerebral I/R injury, and APE1 expression has long been known to decrease following ischemic injury (19). In line with these findings, the present study observed that the APE1 protein and mRNA levels were decreased by OGD/R. In addition, OGD/R increased 8-OHdG and AP site formation, which was in accordance with the findings of a study by Kim *et al* (21). However, these effects were all blocked by curcumin, and the results indicated that curcumin increased APE1 expression and activity in cerebral I/R injury. However, an earlier study revealed that curcumin is an inhibitor of the APE1 redox function that affects numerous genes and pathways, which is contrary to the results of the current study (42). These differences may be a result of the different research systems used: The previous study examined a virus system, while the present study investigated SH-SY5Y cells. Consistent with our present research, a number of other studies have also confirmed that curcumin exerts biological activities via activation of APE1 (43,44). Subsequently, it was revealed that the knockdown of APE1 by transfection with APE1 siRNA reversed the curcumin-induced protective effects against OGD/R injury in SH-SY5Y cells, suggesting the contribution of APE1 to the neuroprotective effects of curcumin. Taken together, these results suggest that APE1 upregulation contributes to the neuroprotective effects of curcumin.

Apoptosis and oxidative stress are prominent features of cerebral ischemia (38,45). Zhao *et al* (46) have proven that curcumin attenuated focal cerebral ischemic injury via anti-oxidative stress and anti-apoptotic mechanisms in rats. The results of a study by Wang *et al* (47) also revealed that the neuroprotective effects of curcumin against I/R-induced neuronal damage were attributed to its antioxidant capacity in decreasing oxidative stress and the associated signaling cascade, leading to apoptotic cell death. Notably, APE1 has been studied for its extended repertoire in controlling the cellular response to apoptosis and oxidative stress (48). APE1 upregulation, either induced endogenously or through transgene overexpression, reduces oxidative DNA damage and prevents hippocampal neuronal apoptosis due to ischemic injury (19). Furthermore, APE1 is required for PACAP-mediated neuroprotection (23). Consistent with these findings, in the present study, it was observed that APE1 knockdown induced by APE1 siRNA transfection further aggravated the OGD/R-induced apoptosis and oxidative stress, while curcumin mitigated the effects of OGD/R. Notably, these protective effects of curcumin were all abolished by APE1 knockdown induced by APE1 siRNA. Overall, these results revealed that APE1 mediates the protective effects of curcumin against apoptosis and oxidative stress in cerebral I/R injury. APE1 has two major functions in

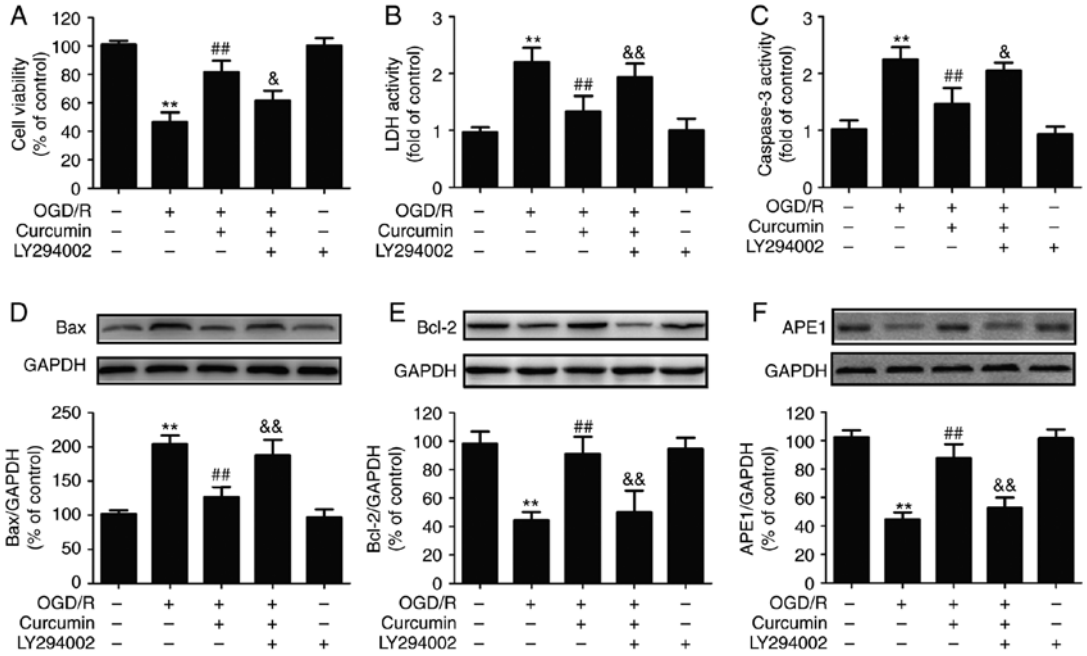

Figure 6. Effects of LY294002 on cytotoxicity, apoptosis and APE1 expression in OGD/R-injured SH-SY5Y cells. Cells were pre-treated with a PI3K/Akt inhibitor (LY294002; 10 μM) for 2 h following incubation with curcumin (10 μl) for 24 h immediately after OGD exposure for 1 h. (A) Cell viability was measured by Cell Counting Kit-8 assay. (B) LDH activity in the culture supernatant was detected using a Cytotoxicity Detection kit. (C) Caspase-3 activity was determined by a caspase-3 activity assay kit. (D) Bax, (E) Bcl-2 and (F) APE1 protein expression levels were detected by western blot analysis. Data are expressed as the mean ± standard deviation of at least three independent experiments. **P<0.01 vs. control; $^{\#\#}$P<0.01 vs. OGD/R exposure alone; $^{\&}$P<0.05 and $^{\&\&}$P<0.01, vs. curcumin + OGD/R co-treatment. OGD/R, oxygen-glucose deprivation/reperfusion; APE1, apurinic/apyrimidinic endonuclease 1; PI3k, phosphatidylinositol 3-kinase; AKT, protein kinase B; LDH, lactate dehydrogenase; Bax, Bcl-2-associated X protein; Bcl-2, B-cell lymphoma 2.

mammalian cells: DNA repair (49) and the redox regulation of gene transcription (50). However, the present study did not determine whether the redox activity of APE1 was less instrumental in the neuroprotective effects of curcumin than its DNA repair activity. These aspects remain to be investigated in the future using APE1 functional mutant (51,52).

The PI3K/AKT pathway has been documented as an essential pathway for cell survival in response to apoptosis and oxidative stress induced by a series of physiological and pathological or exogenous stimuli (53,54), while this pathway is also significantly involved in cerebral ischemia (55). To date, a number of studies have confirmed that the PI3K/AKT pathway serves an important role in the neuroprotective effects of curcumin by inhibiting apoptosis, oxidative stress and inflammation (56-58). However, there are few reports available to date on the roles of the PI3K/AKT pathway in the neuroprotective effects of curcumin against cerebral I/R injury. In the present study, it was further revealed that curcumin increased the ratio of p-PI3K/PI3K and p-AKT/AKT proteins, whereas pharmacological treatment with LY294002, a PI3K inhibitor, reversed the curcumin-induced cytoprotective and anti-apoptotic effects on OGD/R-injured SY-SH5Y cells. It should be noted that LY294002 was also found to block the curcumin-induced upregulation of APE1 expression under OGD/R conditions. These results suggested that the PI3K/AKT pathway contributes to the neuroprotective effects of curcumin by regulating APE1 in cerebral I/R injury.

However, the present study also has certain limitations. Firstly, only one cell line was used to demonstrate the potential mechanism of neuroprotection of curcumin, and the use of other cell lines or cultured neurons would provide further convincing results. Secondly, no data from an *in vivo* animal model were provided, which is important to verify the validity of curcumin concentration *in vitro* in the present study. These two issues will be addressed in our future experiments. Finally, the present study lacked the results from caspase-3 expression and cleaved caspase-3 protein expression, which should be noted in future research.

In conclusion, the results of the present study demonstrated that curcumin confers neuroprotective effects against apoptosis and oxidative stress in cerebral I/R injury by increasing the APE1 level and activity, which may be mediated via the PI3K/AKT signaling pathway. To the best of our knowledge, the present study is the first to suggest that curcumin may be a potential therapeutic agent against cerebral I/R injury. The present study also identified APE1 as a major therapeutic target of the neuroprotective effects of curcumin.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

LW participated in performing the experiments and writing of the paper. CJ and YK contributed to data analysis. YD and WF performed the experiments. PH designed the study and revised the paper. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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