

Curcumin activates autophagy and attenuates oxidative damage in EA.hy926 cells via the Akt/mTOR pathway

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Abstract. Curcumin, which is the effective component of turmeric (*Curcuma longa*), has previously been shown to exert potent antioxidant, antitumor and anti-inflammatory activities *in vitro* and *in vivo*. However, the mechanism underlying the protective effects of curcumin against oxidative damage in endothelial cells remains unclear. The present study aimed to examine the effects of curcumin on hydrogen peroxide (H₂O₂)-induced apoptosis and autophagy in EA.hy926 cells, and to determine the underlying molecular mechanism. Cultured EA.hy926 cells were treated with curcumin (5-20 μ mol/l) 4 h prior to and for 4 h during exposure to H₂O₂ (200 μ mol/l). Oxidative stress resulted in a significant increase in the rate of cell apoptosis, which was accompanied by an increase in the expression levels of caspase-3 and B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), and a decrease in the expression levels of Bcl-2. Treatment with curcumin (5 or 20 μ mol/l) significantly inhibited apoptosis, and reversed the alterations in caspase-3, Bcl-2 and Bax expression. Furthermore, curcumin induced autophagy and microtubule-associated protein 1A/1B-light chain 3-II expression, and suppressed the phosphorylation of Akt and mammalian target of rapamycin (mTOR). These results indicated that curcumin may protect cells against oxidative stress-induced damage through inhibiting apoptosis and inducing autophagy via the Akt/mTOR pathway.

Introduction

Curcumin (diferuloylmethane), which is the main component extracted from turmeric (*Curcuma longa*), is a traditional medicinal plant that exerts various biological functions (1-3), including anti-inflammatory, antioxidant, anticancer and cardio-protective effects. The exact mechanism by which curcumin exerts these effects remains to be elucidated; however, the antioxidant activity of the hydrophobic polyphenol appears to be the essential component underlying its pleiotropic biological effects (4). Previous studies have demonstrated that curcumin is able to ameliorate the production of reactive oxygen species (ROS) and lipid peroxidation in various models of oxidative damage in cardiac tissue (5,6). Endothelial cell injury is a critical step in the development of atherosclerosis and hypertension (7). In addition, it has previously been reported that curcumin may attenuate oxidative damage in endothelial cells (8); however, the underlying mechanism remains unclear.

Apoptosis, which is the process of programmed cell death, leads to the rapid degradation of cellular structures and organelles (9). Furthermore, autophagy is a highly conserved cellular process that comprises bulk degradation and recycling of cytoplasmic components, including long-lived proteins and organelles (10). The functional relationship between apoptosis and autophagy is complex. Autophagy-induced cytoprotection is the basic cellular function of autophagy in eukaryotic cells; however, some stressors, including oxidative stress, induce excessive autophagy that may result in heart disease (11). Active autophagy can be either pro-survival (adaptive) or anti-survival (maladaptive). Complete abrogation of cardiomyocyte autophagy is detrimental to cardiac homeostasis under basal conditions, whereas upregulation of autophagy in failing heart tissue is an adaptive response that protects cells from hemodynamic stress (12). Autophagy has distinct roles in various types of heart disease, in certain conditions, the response is beneficial, in other cases, it can promote disease progression. Targeting autophagy in the cardiovascular system may be therapeutically relevant. It has previously been hypothesized that induction of autophagy and inhibition of apoptosis may be the mechanism underlying the protective effects of curcumin against oxidative stress.

The phosphoinositide 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway is closely associated

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with the regulation of autophagy for its role in cell survival, proliferation and differentiation (13). mTOR, which is an amino acid, ATP and hormone receptor, may inhibit autophagy. Conversely, inhibition of mTOR by nutritional deficiency or the direct use of rapamycin may activate autophagy-related 1, and thus promote autophagy (14,15). By modulating the mTOR signaling pathway, cell apoptosis and autophagy can be adjusted in numerous cells.

The aim of the present study was to determine the molecular mechanism of action of curcumin in H₂O₂-treated EA.hy926 cells. The EA.hy926 human umbilical vein endothelial cell line was pretreated with various concentrations of curcumin prior to hydrogen peroxide (H₂O₂) stimulation, in order to explore the potential underlying mechanism. Alterations in the expression of autophagy and apoptosis-related proteins, cell viability, and activation of the Akt/mTOR pathway in curcumin-pretreated EA.hy926 cells were determined following H₂O₂ stimulation.

Materials and methods

Cell culture and induction of oxidative stress. The EA.hy926 cell line was purchased from the Cell Bank of the Chinese Academy of Science (Shanghai, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.), penicillin (10,000 units/l; Wisent Inc., Quebec, Canada) and streptomycin (100,000 µg/l) at 37°C in an atmosphere containing 95% air/5% CO₂. The media were changed every 2 days. The cells were allowed to grow to 80% confluence within 24 h prior to drug treatment. The cells were pretreated with curcumin (5–20 µmol/l; Sigma-Aldrich, St. Louis, MO, USA) for 4 h, after which the medium was removed and replaced with medium containing various concentrations of curcumin alongside 200 µmol/l H₂O₂ (Nanjing Chemical Reagent, Co., Ltd., Nanjing, China). The medium of the control group was changed at 4 h and cells were not treated with curcumin or H₂O₂. Following an additional 4 h incubation at 37°C, the cells were assessed.

Cytotoxicity assays. Cell viability was determined using the Cell Counting kit-8 (CCK-8) colorimetric assay (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). Briefly, the cells were seeded into a 96-well cell culture plate (1×10⁴ cells/well), and were pretreated with various concentrations of curcumin and H₂O₂. To measure cell viability, 10 µl CCK-8 assay solution was added to each well, which contained 100 µl medium, and the cells were incubated at 37°C for a further 4 h. Subsequently the optical densities of the wells were measured at 540 nm using a microplate reader (ELx800; BioTek Instruments, Inc., Winooski, VT, USA).

Measurement of apoptosis using Hoechst 33258 staining. Chromatin condensation was detected by nuclear staining using Hoechst 33258. After pretreatment, the medium was removed and the cells were fixed with 500 µl methyl hydrate at room temperature for 15 min, before being washed three times with phosphate-buffered saline (PBS). The cells were then stained with 1 µl Hoechst 33258 (5 mg/ml; Sigma-Aldrich) in 1 ml basal medium and incubated at room temperature in the

dark for 20 min. Stained cells were visualized under a fluorescent microscope (excitation, 350 nm; emission, 460 nm; BX51; Olympus, Tokyo, Japan).

Determination of autophagosome formation. Autophagy is controlled by autophagosome formation (input) and autophagosome degradation (output), and the speed of autophagosome turnover is defined as autophagic flux. A fluorescein isothiocyanate (FITC)-labeled-microtubule-associated protein 1A/1B-light chain 3 (LC3) antibody (CYTO-ID® Autophagy Detection kit) was used to detect autophagosome formation using the CYTO-ID® Autophagy Detection kit (Enzo Life Sciences, Inc., Farmingdale, NY, USA), according to the manufacturers' protocol. The autophagy inducer rapamycin (500 nmol/l; CYTO-ID® Autophagy Detection kit), which is often used as a positive control of autophagy, was added to the cells for 18 h at 37°C. The nuclei were then stained using 4',6-diamidino-2-phenylindole (Beyotime Institute of Biotechnology, Haimen, China). Subsequently, the cells were washed twice with 1X Assay Buffer provided in the kit. Images of autophagic cells were captured using a fluorescent microscope (BX51; Olympus) with a FITC filter (excitation, 480 nm; emission, 530 nm).

Western blot analysis. For whole cell lysate preparations, cultured cells were washed twice with cold PBS and immersed in radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology). The cell lysate was harvested and centrifuged at 12,000 × g for 10 min at 4°C, and the supernatant was subsequently collected. The protein concentration was determined using the bicinchoninic acid protein assay (Beyotime Institute of Biotechnology). Identical protein samples (20 µg/µl) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Billerica, MA, USA). The membrane was blocked with 5% fat-free milk at room temperature for 1 h, and was then incubated with the following primary antibodies: Rabbit anti-B-cell lymphoma 2 (Bcl-2; 1:800; cat. no. sc-492; Bioworld Technology Inc., St. Louis Park, MN, USA), rabbit anti-Bcl-2-associated X protein (Bax; 1:800; cat. no. BS1030; Bioworld Technology Inc.), rabbit anti-caspase-3 (1:1,000; cat. no. ab52314; Abcam, Cambridge, MA, USA), rabbit monoclonal anti-human cleaved caspase-3 (1:1,000; Abcam; cat. no. ab184787), anti-LC3 (1:1,000, cat. no. 3868; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit anti-Akt (1:1,000; cat. no. 4691; Cell Signaling Technology, Inc.), rabbit anti-phosphorylated (p)-Akt (1:1,000; cat. no. 4060; Cell Signaling Technology, Inc.), rabbit anti-mTOR (1:1,000; Abcam; cat. no. ab2732), rabbit anti-p-mTOR (1:1,000; cat. no. ab84400; Abcam) and anti-β-actin (1:800; cat. no. AP0733; Bioworld Technology, Inc.) at 4°C overnight. The membrane was subsequently washed three times for 15 min with Tris-buffered saline containing 0.1% Tween, and incubated with horseradish peroxidase-conjugated secondary antibodies (1:500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) for 1 h at room temperature. The membrane was visualized using a western blotting detection system (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The digital image was analyzed for densitometry using ImageJ (version 1.49; National Institutes of Health, Bethesda, MD, USA).

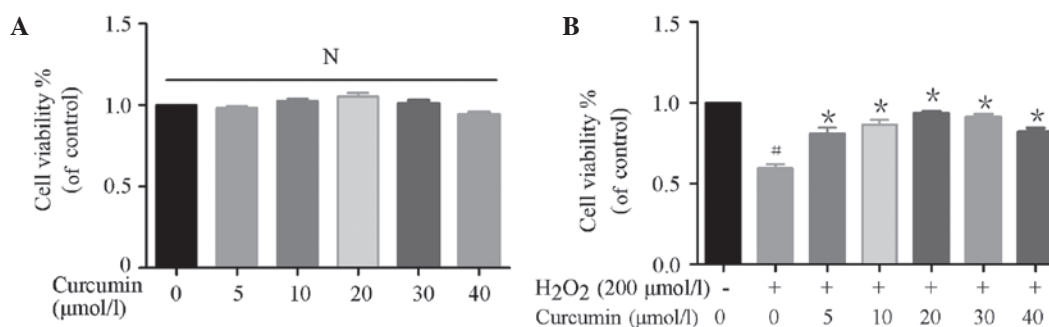


Figure 1. Detection of EA.hy926 cell viability. (A) Curcumin (0–40 μmol/l) did not significantly affect cell viability, as determined by Cell Counting kit-8 assay. (B) Curcumin (5–40 μmol/l) prevented hydrogen peroxide (H₂O₂)-induced cytotoxicity in a dose-dependent manner. Data are presented as the mean ± standard deviation. N, not significant as compared with the control; #P<0.05 vs. the control group; *P<0.05 vs. the H₂O₂-treated group.

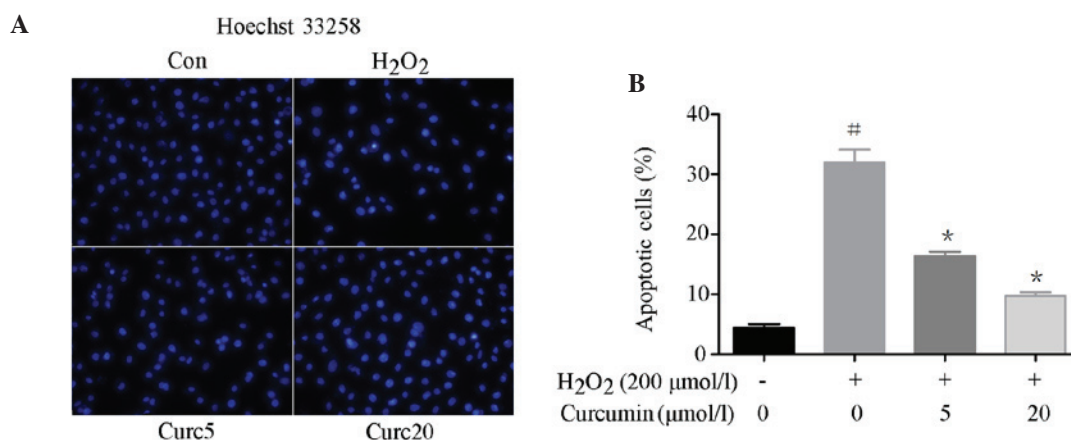


Figure 2. Hoechst 33258 staining of apoptotic cells. (A) Hoechst 33258 staining. Bright blue fluorescence represents apoptosis (magnification, x200). (B) Analysis of the percentage of apoptotic cells. The apoptotic index was significantly lower in the curcumin-pretreated cells, as compared with the hydrogen peroxide (H₂O₂)-treated cells in a concentration-dependent manner. Data are presented as the mean ± standard deviation. #P<0.05 vs. the control group; *P<0.05 vs. the H₂O₂-treated group. Con, control group; H₂O₂, H₂O₂-treated group; Curc5, 5 μmol/l curcumin-treated group; Curc20, 20 μmol/l curcumin-treated group.

Statistical analysis. GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) and SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA) were used for statistical analysis and graphing. All data are presented as the mean ± standard deviation. Statistical differences among groups were analyzed by one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Curcumin protects against H₂O₂-induced cytotoxicity in EA.hy926 cells. EA.hy926 cells were pretreated with curcumin (0, 5, 10, 20, 30 or 40 μmol/l) for 4 h, and were then co-incubated with 200 μmol/l H₂O₂ for an additional 4 h, in order to determine the effects of curcumin on H₂O₂-induced cell death. As shown in Fig. 1A, curcumin (0–40 μmol/l) did not significantly affect viability of the EA.hy926 cells. In addition, curcumin (5–40 μmol/l) prevented H₂O₂-induced cytotoxicity (Fig. 1B).

Curcumin mitigates H₂O₂-induced apoptosis in EA.hy926 cells. To examine the effects of curcumin on the apoptosis of EA.hy926 cells, Hoechst 33258 staining was conducted and the expression levels of apoptosis-associated proteins were detected following curcumin pretreatment. Hoechst 33258 staining was

used to assess DNA fragmentation. As shown in Fig. 2A and B, the percentage of apoptotic cells was significantly reduced in the curcumin-pretreated cells in a concentration-dependent manner, as compared with the H₂O₂-treated cells. The expression levels of apoptosis regulatory proteins, including caspase-3, Bax and Bcl-2 were detected, in order to confirm the anti-apoptotic effect of curcumin on H₂O₂-induced cells. As shown in Fig. 3A–G, the expression levels of cleaved caspase-3 and Bax were increased following treatment with H₂O₂, as compared with the control. Curcumin pretreatment significantly decreased the expression levels of H₂O₂-induced cleaved caspase-3 and Bax in EA.hy926 cells. In addition, the expression levels of the anti-apoptotic protein Bcl-2 were decreased following H₂O₂ treatment, and were increased in a concentration-dependent manner following curcumin pretreatment. The increase in Bax/Bcl-2 ratio induced by H₂O₂ was inhibited by curcumin.

Curcumin promotes autophagy in H₂O₂-treated EA.hy926 cells. In order to detect autophagy and evaluate the extent of autophagosome formation in H₂O₂-treated cells, a FITC-labeled LC3 antibody from the CYTO-ID® Autophagy Detection kit (Enzo Life Sciences, Inc.) was used. When autophagy takes place in mammalian cells, LC3 content, particularly LC3-II

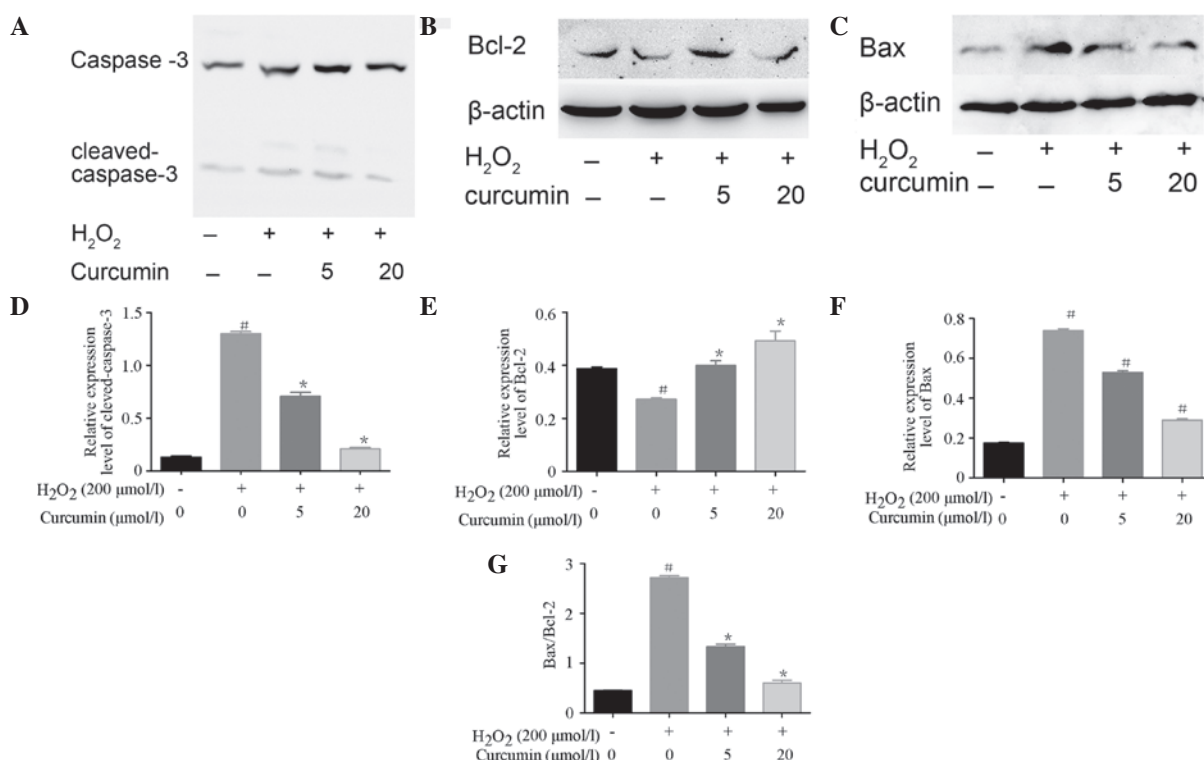


Figure 3. Expression levels of apoptosis regulatory proteins. (A-C) Western blot analysis of cleaved caspase-3, B-cell lymphoma 2 (Bcl-2) and Bcl-2-associated X protein (Bax). β -actin was used as an internal control. (D-F) Densitometric analysis of cleaved caspase-3, Bcl-2 and Bax normalized to β -actin. (G) Analysis of the ratio between the expression of Bcl-2 and Bax. Data are presented as the mean \pm standard deviation. * $P < 0.05$ vs. the control group; # $P < 0.05$ vs. the H_2O_2 -treated group.

content, is significantly increased. LC3-II is considered an appropriate marker of autophagy activation, since it is a vital protein in autophagosome formation (16). As shown in Fig. 4A and B, as compared with the control group, there was no significant difference in LC3-II content in the H_2O_2 -treated cells; however, there was a significant increase in LC3 expression in the rapamycin-treated cells, and a slight increase in expression in the curcumin-pretreated cells. Lipidation of LC3 is essential for autophagy to proceed. As shown in Fig. 4C and D, following an increase in autophagy, the protein expression levels of LC3-II were significantly higher in the curcumin-pretreated cells compared with in the control and H_2O_2 -treated cells. Furthermore, the results of western blotting indicated that the protein expression levels of LC3-II were significantly higher in the curcumin-pretreated cells, as compared with in the control and H_2O_2 -treated cells.

Curcumin downregulates the Akt/mTOR signaling pathway in H_2O_2 -treated EA.hy926 cells. To further evaluate the mechanisms and signaling pathways underlying curcumin-induced autophagy, the activation status of mTOR and its upstream regulator Akt were determined by western blotting. As shown in Fig. 5, the expression levels of p-Akt and p-mTOR were increased in the H_2O_2 -treated cells. However, the expression levels of p-Akt and p-mTOR were significantly decreased in the curcumin-pretreated cells in a concentration-dependent manner. These results indicate that curcumin was able to inhibit the expression of the autophagy suppressor mTOR in EA.hy926 cells, and this was associated with the cell-specific modulation of the mTOR upstream regulator, Akt.

Discussion

The results of the present study demonstrated that pretreatment with curcumin alleviated H_2O_2 -induced cytotoxicity in EA.hy926 cells. In addition, pretreatment with curcumin inhibited oxidative stress-induced apoptosis and activated adaptive autophagy in EA.hy926 cells. These results indicated that the protective effects of curcumin were dependent on regulation of the Akt/mTOR signaling pathway.

The EA.hy926 human umbilical vein cell line is produced by hybridizing human umbilical endothelial cells (HUVECs) with the A549 epithelial cell line. EA.hy926 cells are widely used as a replacement for HUVECs in *in vitro* experiments, and H_2O_2 is widely used to generate models of oxidative stress. Oxidative stress is capable of activating the mitochondrial signaling pathway and has a pivotal role in the pathogenesis of cardiovascular disease, including ischemic heart disease, hypertension and heart failure (17). Therefore, the elimination of excessive intracellular ROS and prevention of oxidative stress may be an effective intervention for the treatment of these diseases.

Curcumin, which is a major effective ingredient extracted from a traditional Chinese herbal medicine, exerts potent antioxidative effects and has been widely used to prevent and treat cardiovascular disease, including atherosclerosis (18,19). However, the mechanisms underlying the protective effects of curcumin vary considerably between studies. Previously, Notch, Toll-like receptor (TLR)2, ROS-related TLR4-mitogen-activated protein kinases/nuclear factor- κ B, glycogen synthase kinase-3 β , peroxisome proliferator-activated

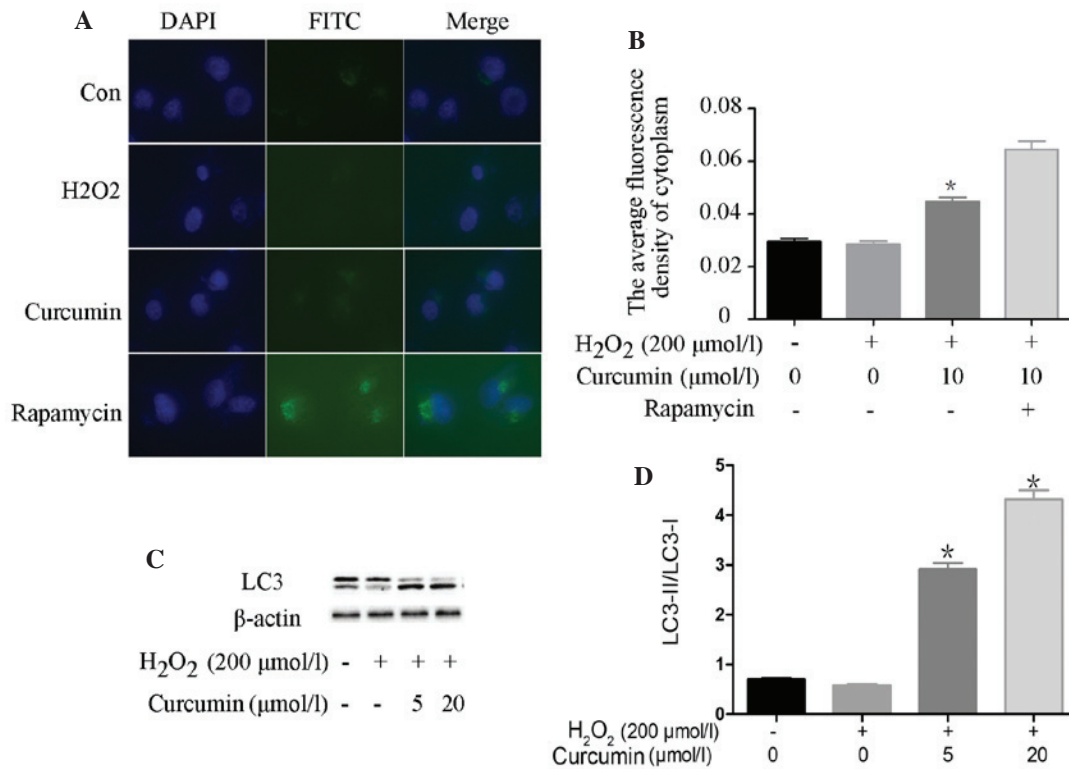


Figure 4. Treatment with curcumin induced autophagy in hydrogen peroxide (H₂O₂)-treated EA.hy926 cells. (A) Detection of autophagosome formation. Blue fluorescence represents the nucleus and green fluorescence represents autophagy (magnification, x200). Rapamycin was used as a positive control. (B) Analysis of autophagosome formation. (C and D) Western blot analysis of microtubule-associated protein 1A/1B-light chain 3 (LC3) normalized to β-actin. Data are presented as the mean ± standard deviation. *P<0.05 vs. the control group; *P<0.05 vs. the H₂O₂-treated group. Con, control group.

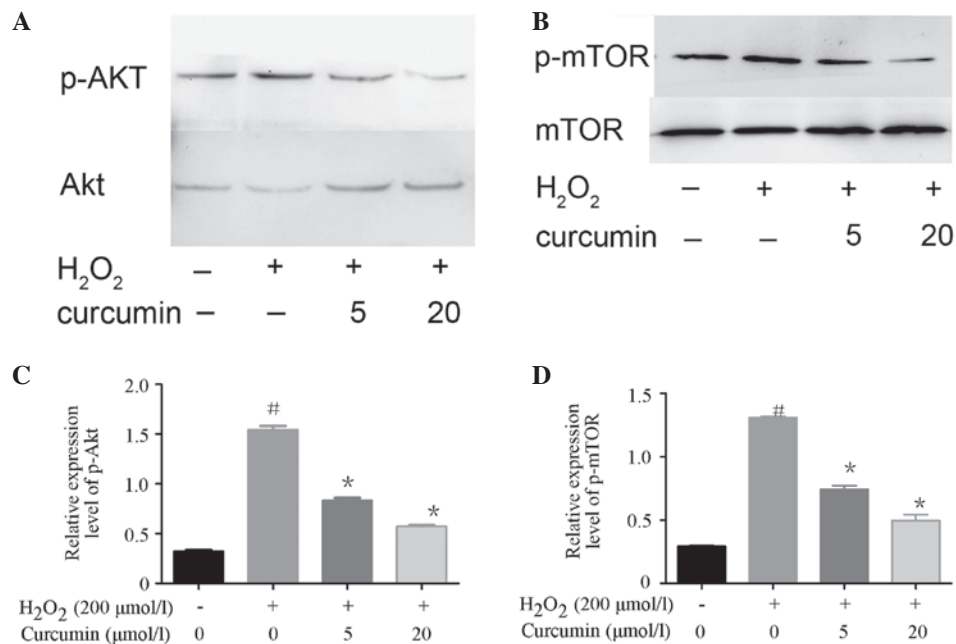


Figure 5. Phosphorylated (p)-Akt and p-mammalian target of rapamycin (mTOR) protein expression in EA.hy926 cells. (A) Western blot analysis of total Akt and p-Akt, and (B) total mTOR and p-mTOR expression. Densitometric analysis of (C) p-Akt and (D) p-mTOR expression. Data are presented as the mean ± standard deviation. #P<0.05 vs. the control group; *P<0.05 vs. the H₂O₂-treated group.

receptor γ, and Sirtuin 1 (2,20-24) have been reported to have a role in the protective effects of curcumin on oxidative stress-injured endothelial cells. In our previous study, curcumin was shown to mitigate H₂O₂-induced myocardial damage

by inhibiting the expression of monocyte chemoattractant protein-1 (25); however, the role of autophagy in this process has not been reported. To the best of our knowledge, no studies have yet reported that curcumin activates adaptive autophagy

in oxidative stress-injured endothelial cells; however, considerable evidence has demonstrated that curcumin induces cancer cell apoptosis via autophagy activation (26-31). Curcumin, or its analogue, have previously been reported to induce cell death in colon cancer, uterine leiomyosarcoma, astrocytoma, ovarian cancer, lung adenocarcinoma and cutaneous T-cell lymphoma cells (26-31) by activating autophagy. In the present study, curcumin attenuated H₂O₂-induced cell death by activating adaptive autophagy in EA.hy926 cells.

Autophagy and apoptosis are two closely regulated pathways through which superfluous, damaged, or aged cells or organelles are eliminated. In addition, autophagy is a process by which cells adapt their metabolism to environmental or intracellular stress conditions, including starvation, endoplasmic reticulum stress, hypoxia, ischemia/reperfusion injury, pathogens and oxidative stress. Autophagy generates metabolic substrates that meet the bioenergetic needs of cells, and thereby allows for adaptive protein synthesis by the catabolism of macromolecules (32-34). Autophagy enables cells to adapt to stress, in order to avoid cell death; however, autophagy is also an alternative pathway that may lead to cell death (35-37). Autophagy and apoptosis can be initiated in response to similar stimuli, whereas in some situations autophagy and apoptosis are initiated in a mutually exclusive manner. The relationship between autophagy and apoptosis is complex. There are several examples in which the induction of autophagy promotes the activation of apoptosis; however, autophagy also arises from the inhibition of apoptosis and protects cells from cell death (38), and sometimes autophagy inhibits the induction of apoptosis. High dose H₂O₂ can induce cellular damage due to oxidative stress. Oxidative stress increases the permeability of the lysosomal membrane, and induces the uncoupling of oxidation and phosphorylation reactions in the mitochondria, resulting in the activation of various cell death programs (39). At a low level of oxidative stress, autophagy protects the cell against major harm by degrading damaged mitochondria prior to cytochrome *c* release (40). In the present study, a significant decrease in cell viability was detected in the H₂O₂-treated (200 μ mol/l) EA.hy926 cells. In addition, the levels of apoptosis were increased, whereas autophagy exhibited no significant change. When pretreated with curcumin, H₂O₂-induced EA.hy926 cell death was reduced, the rate of apoptosis was reduced and autophagy was increased. Therefore, these results suggested that H₂O₂ (200 μ mol/l) was able to induce EA.hy926 cell apoptosis but had no effect on autophagy, whereas curcumin pretreatment attenuated apoptosis in H₂O₂-treated EA.hy926 cells by activating autophagy. These data demonstrated that curcumin may induce autophagy, in order to protect endothelial cells.

The Bcl-2 protein family, which is comprised of anti- and pro-apoptotic members, consists of important mitochondrial regulators during cell apoptosis. As compared with Bax, Bcl-2 blocks cytochrome *c* release, inhibits caspase activity and suppresses cell apoptosis (41). Therefore, alterations to the Bcl-2/Bax ratio influences apoptotic balance. In the present study, curcumin significantly inhibited Bax expression, and increased Bcl-2 expression in cells undergoing oxidative stress, resulting in a reduced Bax/Bcl-2 ratio and increased cell viability.

The regulation of autophagy is complex and involves numerous pathways. The mTOR pathway is the most extensively studied network with regards to autophagy regulation due to its ability to sense nutrient state, growth factor availability and stress (42). Upstream of mTOR complex 1 (mTORC1) is the tuberous sclerosis complex (TSC)1-TSC2 inhibitory complex, which functions as an upstream activator of mTOR. The TSC1-TSC2 complex inactivates Rheb, which inhibits mTOR signaling, leading to the subsequent activation of autophagy. mTORC1 is able to directly sense amino acid concentration and energy state, whereas the PI3K/Akt axis can sense growth factor status. mTOR complex 2 is an inhibitor that decreases the extent of Akt-induced mTORC1 activation (43). The present study demonstrated that H₂O₂ induced the phosphorylation of mTOR, and this activation was inhibited by curcumin. These findings are consistent with the view that mTOR regulates autophagy by controlling phosphorylation (44). Indeed, curcumin pretreatment led to decreased Akt phosphorylation, which was associated with mTOR inhibition and autophagy. These results indicated that Akt is involved in curcumin-induced mTOR suppression and autophagy. However, the underlying mechanisms of action require further study.

In conclusion, the results of the present study demonstrated that oxidative stress may promote cell death in EA.hy926 cells, and pre-treatment with curcumin suppresses cell death by inducing autophagy via regulation of the Akt/mTOR pathway.

Acknowledgements

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