SPANDIDOS PUBLICATIONS

Bisdemethoxycurcumin protects endothelial cells against *t*-BHP-induced cell damage by regulating the phosphorylation level of ERK1/2 and Akt

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Abstract. Curcuminoids are the major active components extracted from *Curcuma longa* and are well known for their antioxidant effects. Previous studies have reported that the antioxidant properties of curcuminoids are mainly attributed to their free radical scavenging abilities. However, whether there are other mechanisms besides the non-enzymatic process and how they are involved, still remains unknown. In the present study, we explored the protective effects of bisdemethoxycurcumin (Cur3) against *tert*-butyl hydroperoxide (*t*-BHP)-induced cytotoxicity in human umbilical vein endothelial cells (HUVECs), focusing on the effect of Cur3 on the regulation of the phosphatidylinositol 3-kinase (PI3K)/Akt and the mitogen-activated protein kinase (MAPK) pathways. The pre-treatment with Cur3 inhibited *t*-BHP-induced cell damage dose-dependently, which was evident by the increased cell viability and the corresponding decrease in lactate dehydrogenase release. The pre-treatment with Cur3 also attenuated *t*-BHP-induced cell morphological changes and apoptosis. MAPKs, including p38, c-Jun N-terminal kinase (JNK), extracellular signal-regulated protein kinase 1/2 (ERK1/2), as well as PI3K/Akt have been reported to be involved in proliferation, apoptosis and differentiation under various stress stimulations. The pre-treatment with Cur3 decreased *t*-BHP-induced ERK1/2 phosphorylation and increased *t*-BHP-induced Akt phosporylation but did not affect the phosphorylation of p38 or JNK. In addition, the Cur3-induced increase in cell viability was attenuated by the treatment with wortmannin or LY294002, the upstream inhibitors of Akt, and was enhanced by the treatment with 2-[2'-amino-3'-methoxyphenyl]-oxanaphthalen-4-one (PD98059), an upstream inhibitor of ERK1/2. These results suggest that the ERK1/2 and PI3K/Akt signaling pathways could be involved in the protective effects of Cur3 against *t*-BHP-induced damage in HUVECs.

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Key words: curcuminoids, oxidative stress, endothelial cells, mitogen-activated protein kinase, Akt

Introduction

Curcuma longa has been used in traditional medicine to treat mild or moderate human diseases in India, China and other Asian countries for centuries (1-3). Over the years, it has sparked increasing scientific curiosity due to its broad range of potent pharmacological effects and its almost non-existent toxicity even at high doses (4). Curcuminoids, including curcumin (Cur1), demethoxycurcumin (Cur2) and bisdemethoxycurcumin (Cur3), are the main active components extracted from the rhizome of *Curcuma longa*, and have been demonstrated to exert various therapeutic, including anti-inflammatory, anti-carcinogenic, anti-infectious, as well as neuro- and cardioprotective effects (1,4-7).

It is known that the structure and functional integrity of endothelial cells play very important roles in the maintenance of healthy vessel walls and circulatory functions (8). Oxidative stress is regarded as one of the major causes of endothelial cell damage (9), and thereby contributes to most major cardiovascular diseases, such as atherosclerosis, hypertension and thrombosis (10). Curcuminoids are well known for their potent protective effects against oxidative stress, and have therefore been suggested as attractive therapeutic agents for the prevention and treatment of free radical-related diseases, including cardiovascular diseases. However, even though data obtained from laboratory experiments and clinical trials have shown promising antioxidant activities of curcuminoids, the underlying mechanisms have yet to be fully clarified. In previous studies, the protective effects of curcuminoids have mainly been attributed to the free radical scavenging ability of the phenolic hydroxyl group in their molecular structures (11-13). However, other studies have suggested that more complex mechanisms are involved. It has been demonstrated that curcuminoids can induce the expression of heme oxygenase-1 (HO-1), which contributed to their cytoprotective effects in response to oxidative injuries. This effect was associated with increased Nrf2 binding to the resident *ho-1* antioxidant-responsive element as well as with the activation of mitogen-activated protein kinases (MAPKs) (14,15). Moreover, curcumin has been reported to increase the

expressions of other antioxidant enzymes such as superoxide dismutase, glutathione S-transferase and aldo-keto reductase, and the phosphatidylinositol 3-kinase (PI3K)/Akt and the p38 MAPK pathways have also been shown to be involved in the regulations of some of these enzymes (16,17).

In view of the evidence that the MAPK and Akt pathways are two of the most commonly involved signal transduction pathways in response to oxidative challenges by the regulation of cell proliferation, apoptosis and death (18,19), we postulated that the MAPK and PI3K/Akt pathways could also play significant roles in the underlying mechanisms of the protective effects of curcuminoids against oxidative injuries. In order to test this hypothesis, exogenous *tert*-butyl hydroperoxide (*t*-BHP) was used to induce endothelial cell damage. The effects of curcuminoids against oxidative stress were examined and possible pathways were analyzed. The results suggest that Cur3 exhibited a potent protective effect against *t*-BHP-induced cell damage in human umbilical vein endothelial cells (HUVECs) which could be mediated through the regulation of the extracellular signal-regulated protein kinase 1/2 (ERK1/2) and PI3K/Akt pathways.

Materials and methods

Materials. Kaighn's modification of Ham's F12 medium (F-12K), fetal bovine serum (FBS), phosphate buffered saline (PBS), penicillin, streptomycin, and 0.25% (w/v) trypsin/1 mM EDTA, were purchased from Invitrogen (Carlsbad, CA, USA). Endothelial cell growth supplement, heparin, gelatin and *t*-BHP solution 70% in H_2O were supplied by Sigma (St. Louis, MO, USA). MTT was purchased from USB (OH, USA). The Cytotoxicity Detection Kit was purchased from Roche Applied Science (Germany). Hoechst 33342 dye was purchased from Molecular Probe (Eugene, OR, USA). All antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). PD98059, LY294002 and wortmannin were purchased from Merck (Germany). RIPA lysis buffer, phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail and the BCA™ Protein Assay kit were purchased from Pierce Biotechnology (Rockford, IL, USA). The ECL advanced Western blotting detection kit was purchased from Amersham (UK)

Drugs. Pure curcumin (MW 368), demethoxycurcumin (MW 338) and bisdemethoxycurcumin (MW 308) were separated from *Curcuma longa* (turmeric). Their structures were deduced on the basis of their physicochemical properties and spectral data (Fig. 1), and the purity of the compounds was >98%. The purity was verified by HPLC-DAD.

Cell culture. HUVECs were purchased from ATCC (Manassas, USA), and maintained at 37˚C and 5% CO_2 in F-12K medium supplemented with 2 mM L-glutamine, 1.5 g/l sodium bicarbonate, 30 mg/l ECGS, 10% (v/v) FBS, 100 mg/l heparin, 100 U/ml penicillin and 100 μg/ml streptomycin. All experiments were performed with cells between passages 2 to 5.

Cell viability assays. HUVECs were seeded at $5x10^3$ cells/well in 96-well plates. After 24 h of pre-treatment with

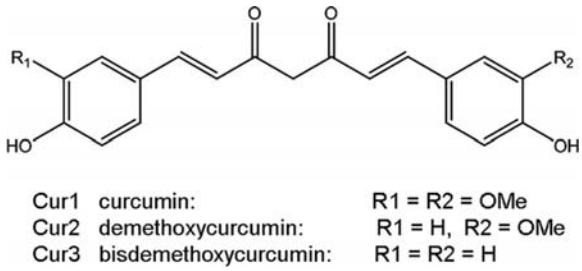

Figure 1. The chemical structures of curcumin, demethoxycurcumin and bisdemethoxycurcumin.

curcuminoids and 1 h *t*-BHP exposure, cell viability was evaluated by MTT and lactate dehydrogenase (LDH) assays. In the MTT assay, the HUVECs were incubated with 100 μl F-12K medium containing 0.5 mg/ml MTT. After 4 h incubation at 37˚C, the cell supernatants were discarded, the MTT crystals were dissolved in 100 μl DMSO and the absorbance was measured at 570 nm using a multilabel counter (Perkin Elmer, 1420 Multilabel Counter Victor3, Wellesley, MA, USA). In the LDH assay, cell injury was assessed by measuring the amount of LDH released into the medium from damaged cells. LDH activity in the cell culture supernatants was determined using the Cytotoxicity Detection Kit according to the manufacturer's instructions. The release of LDH was measured at 490 nm with 690 nm as the reference wavelength.

Hoechst labeling. Hoechst 33342 staining was used to observe the chromosome condensation and morphological changes of the cells. HUVECs were cultured in 24-well plates. The cells were treated with 30 μM Cur3 for 24 h, followed by the stimulation of 300 μM *t*-BHP for 1-2 h. The cells were fixed with 4% paraformaldehyde and washed with PBS, and then stained with Hoechst 33342 (10 μg/ml) with RNase (5 μg/ml) for 30 min at room temperature. After 3 washes with PBS, stained cells were visualized under UV illumination using a fluorescent microscope (Carl Zeiss, Axiovert 200, USA).

Western blot analysis. Protein was extracted using RIPA lysis buffer with 1% PMSF and 1% protease inhibitor. Lysates were centrifuged at 12,000 x g for 20 min at 4˚C and the supernatant was collected. Total protein concentrations were determined using the BCA™ Protein Assay kit. Supernatants containing 40 μg of protein/lane were separated by 12% SDS-PAGE gels. After electrophoresis, the separated proteins were electrically transferred onto polyvinylidene difluoride membranes. The membrane was probed with a primary antibody followed by a second antibody and visualized using an ECL advanced Western blotting detection kit. Photos of protein bands were taken using the Molecular Imager ChemiDoc XRS (BioRad, USA). Densitometric measurements of the band intensities on the Western blots were performed using Quantity One Software.

Statistical analysis. Results were presented as the means ± SD of at least 3 independent experiments. Data were analyzed by

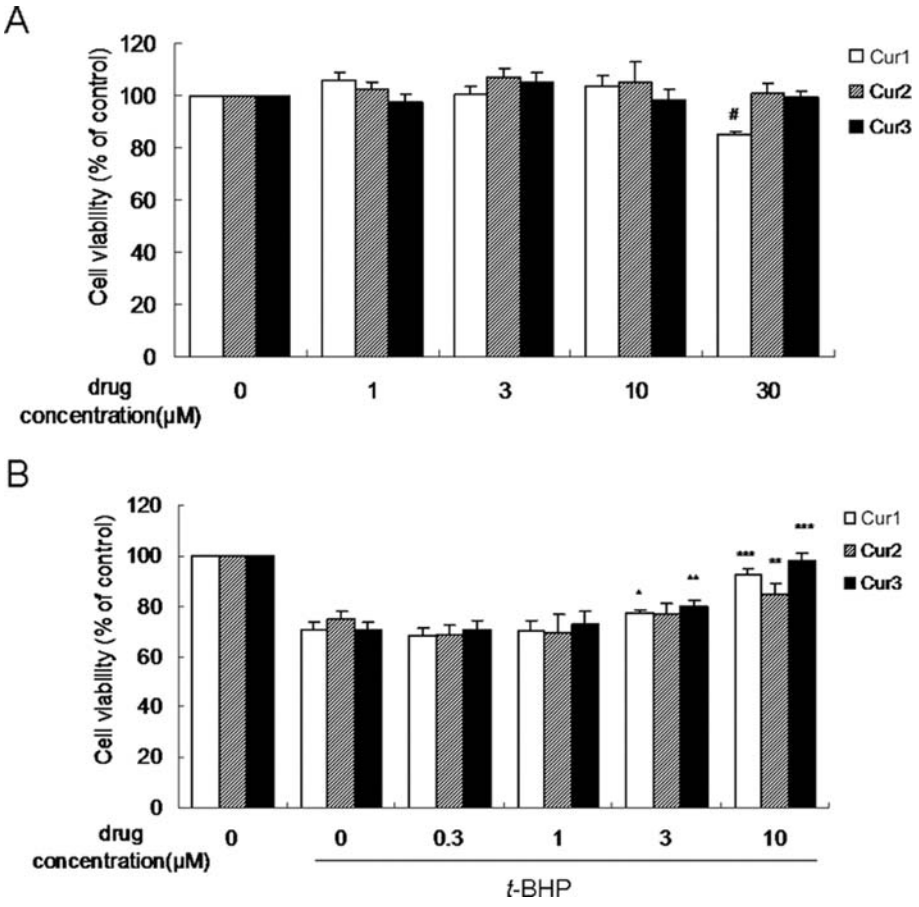

Figure 2. Cytotoxicity and protective effects of curcuminoids against *t*-BHP-induced cell damage in HUVECs. (A) Effects of curcuminoids on the viability of HUVECs. The cells were exposed to various concentrations of Cur1, 2 or 3 (1-30 μM) for 24 h, then the cell viability was determined by MTT assay. (B) Protective effects of curcuminoids against *t*-BHP-induced cell damage. The cells were pre-treated with or without Cur1, 2 and 3 for 24 h, followed by 300 μM *t*-BHP stimulation for 1 h. The results are presented as a percentage of the control. $^{\#}$P<0.05 compared to the untreated controls, *P<0.05, **P<0.01 and ***P<0.001 compared to the *t*-BHP alone group. The data shown here are the means ± SD of 3 independent experiments.

the Student's t-test. A value of P<0.05 was considered to be statistically significant.

Results

Curcuminoids protected HUVECs against t-BHP-induced cytotoxicity. We first examined and compared the cytotoxicity and protective effects of 3 curcuminoids, Cur1, 2 and 3, against *t*-BHP-induced cell damage. HUVECs were treated with different doses of Cur1, 2 or 3 for 24 h followed by MTT assay. The results show that Cur2 and 3 did not exhibit any cytotoxicity up to a dose of 30 μM, while Cur1 slightly decreased the cell viability at the highest concentration used (30 μM, 24 h) (Fig. 2A). The protective effects of these 3 curcuminoids were further explored by exposing HUVECs to *t*-BHP (300 μM) for 1 h with or without the pre-treatment (24 h) with curcuminoids. As shown in Fig. 2B, the pre-treatments with Cur1 and 3 attenuated *t*-BHP-induced cell damage in a dose-dependent manner and they reached their maximal effects at a concentration of 10 μM (P<0.001). The pre-treatment with Cur2 also modestly protected the cells against the oxidative damage elicited by *t*-BHP in a dose-dependent manner with a maximal effect at a dose of 10 μM (P<0.01). Considering that Cur3 exhibited the best performance among the 3 curcuminoids, it was chosen for further study.

Cur3 protected HUVECs against t-BHP-induced cell death and apoptosis. In order to further verify the cellular protective effect of Cur3, the LDH assay was performed. The release of LDH was dramatically increased to 141.01% of the control when the cells were challenged with *t*-BHP-induced oxidative stress. The pre-treatment with Cur3 decreased LDH release dose-dependently with a maximal effect at a dose of 30 μM (116.74% of the control). The effect of the Cur3 pre-treatment on *t*-BHP-induced morphological changes and apoptosis in HUVECs was also examined. As shown in Fig. 3, *t*-BHP induced various morphological changes, including the loss of cell attachment, cell shrinkage, nucleus pycnosis, and the formation of crescent-shaped condensation or apoptotic bodies which were not observed in the control group. Fig. 3D shows that such morphological changes and apoptotic features were reduced significantly when the cells were pre-treated with 30 μM of Cur3.

t-BHP treatment activated MAPK [c-Jun N-terminal kinase (JNK), p38 and ERK1/2] and Akt pathways. In order to investigate whether the MAPK and Akt pathways were

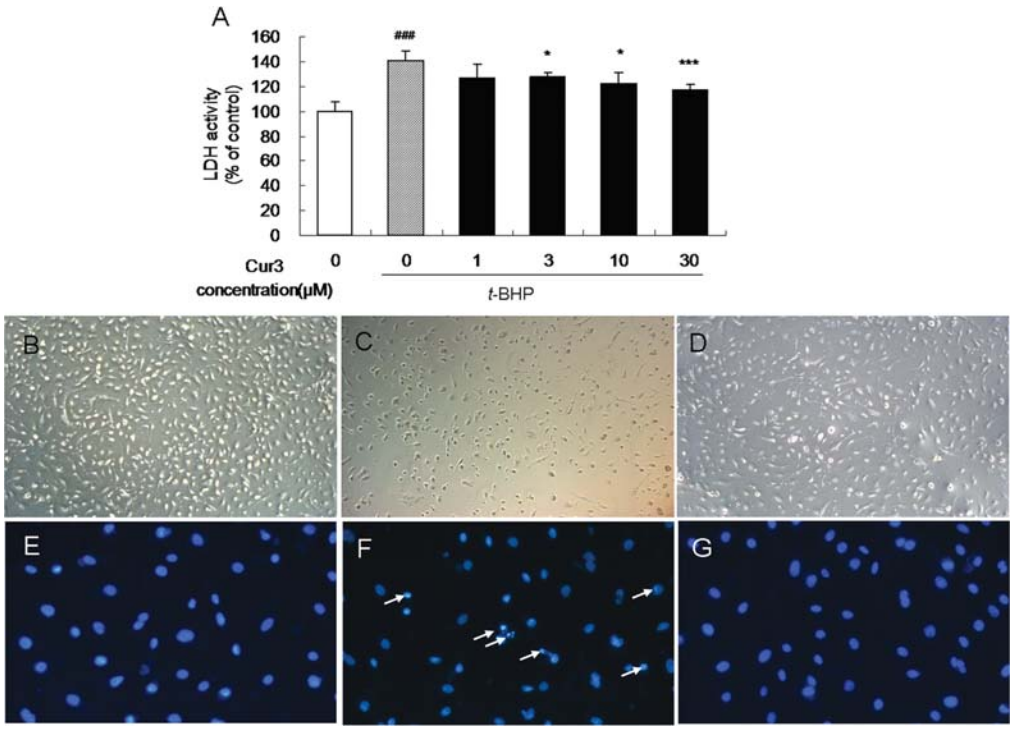

Figure 3. Protective effect of Cur3 against *t*-BHP-induced cell damage in HUVECs. (A) Effect of Cur3 pre-treatment on LDH leakage induced by *t*-BHP in HUVECs. Cells were treated with or without various concentrations of Cur3 for 24 h, followed by *t*-BHP stimulation for 1 h. LDH release was then measured as described in Materials and methods. $^{###}$P<0.001 compared to the untreated control group. *P<0.05, ***P<0.001 compared to *t*-BHP alone group. (B-D) Representative images of HUVECs. (E-G) Representative images of Hoechst 33342 staining. Chromatin condensation and DNA fragments are indicated by the white arrows. (B and E) Untreated control. (C and F) Cells treated with *t*-BHP 300 μM for 1-2 h. (D and G) Cells pre-treated with 30 μM Cur3 for 24 h before being exposed to *t*-BHP.

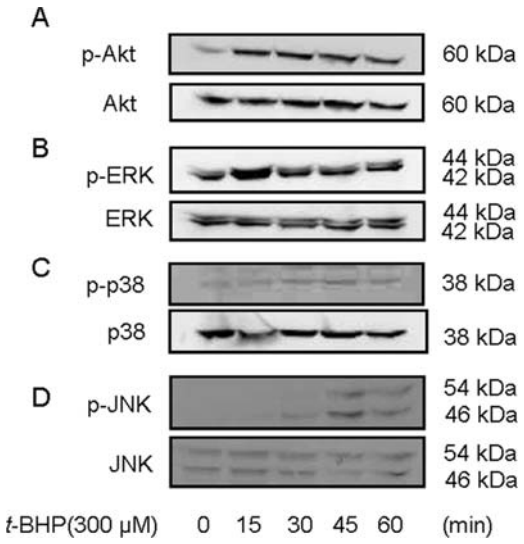

Figure 4. Phosphorylation of Akt, ERK1/2, p38 and JNK induced by *t*-BHP. Cells were treated with *t*-BHP (300 μM) and harvested at the times indicated for Western blot analysis. Antibodies against (A) phospho-Akt (Ser473) and Akt, (B) phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2, (C) phospho-p38 (Thr180/Tyr182) and p38, and (D) phospho-JNK (Thr183/Tyr185) and JNK, were applied as described in Materials and methods.

involved in the *t*-BHP-induced oxidative injury, the phosphorylation levels of JNK (Thr183/Tyr185), p38 (Thr180/Tyr182), ERK1/2 (Thr202/Tyr204) and Akt (Ser473) were examined by Western blot analysis. Fig. 4 shows that *t*-BHP treatments (300 μM) triggered rapid activations of JNK, p38, ERK1/2 and Akt in HUVECs with no significant change in the total protein levels. The maximal levels of phosphorylation for Akt and ERK1/2 were observed at 15 min, while those for p38 and JNK, at 45 min.

The pre-treatment with Cur3 modulated t-BHP-induced ERK1/2 and Akt activation. The pre-treatment with Cur3 modulated the *t*-BHP-induced MAPK and Akt activation. HUVECs were first pre-treated with Cur3 (0.3-30 μM) for 24 h, followed by 300 μM *t*-BHP. Based on the time-response results described earlier (Fig. 4), the cells were exposed to *t*-BHP for 15 min to evaluate the effects on the regulation of ERK1/2 and Akt, and 45 min for JNK and p38, respectively (Fig. 5). The results show that the pre-treatment with Cur3 for 24 h down-regulated the activation of ERK1/2. On the contrary, the phosphorylation level of Akt was increased in a dose-dependent manner. However, the Cur3 pre-treatment did not produce any significant modulation on the *t*-BHP-induced activations of p38 or JNK.

t-BHP-induced cytotoxicity of HUVECs was attenuated by PD98059 and enhanced by wortmannin or LY294002. Specific MEK1 (PD98059) and PI3K inhibitors (wortmannin and LY294002) were used to further clarify whether the *t*-BHP-induced cytotoxicity was mediated through the ERK1/2 or PI3K/Akt signaling pathways. The HUVECs were exposed to PD98059 (10 μM), LY294002 (20 μM) or wortmannin (20 μM) for 1 h before the treatment with *t*-BHP. The cells

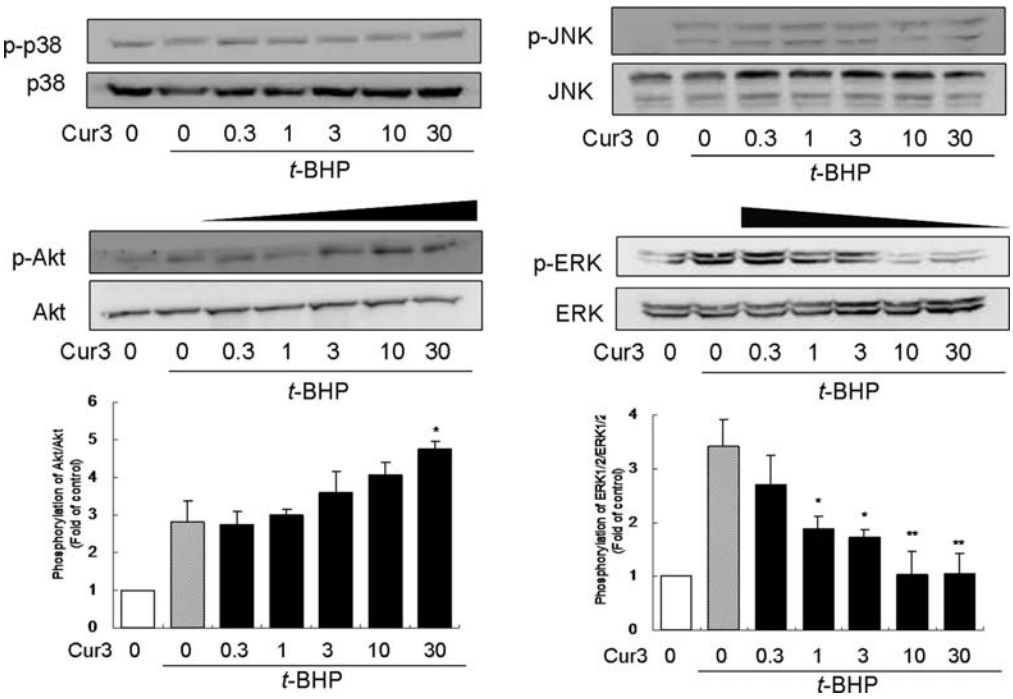

Figure 5. Effects of Cur3 on *t*-BHP-induced phosphorylation of Akt, ERK1/2, p38 and JNK. Cells were pre-treated with various concentrations of Cur3 for 24 h, and then stimulated with *t*-BHP (300 μM) for 15 min for the determination of ERK1/2 and Akt activities, or for 45 min for the determination of p38 and JNK activities. Representative blots are shown. At least 3 independent experiments were done for each investigation.

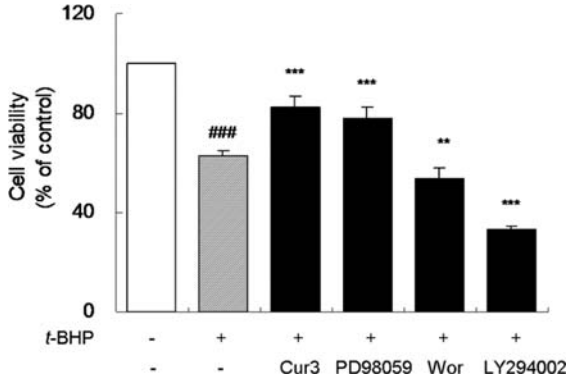

Figure 6. Effects of PD98059, wortmannin and LY294002 on *t*-BHP-induced cell damage. The cells were pre-treated with Cur3 (30 μM, 24 h) PD98059 (10 μM, 1 h), wortmannin (20 μM, 1 h) or LY294002 (20 μM, 1 h), followed by the stimulation of 300 μM *t*-BHP for 1 h. Cell viability was measured by MTT assay. The results are presented as a percentage of the control. $^{\#\#\#}$P<0.001 compared to the untreated controls, *P<0.05, **P<0.01 and ***P<0.001 compared to the *t*-BHP alone group. The data shown here are the means ± SD of 3 independent experiments.

were then further incubated for 1 h in the presence of 300 μM *t*-BHP followed by subsequent MTT assay. PD98059 (10 μM, 1 h) blocked the phosphorylation of ERK1/2 (Thr202/Tyr204), while wortmannin (20 μM, 1 h) or LY294002 (20 μM, 1 h) blocked the phosphorylation of Akt (Ser473). As shown in Fig. 6, the decrease in cell viability caused by *t*-BHP was markly attenuated by PD98059. In contrast, wortmannin and LY294002 enhanced the *t*-BHP-induced cell damage. On the whole, these results suggest that the protective effect of Cur3 against *t*-BHP-induced damage in HUVECs could be mediated through the ERK1/2 and PI3K/Akt pathways.

Discussion

In the present study, we explored the protective effects of curcuminoids against *t*-BHP-induced damage in HUVECs. *t*-BHP, a reactive hydroperoxide produced from lipid peroxidation, has been shown to be a major player in the activation of the redox-sensitive transcription factors (20), and therefore has frequently been used in the investigation of cellular alterations resulting from free radical actions in various cell types, such as hepatocyte (21), auditory (22) retinal pigmented epithelial (23), and endothelial cells (20). Consistent with previous reports, *t*-BHP induced cell damage and even cell death, which was evident by the decreased cell viability and increased LDH release. Cur1, 2 and 3, which are 3 active curcuminoids extracted from the rhizome of *Curcuma longa*, significantly protected HUVECs from *t*-BHP-induced oxidative damage. Among them, Cur3 exerted the best protective effect with the lowest cytotoxicity. In addition, Cur3 attenuated *t*-BHP-induced cell morphological changes and apoptosis, such as the loss of cell attachment, cell shrinkage, nucleus pycnosis, and the formation of crescent-shaped or apoptotic bodies in HUVECs. Therefore, Cur3 was selected for further study in order to explore the underlying mechanisms in further detail.

Oxidative stress triggered various kinds of signaling pathways, among which MAPK and Akt have been proven to play important roles in damages caused by ROS (24). Our results show that the exposure of HUVECs to *t*-BHP rapidly activates JNK, p38, ERK1/2 and Akt without changing the total protein levels. Furthermore, it was found that Cur3 dose-dependently decreased the ERK1/2, whereas it increased the Akt phosphorylation level. These results indicate the possibility of the involvement of ERK1/2 and Akt in the

protective effects of Cur3 against *t*-BHP-induced cell damage in HUVECs.

The ERK1/2 pathway, as a subfamily of MAPK whose function and regulation are evolutionarily conserved, has been reported to be involved in many cellular processes such as growth, proliferation, differentiation and death. In addition, the ERK1/2 pathway has been reported to be rapidly activated in response to various cellular stimuli, including oxidative stress. Our results confirm that *t*-BHP increased the phosphorylation level of ERK1/2 which peaked at 15 min. In order to clarify the role of ERK1/2 in the process of *t*-BHP-induced damage, PD98059, which is a specific inhibitor of the upstream factor of ERK, was used. The results demonstrate that when the ERK pathway was blocked by the inhibitor, the cell damage caused by *t*-BHP stimulation, was attenuated. This observation is not consistent with previous studies which generally consider ERK1/2 to be a cell survival signal in the process of ROS-triggered apoptosis. However, other evidence has suggested that the activation of ERK1/2 also mediates cell damage (25). It has been reported that blocking the ERK pathway by using MEK1 inhibitors rescues cells from injury induced by various stimuli (26,27). This observation was further confirmed by the observation that the transient transfection of cells with constitutively active MEK1 increased the cisplatin-induced apoptosis, whereas the transfection with a dominant-negative mutant of MEK1, decreased it (28). Additionally, the inhibition of ERK activation has been implicated in the protective effects of drugs such as salvianolic acid B against hydrogen peroxide-induced endothelial cell apoptosis (29). These results suggest that the decrease in the phosphorylation level of ERK1/2 could be beneficial to cell survival in some cell types and organs under certain conditions.

Growing evidence indicates that the Akt signaling pathway plays a crucial role in cell survival in response to oxidative stress. Several downstream targets of Akt have been found to be involved in the regulation of cell apoptosis, DNA repair, stress resistance and metabolism. For example, it has been reported that Akt can phosphorylate the Bcl-2 family member, BAD, both *in vivo* and *in vitro*, and promotes cell survival and suppresses BAD-mediated cell death (30). NF-κB and caspase-9 have also been identified as functional targets of Akt (31). Our results show that the *t*-BHP-induced cell death of HUVECs was accompanied with an increased level of Akt phosphorylation. However, the Akt phosphorylation reached its maximal level at 15 min after *t*-BHP exposure, but was not maintained and started to decline soon afterwards. This could be explained by the effect of stress response which occurred instantly and only temporarily as the first call of defense of the cells against injuries. Nevertheless, our results clearly show that the pre-treatment with Cur3 significantly prevented cell loss and dose-dependently increased the level of phosphorylated Akt when the cells were challenged with *t*-BHP, suggesting that Cur3 produced its protective effects through the up-regulation of Akt activation. Furthermore, the *t*-BHP-induced cell damage was enhanced by blocking the PI3K/Akt pathway with wortammin or LY294002. It has been reported that cytotoxicity induced by a variety of apoptotic stimuli can be attenuated effectively by the constitutive activation of Akt, while the transfection with dominant-negative Akt alleles abrogates the ability of various growth factors to promote cell survival (32-34). Consistent with previous findings, it is evident that the PI3K/Akt pathway is essential for cell survival and apoptosis, and that the up-regulation of Akt activation could be one of the under-lying mechanisms that contribute to the protective effects of Cur3.

In summary, the present study demonstrates that Cur3 protects *t*-BHP-induced endothelial cell damage by down-regulating the activation of ERK1/2 and up-regulating the phosphorylation of Akt. This study provides experimental evidence to support the notion that curcuminoids could have therapeutic and protective applications in free radical-related cardiovascular diseases.

Acknowledgements

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