Baicalein protects *tert*-butyl hydroperoxide-induced hepatotoxicity dependent of reactive oxygen species removal

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Abstract. Baicalein (BA), one of the major bioactive flavonoids isolated from *Scutellariae Radix*, possesses various pharmacological activities. The present study aimed to investigate the protective effects of BA on *tert*-butyl hydroperoxide (*t*-BHP)-induced hepatotoxicity, and to investigate the potential mechanisms in LO2 cells. BA was demonstrated to possess protective properties against *t*-BHP injury in LO2 cells, as evidenced by MTT and lactate dehydrogenase assays. BA significantly prevented *t*-BHP-induced depolarization of mitochondrial membrane potential (MMP), decreased the percentage of apoptotic cells caused by *t*-BHP, and prevented intracellular reactive oxygen species (ROS) generation in LO2 cells. Furthermore, BA slightly triggered autophagy in LO2 cells, as evidenced by the elevation of LC3-II expression, while BA combined treatment with an autophagy inhibitor (chloroquine) or activator (rapamycin) did not alter the hepatoprotective properties. In conclusion, BA may possess a hepatoprotective effect against *t*-BHP-induced liver cell injury, dependent on ROS removal. Therefore, BA may represent a potential drug candidate in protecting hepatotoxicity.

Introduction

The liver is the largest organ which detoxifies various metabolites and produces plasma proteins, and is necessary for the digestive system. However, numerous factors, including pathogen infections, alcohol, harmful chemicals and drug abuse, may lead to liver trauma, such as hepatitis, cirrhosis, fibrosis and hepatic steatosis (1). These may eventually lead to the development of hepatocellular carcinoma (2). Therefore, it is essential to develop novel approaches to protect the liver from injury.

Numerous researchers have selected natural phytochemicals to develop hepatotherapeutic agents (3). These phytochemicals can ameliorate illness and provide a safer way to protect against hepatic injury (4). Baicalein (BA, Fig. 1) is one of the major flavonoids isolated from the dried *Scutellariae Radix* (5). BA exhibits a variety of biological activities, including antibacterial (6), antioxidative (7), cardiovascular protective (8,9), neuroprotective (10) and anticancer (11). Furthermore, treatment with BA may protect against liver ischemia/reperfusion injury (12), polymicrobial sepsis-induced liver injury (13), concanavalin A-induced hepatitis (14) and carbon tetrachloride (CCl_4)-induced liver fibrosis (15).

Certain physiological activities in liver such as detoxification may generate high levels of reactive oxygen species (ROS), which leads to oxidative stress (16). These free radicals are highly unstable and alter membrane permeability, which lead to hepatic tissue injury (17). *Tert*-butyl hydroperoxide (*t*-BHP) is an organic peroxide which can be metabolized to free radical intermediates that subsequently affect cell integrity by initiating lipid peroxidation, resulting in oxidative hepatotoxicity in hepatocytes (18). Alternatively, *t*-BHP as a model compound is often used to investigate toxicity and the mechanisms of liver injury caused by oxidative stress. It may cause a variety of liver injuries such as elevating leakage of liver enzymes, including alanine aminotransferase and aspartate aminotransferase (19), forming malondialdehyde and suppressing glutathione depletion (18). These alterations finally lead to chronic hepatitis and hepatic fibrosis. Therefore, investigation of *t*-BHP-induced hepatotoxicity may be critical to develop hepatoprotective therapies.

Autophagy is a self-digestive process that degrades cellular organelles and proteins in order to maintain cellular homeostasis and ensure cell survival under stressful situations (20). In general, autophagy is an effective cellular defense system against a variety of pathologic diseases (21). Previous evidence indicates that autophagy serves as a cell survival mechanism against multiple liver injuries. Induction of autophagy is a defensive mechanism against usnic acid-induced toxicity in hepatic cells (22). Furthermore, activation of caspase 1 can protect against hypoxia/reoxygenation injury by up-regulating beclin1 and mitochondrial autophagy in the liver (23). In our

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previous study, BA induced protective autophagy in HepG2 hepatocellular carcinoma cells (24). The present study aimed to detect whether BA triggers autophagy and its role in *t*-BHP-induced liver injury in LO2 cells.

Materials and methods

Materials. Baicalein (≥98.5%, Zelang Group, Nanjing, China) was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) as a stock solution. Phosphate-buffered saline (PBS) powder, RPMI 1640 medium, fetal bovine serum (FBS), penicillin/streptomycin and trypsin-EDTA were obtained from Gibco; Thermo Fisher Scientific (Waltham, MA, USA). Tetraethylbenzimi-dazolylcarbocyanine iodide (JC-1) dye was purchased from Thermo Fisher Scientific, Inc. 2,7'-Dichlorodihydrofluorescein diacetate (H_2DCF-DA), *t*-BHP and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-2Htetrazolium bromide (MTT) were obtained from Sigma-Aldrich; Merck KGaA. The Cytotoxicity Detection kit (lactate dehydrogenase, LDH) was obtained from Roche Diagnostics GmbH (Mannheim, Germany). Rabbit primary antibodies against microtubule-associated protein 1A/1B-light chain 3 (LC3; cat. no. 2775s), cleaved-PARP (c-PARP; cat. no. 9532), B-cell lymphoma 2 (Bcl-2; cat. no. 4223s), Bcl-2 associated X protein (Bax; cat. no. 5023s), survivin (cat. no. 2808) and GAPDH (cat. no. 2118), and the horseradish peroxidase-conjugated goat anti-rabbit secondary IgG antibody (cat. no. 7074) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture. The LO2 immortalized healthy human liver cell line (25,26) was obtained from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in an incubator with 5% CO_2 at 37˚C. During treatments, cells were cultured in RPMI 1640 medium with 0.5% FBS and 100 U/ml penicillin and 100 μg/ml streptomycin with or without indicated treatments.

MTT assay. LO2 cells were seeded into 96-well plates at a density of $5x10^3$ cells/well and cultured at 37˚C for 24 h. LO2 cells were pretreated with 0, 12.5, 25 or 50 μM BA for 12 h and exposed to 1 mM *t*-BHP for 4 h. Cell proliferation was determined by adding 1 mg/ml MTT-containing medium for 4 h, followed by 100 μl DMSO to solubilize the formazan, and agitation for 10 min in the dark. The absorbance at a wavelength of 570 nm using a SpectraMax M5 Microplate Reader (Molecular Devices, LLC, Sunnyvale, CA, USA). The results were analyzed based on at least three independent experiments.

Lactate dehydrogenase (LDH) assay. LO2 cells were seeded into 96-well plates at a density of $8x10^3$ cells/well and cultured at 37˚C for 24 h. LO2 cells were pretreated with 0, 12.5, 25 or 50 μM BA for 12 h and exposed to 1 mM *t*-BHP for 4 h. Cell injury was determined by measuring the quantity of LDH released into the incubation medium. A Cytotoxicity Detection kit was used to detect released LDH activity, according to the manufacturer's protocol. The absorbance was measured at a wavelength of 490 nm using a SpectraMax M5 Microplate Reader.

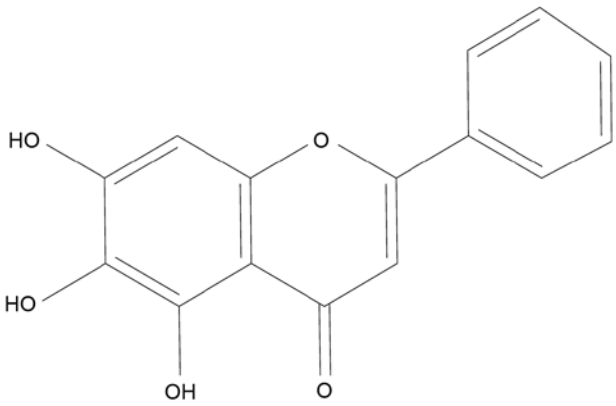

Figure 1. Chemical structure of baicalein.

Measurement of mitochondrial membrane potential (MMP). The MMP was determined by fluorescent probe JC-1 staining assay. Briefly, LO2 cells were pretreated with various concentrations of BA (0, 12.5, 25 or 50 μM) for 12 h, followed by treating with 1 mM *t*-BHP for 30 min. Following this, the cells were stained with JC-1 (1 μg/ml) for a further 30 min. The fluorescence of JC-1 was observed using an IN Cell Analyzer 2000 (GE Healthcare Life Sciences, Little Chalfont, UK).

Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) staining analysis. An apoptosis detection kit (BioVision, Inc. Milpitas, CA, USA) containing annexin V-FITC and PI was used to identify apoptotic cells, according to the manufacturer's protocol. Briefly, LO2 cells were seeded into 6-well plates at a density of $2x10^6$ cells/well. Following a 2-h incubation with *t*-BHP in the presence or absence of BA (25 μM) for 24 h, LO2 cells were harvested, washed and resuspended in 500 μl binding buffer containing 5 μl annexin V-FITC and 5 μl PI for 30 min at 25˚C. A total of 10,000 cells were analyzed by using a flow cytometer (Becton Dickinson FACS Canto, Franklin Lakes, NJ) and FlowJo software (version 7.6.1; Tree Star, Inc., Ashland, OR, USA).

Detection of intracellular ROS. ROS formation was determined using the probe H_2DCF-DA. Briefly, LO2 cells were pretreated with 25 μM BA for 12 h and incubated with 5 μM CM-H2DCFDA at 37˚C for 15 min. Following this, cells were treated with 1 mM *t*-BHP for another 30 min and harvested for analysis by flow cytometry.

Western blot analysis. Cells were lysed in radioimmunoprecipitation assay lysis buffer (Beyotime, Shanghai, China) for 30 min at 4˚C, and subsequently centrifuged for 20 min (14,000 x g and 4˚C). The supernatant fraction was obtained as protein and the protein concentrations were determined using a Bicinchoninic Acid Protein Assay kit (Pierce, Rockford, IL, USA). Total proteins (25 μg) were separated by 12% SDS-PAGE, followed by transferring onto PVDF membranes. After blocking with 5% nonfat milk at room temperature for 1 h with agitation, the membranes were probed with LC3

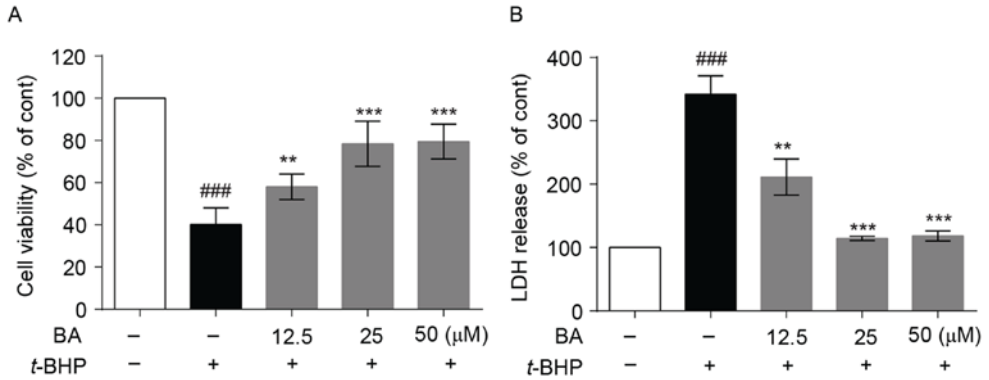

Figure 2. Protective effects of BA on *t*-BHP-induced cytotoxicity. After pretreatment with various concentrations of BA (0, 12.5, 25, and 50 μM) for 12 h, LO2 cells were treated with 1 mM *t*-BHP for another 4 h. (A) Cell viability was assessed by MTT assay. (B) LDH release was assessed by an LDH assay. Data are presented as the mean ± standard deviation of at least three independent experiments. $^{###}$P<0.001 vs. control group; **P<0.01, ***P<0.001 vs. *t*-BHP treatment group. BA, baicalein; *t*-BHP, *tert*-butyl hydroperoxide; LDH, lactate dehydrogenase.

(1:2,000), c-PARP (1:2,000), Bcl-2 (1:2,000), Bax (1:2,000), survivin (1:2,000) and GAPDH (1:2,000) primary antibodies, which were diluted with PBS-0.1% Tween-20, overnight at 4˚C. After washing with PBS with Tween-20 three times for 15 min each, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000) at room temperature for 1 h. Protein bands were visualized using an Enhanced Chemiluminescence advanced western blot detection kit (GE Healthcare Life Sciences).

Chloroquine (CQ) and rapamycin (RAPA) treatment in LO2 cells. LO2 cells were seeded into 96-well plates at a density of $5x10^3$ cells/well (MTT assay) or $8x10^3$ cells/well (LDH assay) and cultured at 37˚C for 24 h. Cells were subsequently pretreated with 10 μM CQ (Sigma-Aldrich; Merck KGaA) or 100 nM RAPA (Sigma-Aldrich; Merck KGaA) at 37˚C for 1 h, followed by treatment with BA (25 μM) at 37˚C for an additional 12 h. Subsequently, the cells were treated with or without 1 mM *t*-BHP at 37˚C for 4 h. The change in cell viability and LDH release were subsequently detected as described above for MTT and LDH release assays, respectively. For western blot analysis, LO2 cells were seeded into 6-well plates at a density of $2x10^6$ cells/wells and cultured at 37˚C for 24 h. Cells were then pretreated with 10 μM CQ or 100 nM RAPA at 37˚C for 1 h, followed by treatment with BA (25 μM) at 37˚C for an additional 12 h. LC3-I and LC3-II protein expression was detected as described above for western blot analysis.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical significance was analyzed by one-way analysis of variance followed by the Tukey post-hoc test using Graph Pad Prism version 5 (GraphPad Software, Inc., La Jolla, CA, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

BA protects against t-BHP-induced LO2 cell injury. To investigate whether BA protects cells from *t*-BHP-induced cytotoxicity, LO2 cells were pretreated with BA for 12 h and then exposed to 1 mM *t*-BHP for another 4 h. The cell viability was assessed by MTT assay. As presented in Fig. 2A, the cell viability of *t*-BHP-treated group decrease to 40.1±4.6% compared with the control group. Following pretreatment with 12.5, 25 and 50 μM BA, cell viability significantly increased to 58.0±3.5, 78.4±6.1 and 79.5±4.8%, respectively. The results of the LDH assay demonstrated that *t*-BHP increased the LDH release rate from the basal level of 100.0 to 341.9±17.0%, which was decreased to 211.3±16.5, 114.4±2.0 and 118.5±4.5% by 12.5, 25 and 50 μM BA pretreatment, respectively (Fig. 2B). The results indicated that BA obviously reduced LDH leakage induced by *t*-BHP in a dose-dependent manner, suggesting that pretreatment with BA may block *t*-BHP-induced cell injury.

BA attenuates t-BHP-induced apoptosis. Annexin V and PI staining were used to detect apoptotic cells. Pretreatment of 25 μM BA significantly decreased percentage of apoptotic cells from 66.4±4.0 to 18.8±5.8 in LO2 cells (Fig. 3A and B). It is well known that depolarization of MMP is an early event in the process of cell apoptosis. To identify the effect of BA on *t*-BHP-induced early apoptosis in LO2 cells, the MMP in LO2 cells was evaluated by JC-1 staining assay. Once the MMP is depolarized, the color of the dye alters reversibly from red to green fluorescence (27). As presented in Fig. 3C, exposure to 1 mM *t*-BHP in LO2 cells resulted in an increase in green fluorescence intensity. Pretreatment with BA (12.5, 25 and 50 μM) attenuated MMP disruption, indicating that BA may protect the cells against *t*-BHP-induced apoptosis. Additionally, the expression levels of numerous proteins associated with apoptosis were detected. Following *t*-BHP treatment, the antiapoptotic proteins Bcl-2 and survivin were markedly downregulated, while the levels of c-PARP and Bax were obviously upregulated. Notably, these effects were reversed by pre-treatment with 25 μM BA (Fig. 3D).

BA decreases ROS generation in LO2 cells. It has been reported that BA has a strong free radical scavenging activity (28). To determine whether the protective effect of BA was attributed to reducing the intracellular ROS levels caused by *t*-BHP, a ROS dye (H_2DCF-DA) was used to detect alterations in intracellular peroxide levels. As presented in Fig. 4A and B, when LO2 cells were exposed to 1 mM *t*-BHP for 30 min, the intracellular ROS level significantly increased compared with

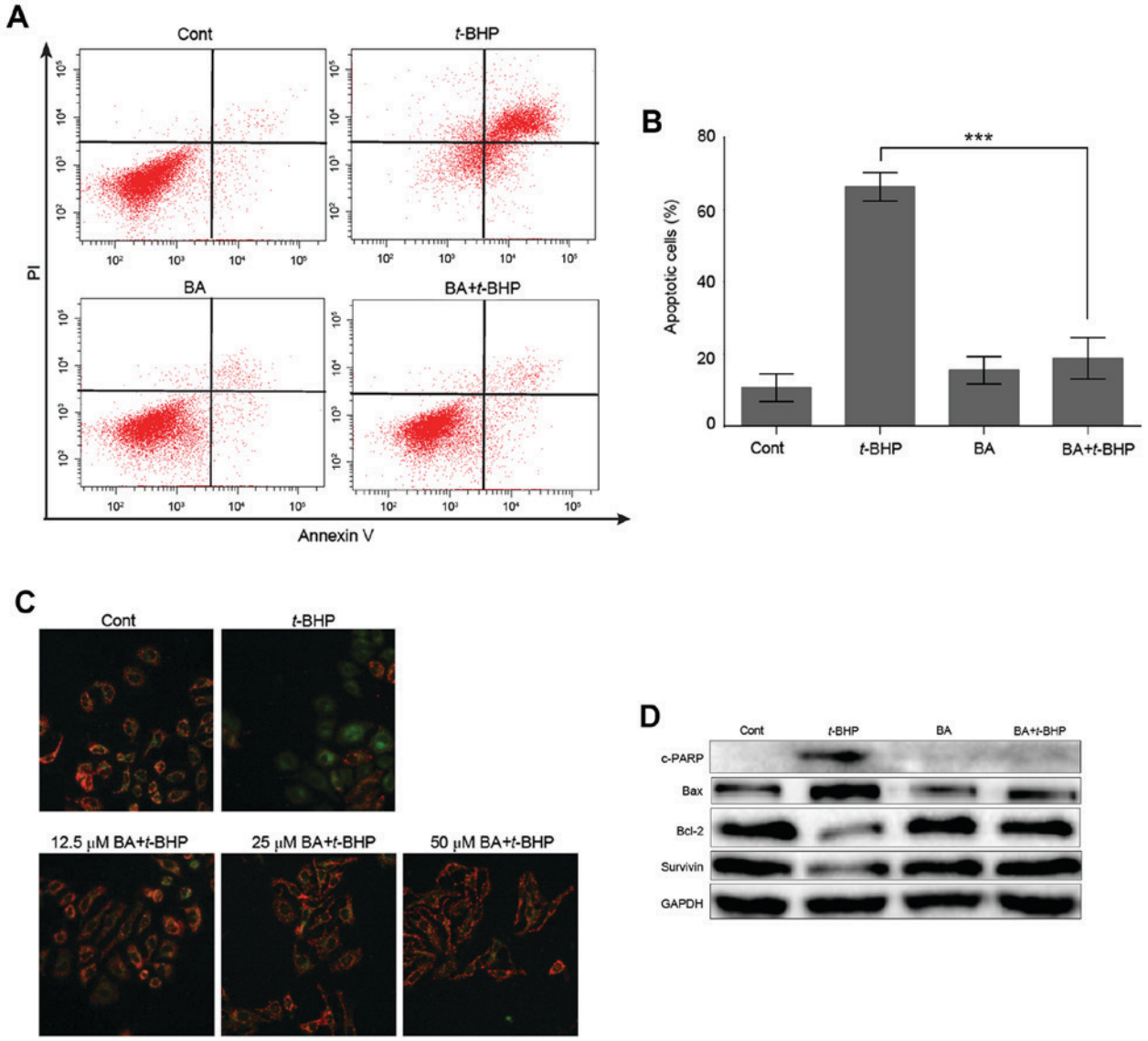

Figure 3. BA attenuates *t*-BHP-induced apoptosis in LO2 cells. (A) After pretreatment with vehicle control or 25 μM BA for 12 h, LO2 cells were treated with 1 mM *t*-BHP for another 2 h and then stained with Annexin V-fluorescein isothiocyanate and PI. Apoptotic cells were quantified by flow cytometry. (B) Quantification of three independent tests. Data are presented as mean ± SD of at least three independent experiments. ***P<0.001 vs. *t*-BHP treatment group. (C) Representative immunofluorescence images of cells stained with JC-1. Once the MMP is depolarized, the color of the dye alters reversibly from red to green fluorescence. (D) Representative western blot images of c-PARP, Bax, Bcl-2 and survivin protein expression levels. GAPDH served as an internal control. Cont, control; BA, baicalein; *t*-BHP, *tert*-butyl hydroperoxide; LC3, microtubule-associated protein 1A/1B-light chain 3; c-PARP, cleaved PARP; Bcl-2, B-cell lymphoma 2; Bax, B-cell lymphoma 2 associated X protein; PI, propidium iodide.

the control group. Pretreatment with BA significantly attenuated the increased ROS level induced by *t*-BHP from 100 to 10.0±2.0% in LO2 cells, indicating that BA protected LO2 cells from *t*-BHP-induced injury by reducing intracellular production of ROS.

Autophagy may not be involved in the protective effect of BA against t-BHP-induced LO2 cell injury. To confirm whether autophagy is triggered in BA-treated LO2 cells, protein expression levels of LC3-II protein, a marker of autophagy (29), were determined by western blot analysis. As presented in Fig. 5A, 25 μM BA slightly increased the expression of LC3-II. To further investigate BA-induced autophagic flux in LO2 cells, CQ, an autophagy inhibitor that blocks autophagosome fusion with the lysosome, causing upregulation of LC3-II protein (30), was used. LO2 cells were treated with 10 μM CQ for 1 h prior to BA treatment. The expression of LC3-II was markedly upregulated following CQ and BA combined treatment. Furthermore, rapamycin, which activates autophagy by inhibition of the mechanistic target of rapamycin signaling pathway (31) was used in these experiments. Following pretreatment with rapamycin, the expression of LC3-II was also increased (Fig. 5A), indicating that BA potentially induces autophagic flux in LO2 cells.

Our previous study indicated that BA induces protective autophagy in HepG2 hepatocellular carcinoma cells (24). In addition, accumulating evidence has indicated that autophagy may be a defense mechanism against liver injury. Therefore, BA-induced autophagy may be involved in the protective effects of BA against LO2 cell injury caused by *t*-BHP. After

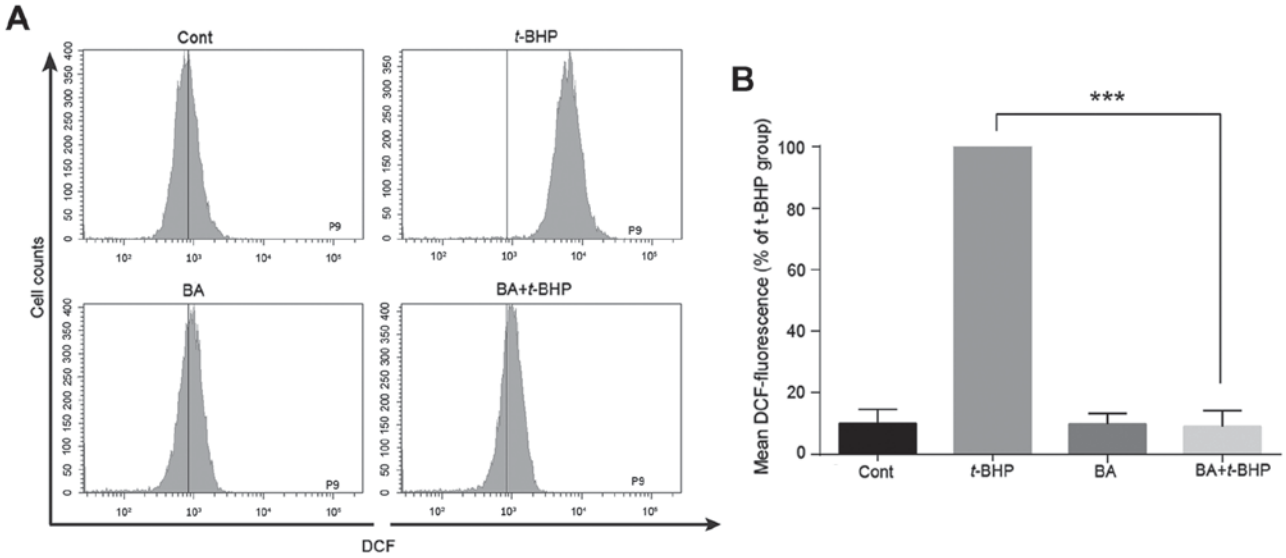

Figure 4. BA attenuates *t*-BHP-induced intracellular oxidant levels in LO2 cells. (A) The intracellular ROS level of LO2 cells was measured by flow cytometry. (B) Data are presented as the mean ± standard deviation of at least three independent experiments. ***P<0.001 vs. *t*-BHP treatment group. BA, baicalein; *t*-BHP, *tert*-butyl hydroperoxide; Cont, control.

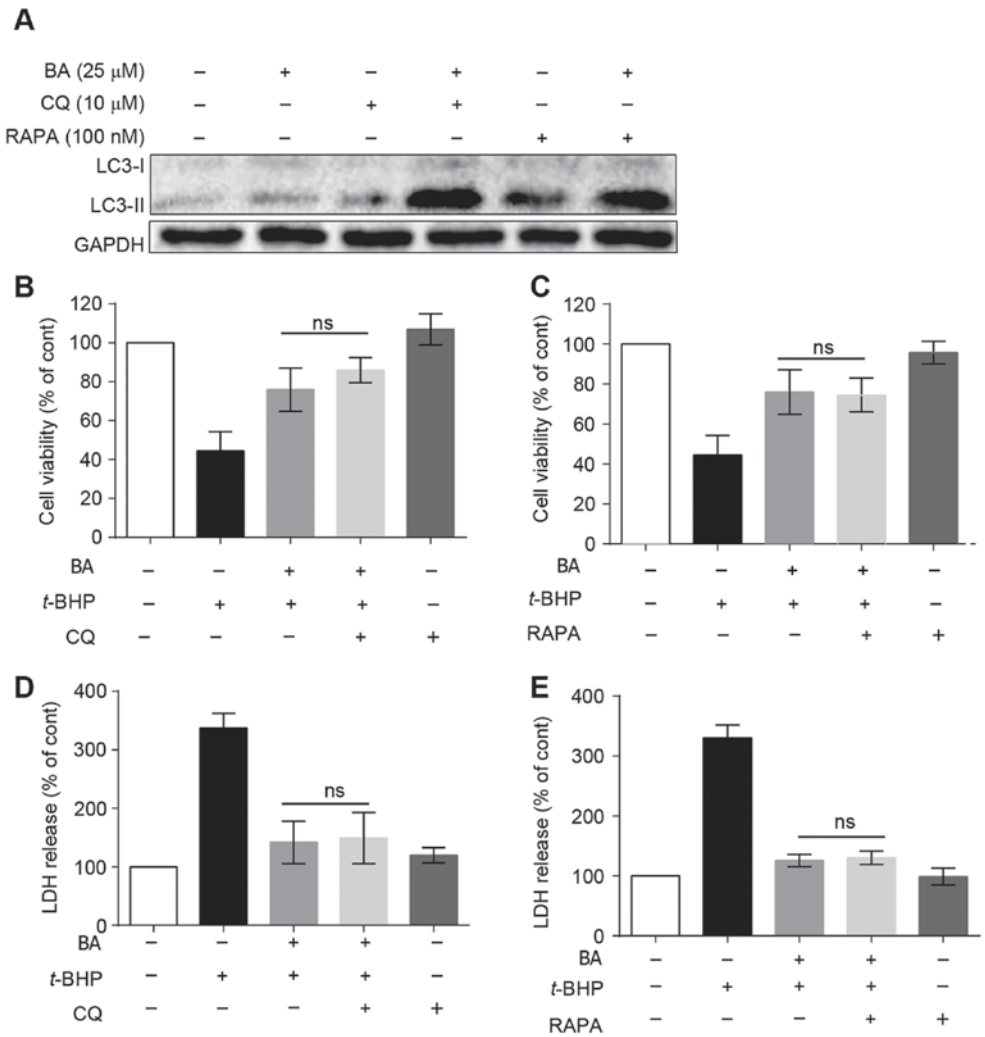

Figure 5. Autophagy may be not involved in the protective effect of BA against *t*-BHP-induced cell injury. (A) Representative western blot images of protein expression levels of LC3-I and -II. Cells pretreated with 10 μM CQ or 100 nM RAPA for 1 h, followed by treatment with BA for an additional 12 h. Cell viability was assessed following additional treatment with (B) CQ and (C) RAPA. LDH release was determined following additional treatment with (D) CQ and (E) RAPA. Data are presented as the mean ± standard deviation of at least three independent experiments. BA, baicalein; *t*-BHP, *tert*-butyl hydroperoxide; cont, control; LC3, microtubule-associated protein 1A/1B-light chain 3; RAPA, rapamycin; CQ, chloroquine; ns, non-significant; LDH, lactate dehydrogenase.

inhibiting or activating autophagy by CQ or rapamycin, cell viabilities detected by MTT were 86.0±3.8 (Fig. 5B) and 74.5±4.9% (Fig. 5C), respectively, which was not significantly different compared with the BA-treated group in *t*-BHP-injured LO2 cells. Similar results were observed in LDH release assay (Fig. 5D and E), suggesting that autophagy may be not involved in the protective effect of BA against *t*-BHP-induced LO2 cell injury.

Discussion

BA exhibits a variety of biological activities, including anti-inflammation (32), antioxidative (33) and hepatic protective effects (12). Furthermore, BA is contained in a traditional Chinese medicine formula named Xiao-Chai-Hu-Tang (34), which has been used to treat chronic hepatitis (35), liver fibrosis and cirrhosis (36) for many years. Thus, much attention has been focused on its protective function on hepatic injury (13). Chronic administration of BA may prevent liver fibrosis induced by CCl_4 in rats (15). BA may also inhibit apoptosis on acute liver failure induced by d-galactosamine (d-GalN)/lipopolysaccharides (37). The present study further elucidated the protective role of BA in LO2 liver cells by suppressing *t*-BHP-induced hepatic damage. Pretreatment with BA significantly protected cell injury caused by *t*-BHP, demonstrated by increased cell viability and reduced leakage of LDH, and reduced apoptosis induced by *t*-BHP, indicating that BA serves a protective role in liver injury in LO2 cells.

Based on its polyphenolic structure, BA has a strong antioxidant and free radical scavenging activity (28). Treatment with BA may protect HT22 neuronal cells by inhibiting production of ROS (7). Through reducing ROS production and calcium overload, BA may also prevent lysophosphatidylcholine-induced cardiac injury (38). Furthermore, *t*-BHP may induce hepatic oxidative damage by inducing cellular oxidative stress, including lipid peroxidation and glutathione levels (39). In the present study, treatment with *t*-BHP increased the production of ROS compared with untreated controls, whereas pretreatment with BA greatly decreased ROS levels, indicating that the protective effect of BA was attributed to reducing intracellular ROS levels caused by *t*-BHP. A recent study demonstrated that BA protects against polymicrobial sepsis-induced liver injury via inhibition of apoptosis (13). In addition, overproduction of ROS may lead to apoptotic cell death (40). The results of the present study also demonstrated that pretreatment with BA attenuates apoptotic cell death and MMP disruption during LO2 cell injury caused by *t*-BHP.

Autophagy is actively involved in liver physiology and pathogenesis (41), and constitutes an effective defense mechanism against multiple pathological insults (42). It has been reported that tonsil-derived mesenchymal stem cells can differentiate into hepatocyte-like cells and exert a protective effect against liver fibrosis through autophagy activation (43). Autophagy can prevent dasatinib-induced hepatotoxicity both *in vitro* and *in vivo* (44). Regulation of autophagy can also alleviate fatty liver conditions and liver injury in mice (45). In our previous study, BA was demonstrated to induce protective autophagy in HepG2 hepatocellular carcinoma cells (25). In the present study, autophagy was triggered in BA-treated LO2 cells, whereas inhibition or activation of autophagy did not affect the protective effect against *t*-BHP induced by BA, suggesting that autophagy may be not involved in the protective effect of BA in LO2 cells. However, other biomarkers, such as p62, require further study to confirm BA-triggered autophagic flux in cells.

In conclusion, BA exerted a strong hepatoprotective effect against *t*-BHP-induced liver cell damage via scavenging ROS generation and inhibiting apoptotic cell death, suggesting that BA holds great potential as a drug candidate in protecting hepatotoxicity.

Acknowledgements

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