Plumbagin protects H9c2 cardiomyocytes against TBHP-induced cytotoxicity by alleviating ROS-induced apoptosis and modulating autophagy

QIANRUI ZHANG[1], HAITAN FU[1], WENJUAN GONG[1], FENG CAO[1], TAO WU[2] and FEI HU[3]

[1]Department of Pharmacy, General Hospital of the Yangtze River Shipping, Wuhan Brain Hospital, Wuhan, Hubei 430014;
[2]Department of Pharmacy, Wuhan Fourth Hospital, Wuhan, Hubei 430000; [3]Department of Neurosurgery,
General Hospital of the Yangtze River Shipping, Wuhan Brain Hospital, Wuhan, Hubei 430014, P.R. China

Abstract. Plumbagin (PLB) has been previously reported to alleviate myocardial ischemia/reperfusion injury *in vivo*. In the present study, the potential of plumbagin to protect against hydrogen peroxide-induced injury in cardiomyocytes was analyzed. Specifically, the cytoprotective effects of PLB were evaluated in H9c2 cardiomyocytes, in which oxidative stress was induced by tertiary butyl hydrogen peroxide (TBHP; 75 μM) treatment. After the cardiomyocytes were treated with different concentrations of PLB, cell viability, creatine kinase (CK) activity and lactate dehydrogenase (LDH) release were determined. The apoptosis rate and reactive oxygen species (ROS) levels were evaluated by flow cytometry. Western blot analyses of cleaved caspase-3, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme 4 (NOX4), and phosphorylated (p)-p38 mitogen-activated protein kinase (MAPK) were performed. PLB pretreatment (5, 10 or 20 μM) restored TBHP-treated H9c2 cell viability (P<0.01). Additionally, PLB significantly decreased CK (P<0.01) and LDH activity (P<0.01). TBHP induced apoptosis and oxidative stress in cardiomyocytes, whereas PLB pretreatment significantly reduced the TBHP-induced apoptosis rate (P<0.01) and ROS levels (P<0.01). Furthermore, PLB resulted in a decrease in the expression of cleaved caspase-3, NOX4, and p-p38 MAPK in TBHP-treated H9c2 cells. The active marker of autophagosomes, LC3-II/LC3-I, was increased following treatment with PLB, indicating the induction of autophagy. The present study revealed the protective role of PLB against TBHP-induced cardiomyocyte injury via the alleviation of ROS-mediated apoptosis and induction of autophagy.

Correspondence to: Dr Tao Wu, Department of Pharmacy, Wuhan Fourth Hospital, 473 Hanzheng Street, Wuhan, Hubei 430000, P.R. China
E-mail: hydrinsk@sina.com

Introduction

Cardiovascular disease (CVD) is a prime cause of death worldwide, accounting for 17.3 million deaths per year (1). Acute myocardial infarction is the most serious type of CVD. There are >3 million patients with acute ST-segment elevation myocardial infarction each year. The most effective treatment for these patients is timely and effective reperfusion therapy (2). Reperfusion therapy improves the myocardial blood supply and is accompanied by a series of pathophysiological reactions, including peroxidation, inflammation, intracellular calcium overload, and finally irreversible apoptosis and necrosis. Myocardial injury resulting from reperfusion is termed reperfusion injury (3), and it presents a clinical problem that urgently needs to be solved.

During myocardial ischemia-reperfusion (I/R), myocardial nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme 4 (NOX4) expression is upregulated, and myocardial metabolic activity is enhanced, producing a large amount of reactive oxygen species (ROS) which contributes to myocardial injury (4). ROS can trigger a variety of signal transduction pathways, including enzyme-coupled receptor signaling pathways and G protein-coupled receptor signaling pathways, among which the MAPK signaling pathways play a key role in numerous cell activities (such as proliferation, differentiation, survival and death) (5). The inhibition of over-activated p38 MAPK can significantly reduce experimental myocardial I/R injury (6).

Plumbagin (PLB; 5-hydroxy-2-methyl-naphthalene-1,4-dione) is a major bioactive compound extracted from the roots of *Plumbago zeylanica* that acts as an inhibitor of NOX4 (7). PLB not only inhibits adenosine diphosphate (ADP)-induced platelet aggregation in the cardiovascular system but also suppresses NOX4 expression, which can significantly improve the redox state imbalance of myocardial I/R (8-10). The aforementioned studies suggest that PLB can alleviate myocardial I/R injury and has robust potential for application in the treatment of CVD. As an organic hydroperoxide, tertiary butyl hydrogen peroxide (TBHP) is more stable than hydrogenperoxide (H_2O_2), and it selectively inhibits mitochondrial function, induces membrane lipid peroxidation, and promotes nucleotide degradation and

adenosine formation (11). Based on the relatively well-defined mechanisms of TBHP action, TBHP-treated H9c2 cells were selected as a biological model likely to result in peroxidation injury in response to oxidative stress. In the present study, the protective effects of PLB in the prevention of TBHP-induced oxidative stress and apoptosis were verified in H9c2 cardiomyocytes.

Materials and methods

Materials. PLB (cat. no. S4777) was obtained from Selleck Chemicals. Anti-microtubule-associated protein 1 light chain 3 (LC3)-II/LC3-I (cat. no. PAB34124), anti-NOX4 (PAB30655), anti-phosphorylated (p)-p38 MAPK (PAB43139-P) and anti-p38 MAPK (PAB40560) were purchased from Bioswamp; Wuhan Bienle Biotechnology Co., Ltd. Anti-cleaved caspase-3 (ab214430) and anti-GAPDH (ab181602) were obtained from Abcam.

Cell culture and treatments. H9c2 cardiomyocytes were obtained from the American Type Culture Collection (ATCC: CRL-1446). Cells were grown in DMEM (product no. SH30022.01B; HyClone; Cytiva) supplemented with 10% FBS (Gibco), 100 μg/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) and maintained at 37°C in a humidified 5% CO_2 incubator. Confluent cardiomyocytes were cultured in DMEM supplemented with 2% FBS for an additional 12 h prior to experimentation. For experiments, cells were preincubated with PLB (5, 10 or 20 μM) for 24 h and TBHP (75 μM) for another 4 h. PLB was dissolved in DMSO and then diluted with DMEM to a final concentration of <0.1% DMSO. TBHP was dissolved in DMEM.

Cell viability assays. Cardiomyocytes were seeded in 96-well plates at a density of 5×10^3 cells/well. Cells were pretreated with PLB (5, 10, and 20 μM) for 24 h and then treated with TBHP for another 4 h. The number of viable cells was determined using a Cell Counting Kit-8 (CCK-8) assay. Briefly, the DMEM culture medium was discarded, and 100 μl CCK-8 reagent (Beyotime Institute of Biotechnology) was added to fresh DMEM. The 96-well plate was placed in a CO_2 incubator for 2 h. The optical density (OD) values were determined at a wavelength of 450 nm. The cell proliferation rate (%) was calculated as follows: (OD value of experimental well - OD value of control well)/OD value of control well x100%. The CCK-8 assay was repeated 3 times for consistency.

Lactate dehydrogenase (LDH) and creatine kinase (CK) leakage. Cytotoxicity was evaluated by detecting plasma membrane damage using commercially available LDH-estimation (cat. no. A020-1) and CK-estimation (cat. no. A032-1-1) kits (both from Beyotime Institute of Biotechnology). For LDH and CK leakage assays, H9c2 cells were grown in 24-well plates at a density of 3×10^5 cells/well, and cells were subjected to further experiments after 24 h. The LDH and CK activities were measured after 24 h of treatment per the manufacturer's protocols.

Estimation of intracellular ROS production. ROS production was determined by detecting the fluorescence intensity of dichlorofluorescin (DCF) via flow cytometry. Cells were treated with 2'-7'dichlorofluorescin diacetate (DCFH-DA) (10 μM) at 37°C in the dark for 20 min. Cells were then collected and suspended in PBS. The DCF fluorescence intensity was analyzed using flow cytometry (ACEA NovoCyte, ACEA Biosciences) at an excitation wavelength of 488 nm and an emission wavelength of 519 nm. Each assay was performed 3 times. Data were analyzed with NovoExpress 1.5 developed by ACEA Biosciences Inc.

Measurement of apoptosis. Following treatment, cardiomyocytes (1.5×10^5-1×10^6) were collected and immobilized in 75% cold ethanol for 12 h at 4°C. Immobilized cells were double-stained with Annexin V-FITC (10 μl) and PI (10 μl; cat. no. 556547, BD Biosciences) in the dark at room temperature for 30 min. The apoptotic rate of cardiomyocytes was analyzed using flow cytometry (ACEA NovoCyte, ACEA Biosciences). Each test was repeated 3 times. Data were analyzed with NovoExpress 1.5 (ACEA Biosciences Inc.).

Western blot analysis. Protein levels were analyzed in whole cardiomyocyte lysates. H9c2 cell lysates were prepared with RIPA buffer containing protease inhibitor cocktail (cat. no. PAB180006; Bioswamp), and protein concentration were determined by BCA Protein Assay Kit. A total of 30 μg of protein was separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore). Membranes were blocked in 5% bovine serum albumin (Beyotime) for 2 h at room temperature and then incubated with the following primary antibodies overnight at 4°C: NOX4, cleaved caspase-3, LC3-II/LC3-I, p-p38 MAPK, p38 MAPK, and GAPDH (all 1:1,000). The following day, the membranes were incubated with HRP-conjugated secondary antibody (cat. no. SAB43714, Bioswamp, 1:10,000) at room temperature for 1.5 h. The secondary antibody was detected by ECL (Beyotime Institute of Biotechnology). The bands were scanned and quantified by densitometry analysis using Tanon GIS software (GIS 1D Ver.4.00, Tanon, Shanghai, China).

Statistical analysis. Data are presented as the mean ± SD. The significant differences between groups were assessed with SPSS version 13.0. Comparisons of results were performed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

PLB protects H9c2 cells from cell death. Cell viability was assessed using the CCK-8 assay, as shown in Fig. 1A. TBHP significantly reduced cell viability compared with the control group (P<0.01). PLB (5, 10 or 20 μM) pretreatment attenuated the TBHP-induced reduction in H9c2 cell viability (P<0.01). Additionally, TBHP increased LDH and CK activities in H9c2 cardiomyocytes, and these increases were reduced by PLB pretreatment (P<0.01; Fig. 1B and C). The chemical structure of PLB is presented in Fig. 1D.

PLB alleviates TBHP-induced increase of ROS. The ROS levels in H9c2 cells were quantified by DCF-DA staining. TBHP treatment significantly increased intracellular
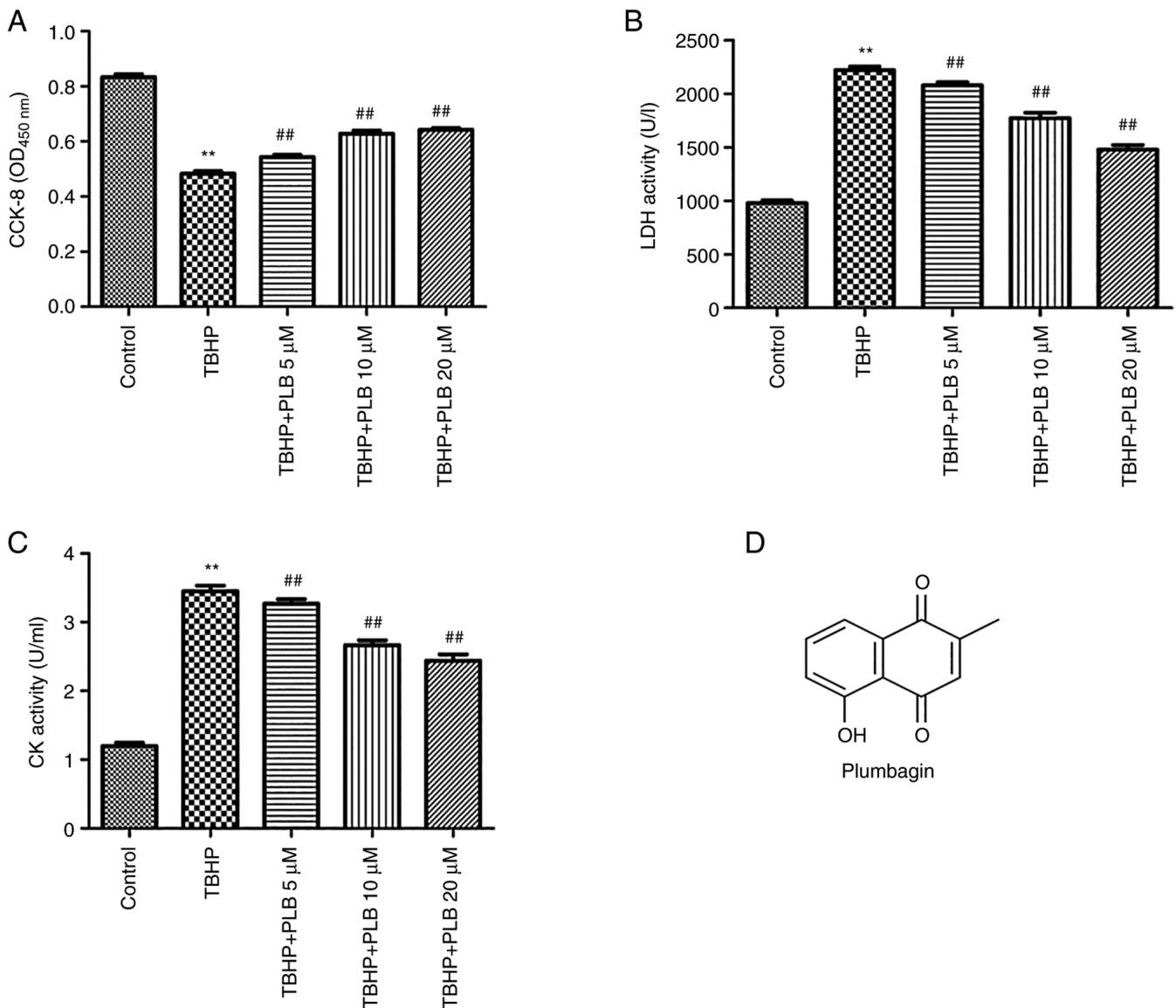
Figure 1. PLB protects H9c2 cells from cell death. (A) Cell viability. (B) LDH activity. (C) CK activity. (D) Chemical structure of PLB. Data are expressed as the mean ± SD; n=6. **P<0.01 vs. control; ##P<0.01 vs. TBHP; determined using one-way ANOVA with Tukey's post hoc analysis. PLB, plumbagin; LDH, lactate dehydrogenase; CK, creatine kinase; TBHP, tertiary butyl hydrogen peroxide; OD, optical density.

ROS levels in H9c2 cells, and pretreatment with PLB (5, 10 and 20 μM) decreased ROS generation (P<0.01; Fig. 2).

PLB prevents TBHP-induced apoptosis. As revealed in Fig. 3, the apoptotic rate was significantly increased in the TBHP group compared with the control group (P<0.01). Pretreatment with PLB (5, 10 or 20 μM) significantly reduced the apoptotic rate compared with TBHP treatment alone (P<0.01).

PLB promotes autophagy in H9c2 cells. LC3-II/LC3-I levels, a marker of active autophagosomes, were analyzed by western blot analysis (Fig. 4). The ratio of LC3-II/LC3-I was decreased in TBHP-treated H2c9 cells, and this decrease was attenuated by PLB pretreatment compared with TBHP treatment alone (P<0.05). These data indicated that PLB induces autophagy in TBHP-treated H9c2 cells.

PLB suppresses the NOX4/p38 MAPK pathway. NOX4, cleaved caspase-3, and p-p38/p38 MAPK protein expression was significantly increased in the TBHP group compared with the control group (P<0.05 and P<0.01). Pretreatment with PLB (5, 10 or 20 μM) suppressed TBHP-induced NOX4, cleaved caspase-3, and p-p38/p38 MAPK protein expression when compared with the TBHP only group (P<0.01 and P<0.05; Fig. 5).

Discussion

Myocardial I/R injury is closely related to oxidative stress. Under physiological conditions, small amounts of oxygen free radicals can be quickly eliminated in the body. However, when cells are ischemic and hypoxic, intracellular metabolism becomes disordered, and oxygen free radical scavenging capacity is insufficient. When blood supply is suddenly restored in previously ischemic tissue, oxygen
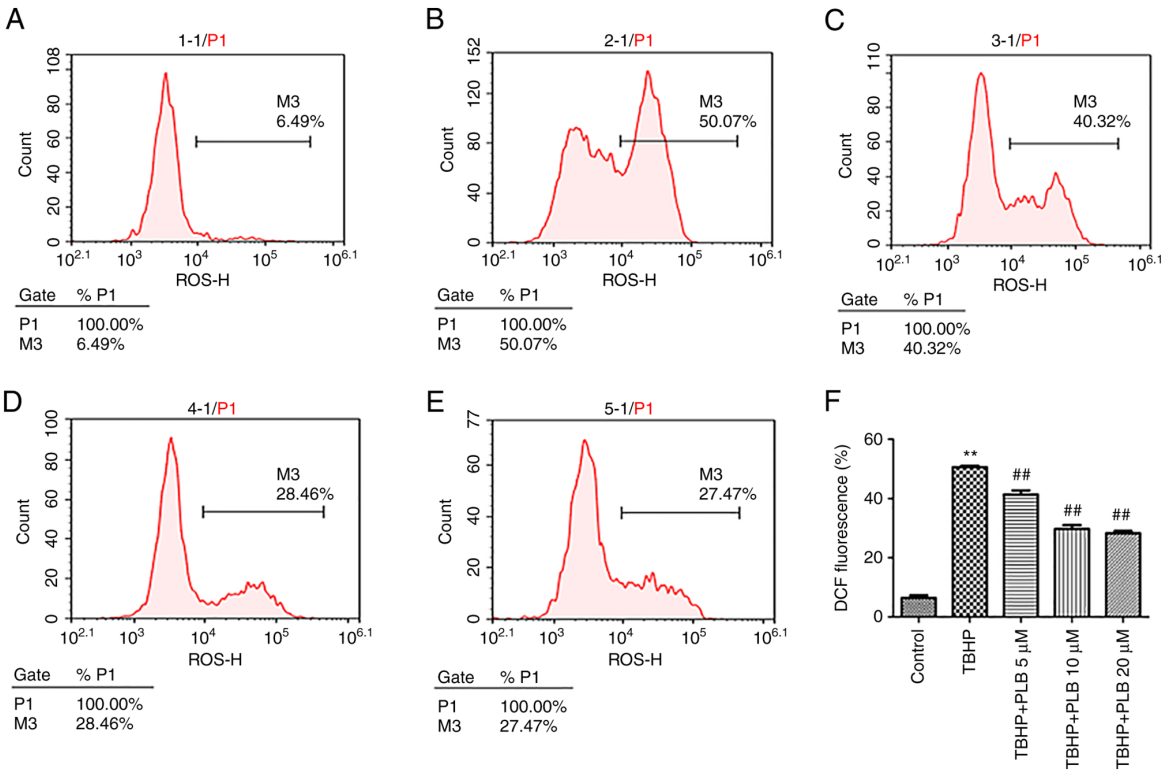
Figure 2. PLB reduces ROS production. ROS levels were determined using DCFH-DA. (A) Control, (B) TBHP, (C) TBHP + PLB (5 μM), (D) TBHP + PLB (10 μM), and (E) TBHP + PLB (20 μM). (F) Bar graph represents DCF fluorescence intensity. Data are expressed as the mean ± SD; n=3. **P<0.01 vs. the control; $^{##}$P<0.01 vs. TBHP; determined using one-way ANOVA with Tukey's post hoc analysis. PLB, plumbagin; ROS, reactive oxygen species; DCFH-DA, 2'-7'dichlorofluorescin diacetate; TBHP, tertiary butyl hydrogen peroxide; DCF, dichlorofluorescin.
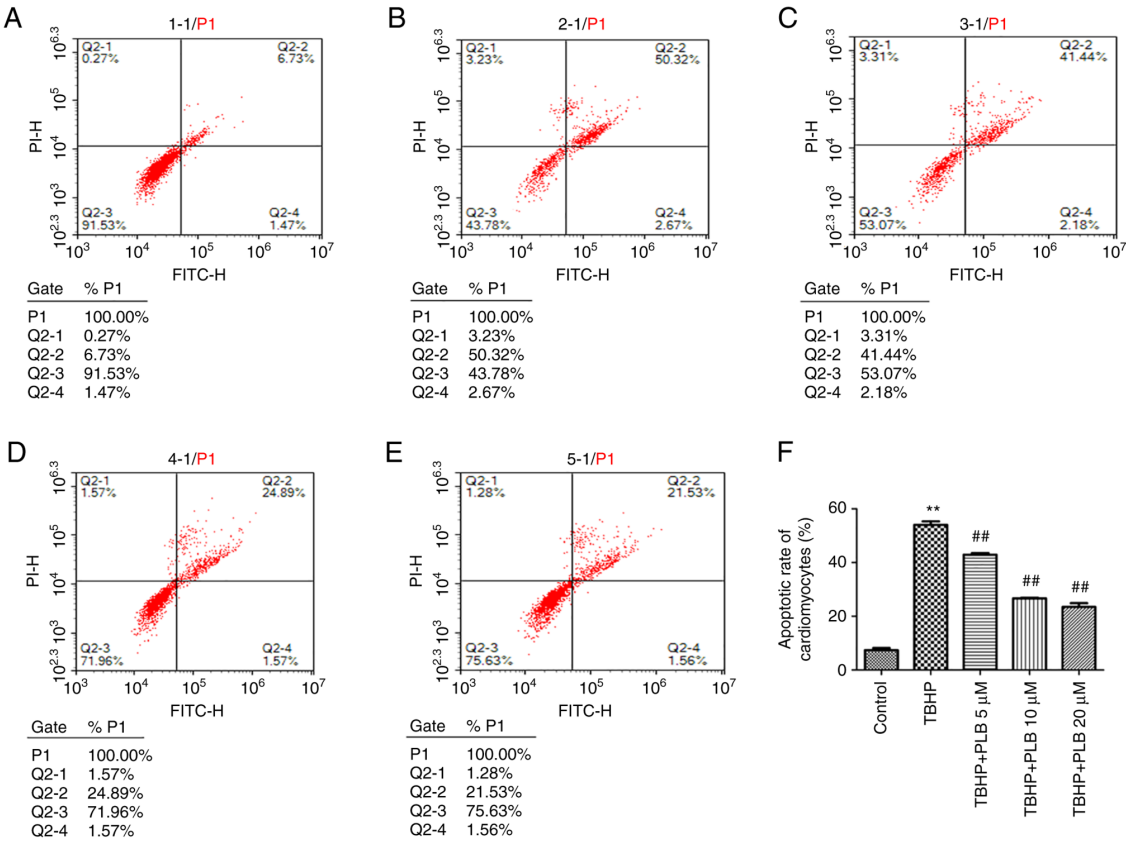

Figure 3. PLB prevents TBHP-induced apoptosis. Apoptotic rates were detected by Annexin V-FITC/PI assay. (A) Control, (B) TBHP, (C) TBHP + PLB (5 μM), (D) TBHP + PLB (10 μM), and (E) TBHP + PLB (20 μM). (F) Bar graph represents the apoptotic rates. Data are expressed as the mean ± SD; n=3. **P<0.01 vs. the control; $^{##}$P<0.01 vs. TBHP; determined using one-way ANOVA with Tukey's post hoc analysis. PLB, plumbagin; TBHP, tertiary butyl hydrogen peroxide.
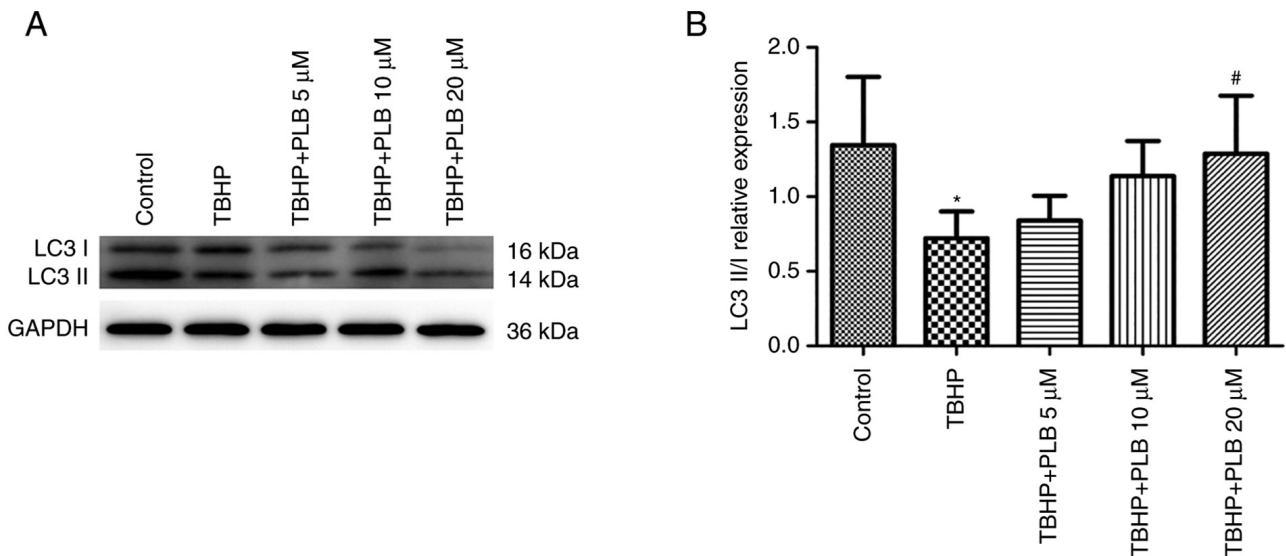
Figure 4. PLB promotes the autophagy of H9c2 cells. (A) Representative western blots for LC3-II/LC3-I. (B) Densitometric analysis for the ratio of LC3-II/LC3-I. Data are expressed as the mean ± SD; n=3. *P<0.05 vs. control; #P<0.05 vs. TBHP; determined using one-way ANOVA with Tukey's post hoc analysis. PLB, plumbagin; LC3, microtubule-associated protein 1 light chain 3; TBHP, tertiary butyl hydrogen peroxide.
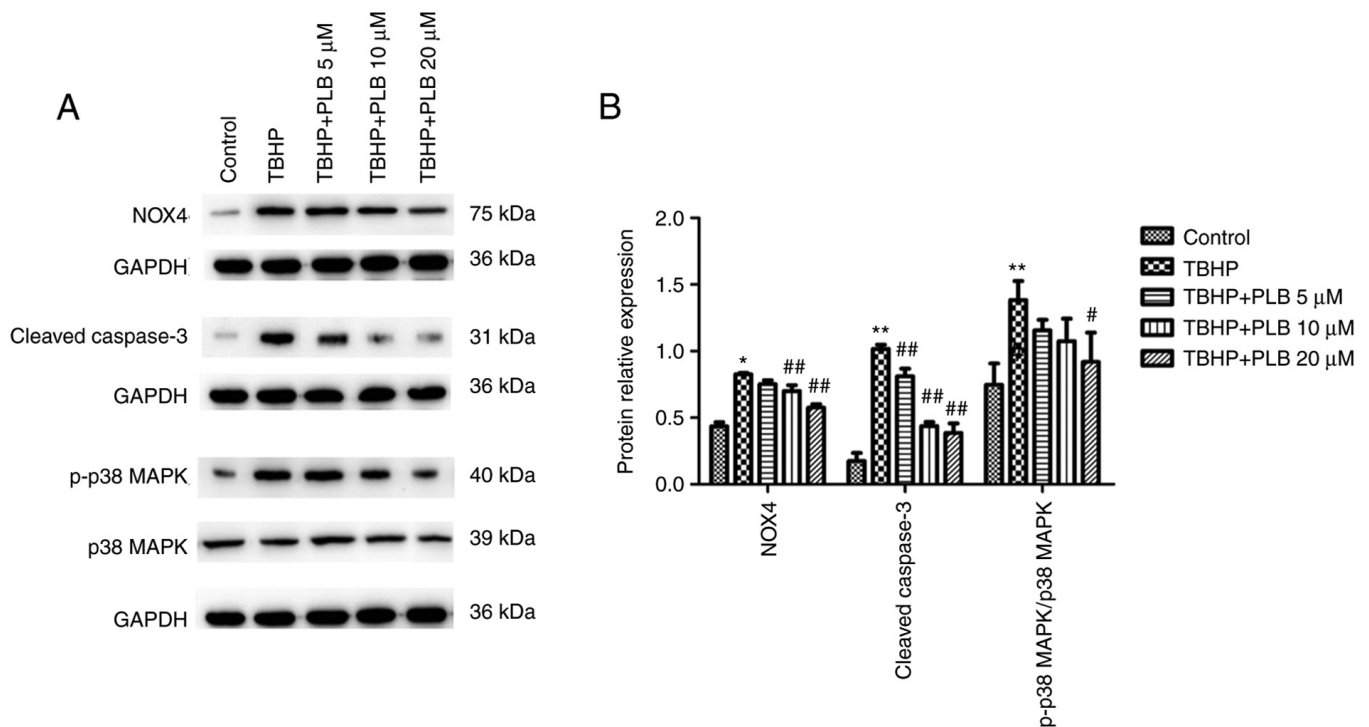

Figure 5. PLB suppresses the NOX4/p38 MAPK pathway. (A) Representative western blots for NOX4, cleaved caspase-3, p-p38/p38 MAPK protein. (B) Densitometric analysis for expression of NOX4, cleaved caspase-3, p-p38/p38 MAPK protein. Data are expressed as the mean ± SD; n=3. *P<0.05 and **P<0.01 vs. the control; #P<0.05 and ##P<0.01 vs. TBHP; determined using one-way ANOVA with Tukey's post hoc analysis. PLB, plumbagin; NOX4, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme 4; p-, phosphorylated; TBHP, tertiary butyl hydrogen peroxide.

free radicals are produced at a level that is unable to be quickly eliminated, causing damage to myocardial tissues and surrounding cells (12-14). PLB is a natural naphthoquinone compound that protects against myocardial damage by modulating cardiac biomarkers, antioxidants, and apoptotic signaling in doxorubicin-induced cardiotoxicity in rats (15). PLB can also inhibit NOX4 and regulate redox signals (16). Thus, in the present study, the effect of PLB on oxidative

stress-induced H9c2 cardiomyocyte injury was investigated. Experimental doses of PLB were based on a previous *in vitro* study (17).

TBHP is a pro-oxidant that increases membrane permeability, lipid peroxidation, ATP consumption, protein thiol group modification, and cytoplasmic calcium ion concentration imbalance by generating tert-butoxy groups (18). TBHP also causes cytotoxicity, mitochondrial-mediated apoptosis,

and necrosis through the loss of membrane integrity, characterized by the release of cytochrome *c*, increased p53 expression, and mitochondrial membrane transformation (19). In the present study, the classic oxidant TBHP was selected to induce oxidative stress damage in the H9c2 cell line and to explore the cytoprotective effects of PLB on TBHP-induced cardiomyocyte injury. The electron carrier 1-methoxy PMS mediates CCK-8 reduction by dehydrogenase in cell mitochondria to form a highly water-soluble yellow formazan. The amount of formazan produced is directly proportional to the number of living cells. Therefore, CCK-8 measurement can indirectly reflect the number of living cells. The results of the present study demonstrated that TBHP reduced H9c2 proliferation rate, suggesting that TBHP-induced oxidative stress decreased cell viability. Pretreatment with PLB reduced the negative impact of TBHP on cellular proliferation and improved cell viability in a dose-dependent manner. LDH and CK are important diagnostic indicators of acute myocardial infarction, angina pectoris, myocarditis, and other myocardial injuries. Following ischemia and reperfusion, the myocardial cell membrane is damaged and its permeability increases, causing leakage of intracellular LDH and other enzymes. As a result, plasma LDH levels increase. CK is an important enzyme in energy metabolism. When various tissues and organs of the body are injured, intracellular CK leaks and cell vitality is reduced, which can particularly be observed with myocardial tissue damage that occurs during ischemia (20). PLB pretreatment reduces LDH and CK activity induced by TBHP, suggesting that PLB may improve TBHP-induced cardiomyocyte damage.

Excessive ROS production during the I/R period leads to oxidative stress, an important pathogenic factor of myocardial I/R injury (21). Under physiological conditions, there is a certain amount of ROS in the myocardium. When myocardial ischemia and hypoxia occur, the function of the ROS scavenging system decreases and the function of the generating system increases. Once the blood oxygen supply is restored, ROS are produced in large quantities and accumulate quickly, causing acute or chronic cardiomyocyte injury (22). During myocardial I/R, ROS is produced in large quantities by myocardial cell mitochondria, vascular endothelial cell purine oxidase and other oxidases, neutrophil respiratory bursts, catecholamines, and other pathways that allow the myocardial cell membrane and subcellular organelle membrane to communicate. Increased permeability and loss of membrane integrity result in membrane dysfunction, allowing a large amount of Ca^{2+} to flood into cells and the direct attack of cell structural proteins and nucleic acids by ROS. Consistent with this, TBHP significantly increased intracellular ROS levels in H9c2 cells in the present study, whereas TBHP-induced ROS production was reduced by PLB pretreatment.

Apoptosis is a main pathogenic mechanism of I/R injury (23,24). Oxidative stress leads to changes in the metabolic and functional properties of mitochondria in ischemic myocardium, thus activating the mitochondrial apoptotic pathway. ROS cause oxidative damage to membrane proteins and lipids, leading to mitochondrial dysfunction and cytochrome *c* release and ultimately activating caspases, particularly caspase-3, that induce apoptosis (25). In the present study, it was determined that PLB reduced the TBHP-induced apoptosis of H9c2 cells by reducing lysed caspase-3, indicating that PLB prevents apoptosis by inhibiting the intrinsic apoptotic pathway mediated by mitochondria.

Autophagy is a ubiquitous protein degradation process that removes abnormal proteins and organelles to promote energy recycling (26). The specific process is divided into induction of macroautophagy, formation of autophagosomes, autophagosome docking and fusion, and autophagic body breakdown (27). Autophagy occurs at a basic level to allow sustained metabolic recycling of intracellular components in most tissues. Under pathological conditions, autophagy can act as a cytoprotective mechanism to degrade and recycle defective cytoplasmic proteins (28). Autophagy inhibition in cardiomyocytes has adverse effects (29). Additionally, the dual effect of autophagy in CVD has been investigated. An increasing number of investigations have confirmed that autophagy stimulates the inflammatory response and is responsible for ceroid formation in atherosclerosis (30-33). LC3 plays a key role in autophagosome formation during autophagy. Activated LC3-I conjugates with the target lipid phosphatidylethanolamine (PE) on the outer membrane, forming LC3-II. LC3-II is cleaved from LC3-I and released back to the cytosol or degraded upon autophagosome maturation (34). Thus, expression of LC3-II/LC3-I has been regarded as a classic autophagy marker. In the present study, PLB induced autophagy in H9c2 cells, as evidenced by the increased LC3-II/LC3-I ratio determined by western blot analysis. Crosstalk between apoptosis (type 1 cell death) and autophagy (type 2 cell death) occurs in myocardial injury. When autophagy is promoted, cell death via apoptosis is inhibited (35). Likewise, results of the present study demonstrated that TBHP enhanced apoptosis in H9c2 cells, and PLB abolished this apoptosis when autophagy was induced.

MAPK activation constitutes a pattern of intracellular signaling and participates in myocardial I/R injury (36). The p38 MAPK pathway regulates the cellular response to growth, apoptosis, and stress signals in different cell models. Phosphorylation of threonine and tyrosine residues in p38 leads to conformational changes, thereby increasing accessibility of the p38 active site and enhancing catalysis (37). Some researchers have suggested that autophagy may be induced by activating p38 MAPK and that upregulating autophagy via the p38 MAPK pathway may protect H9c2 cells from oxidative stress (38,39). In addition, the downstream effects of p38 MAPK pathway activation depends on the assembly of Nox subunits into the NAPDH oxidase complex responsible for ROS production (40). Considering that ROS leads to p38 MAPK phosphorylation and cytotoxicity, the effect of PLB treatment on p38 MAPK phosphorylation was evaluated in the present study. Compared with the use of TBHP alone, PLB treatment significantly reduced TBHP-induced phosphorylation of p38 MAPK in H9c2 cells.

Scavenging ROS fails to effectively prevent CVD progression (41). Large-scale multicenter clinical studies (HOPE, SECURE, GISSI and HPS) (42-44) have not confirmed the therapeutic value of vitamin E (a free radical scavenger) in slowing atherosclerosis or in reducing major cardiovascular events (45). This may be due to the fact that simply administering antioxidant vitamins fails to eliminate the root cause of large amounts of ROS-oxidase, especially NOX (46).

The NOX family is a tissue-specific homolog of NADPH oxidase, which exists on the plasma membrane of various nonphagocytic cells. Among the NOX family members, NOX1 and NOX5 are mainly expressed in smooth muscle and endothelial cells, respectively. NOX2 and NOX4 are found in endothelial cells, adventitia fibroblasts, and vascular smooth muscle cells, and NOX4 is the main source of intracellular ROS (47). The expression and activity of NOX4 are increased in myocardial I/R, leading to increased ROS and representing an important mechanism of myocardial injury (48). The results of the present study demonstrated that PLB suppressed NOX4 expression induced by TBHP in H9c2 cells, indicating that PLB may help reduce ROS production. Even though H9c2 cardiomyocytes have been widely used to investigate the molecular mechanisms underlying the cellular response to oxidative stress, it may not be fully consistent with other models. Thus, further studies based on *in vivo* models are still ongoing.

In summary, the present study suggested that PLB alleviated TBHP-induced cytotoxicity by reducing ROS-induced apoptosis and modulating autophagy in cardiomyocytes.

Acknowledgements

Not applicable.

Funding

Availability of data and materials

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

Authors' contributions

QZ and TW designed the study and wrote the manuscript. HF, WG and FC performed some of the experiments and collected the main data. FH conducted the statistical analysis. TW conceived the study. QZ and TW confirm the authenticity of all the raw data. All authors have read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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.

References

1. Syama HP, Arya AD, Dhanya R, Nisha P, Sundaresan A, Jacob E and Jayamurthy P: Quantification of phenolics in Syzygium cumini seed and their modulatory role on tertiary butyl-hydrogen peroxide-induced oxidative stress in H9c2 cell lines and key enzymes in cardioprotection. J Food Sci Technol 54: 2115-2125, 2017.
2. Arslan F, Bongartz L, Ten Berg JM, Jukema JW, Appelman Y, Liem AH, de Winter RJ, van 't Hof AWJ and Damman P: 2017 ESC guidelines for the management of acute myocardial infarction in patients presenting with ST-segment elevation: Comments from the Dutch ACS working group. Neth Heart J 26: 417-421, 2018.
3. Neri M, Riezzo I, Pascale N, Pomara C and Turillazzi E: Ischemia/Reperfusion injury following acute myocardial infarction: A critical issue for clinicians and forensic pathologists. Mediators Inflamm 2017: 7018393, 2017.
4. Siu KL, Lotz C, Ping P and Cai H: Netrin-1 abrogates ischemia/reperfusion-induced cardiac mitochondrial dysfunction via nitric oxide-dependent attenuation of NOX4 activation and recoupling of NOS. J Mol Cell Cardiol 78: 174-185, 2015.
5. Kim EK and Choi EJ: Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta 1802: 396-405, 2010.
6. Zhou QL, Teng F, Zhang YS, Sun Q, Cao YX and Meng GW: FPR1 gene silencing suppresses cardiomyocyte apoptosis and ventricular remodeling in rats with ischemia/reperfusion injury through the inhibition of MAPK signaling pathway. Exp Cell Res 370: 506-518, 2018.
7. Yong R, Chen XM, Shen S, Vijayaraj S, Ma Q, Pollock CA and Saad S: Plumbagin ameliorates diabetic nephropathy via interruption of pathways that include NOX4 signalling. PLoS One 8: e73428, 2013.
8. Zhang Q, Liao X and Wu F: The naphthoquinone plumbagin suppresses ADP-induced rat platelet aggregation through P2Y1-PLC signaling pathway. Pak J Pharm Sci 30 (2(Suppl.)): S573-S578, 2017.
9. Guida M, Maraldi T, Resca E, Beretti F, Zavatti M, Bertoni L, La Sala GB and De Pol A: Inhibition of nuclear Nox4 activity by plumbagin: Effect on proliferative capacity in human amniotic stem cells. Oxid Med Cell Longev 2013: 680816, 2013.
10. Wang SX, Wang J, Shao JB, Tang WN and Zhong JQ: Plumbagin mediates cardioprotection against myocardial ischemia/reperfusion injury through Nrf-2 signaling. Med Sci Monit 22: 1250-1257, 2016.
11. Luo C, Li Y, Wang H, Feng Z, Li Y, Long J and Liu J: Mitochondrial accumulation under oxidative stress is due to defects in autophagy. J Cell Biochem 114: 212-219, 2013.
12. Zorov DB, Juhaszova M, Yaniv Y, Nuss HB, Wang S and Sollott SJ: Regulation and pharmacology of the mitochondrial permeability transition pore. Cardiovasc Res 83: 213-225, 2009.
13. Kurian GA, Rajagopal R, Vedantham S and Rajesh M: The role of oxidative stress in myocardial ischemia and reperfusion injury and remodeling: Revisited. Oxid Med Cell Longev 2016: 1656450, 2016.
14. Granger DN and Kvietys PR: Reperfusion injury and reactive oxygen species: The evolution of a concept. Redox Biol 6: 524-551, 2015.
15. Li Z, Chinnathambi A, Ali Alharbi S and Yin F: Plumbagin protects the myocardial damage by modulating the cardiac biomarkers, antioxidants, and apoptosis signaling in the doxorubicin-induced cardiotoxicity in rats. Environ Toxicol 35: 1374-1385, 2020.
16. Ding Y, Chen ZJ, Liu S, Che D, Vetter M and Chang CH: Inhibition of Nox-4 activity by plumbagin, a plant-derived bioactive naphthoquinone. J Pharm Pharmacol 57: 111-116, 2005.
17. Zhang Q, Zhao S, Zheng W, Fu H, Wu T and Hu F: Plumbagin attenuated oxygen-glucose deprivation/reoxygenation-induced injury in human SH-SY5Y cells by inhibiting NOX4-derived ROS-activated NLRP3 inflammasome. Biosci Biotechnol Biochem 84: 134-142, 2020.
18. Chang G, Zhang D, Yu H, Zhang P, Wang Y, Zheng A and Qin S: Cardioprotective effects of exenatide against oxidative stress-induced injury. Int J Mol Med 32: 1011-1020, 2013.
19. T MM, Anand T and Khanum F: Attenuation of cytotoxicity induced by tBHP in H9C2 cells by Bacopa monniera and Bacoside A. Pathophysiology 25: 143-149, 2018.

20. Callegari GA, Novaes JS, Neto GR, Dias I, Garrido ND and Dani C: Creatine kinase and lactate dehydrogenase responses after different resistance and aerobic exercise protocols. J Hum Kinet 58: 65-72, 2017.
21. Inafuku H, Kuniyoshi Y, Yamashiro S, Arakaki K, Nagano T, Morishima Y and Kise Y: Determination of oxidative stress and cardiac dysfunction after ischemia/reperfusion injury in isolated rat hearts. Ann Thorac Cardiovasc Surg 19: 186-194, 2013.
22. Ruiz-Ginés JA, López-Ongil S, González-Rubio M, González-Santiago L, Rodríguez-Puyol M and Rodríguez-Puyol D: Reactive oxygen species induce proliferation of bovine aortic endothelial cells. J Cardiovasc Pharmacol 35: 109-113, 2000.
23. Jeremias I, Kupatt C, Martin-Villalba A, Habazettl H, Schenkel J, Boekstegers P and Debatin KM: Involvement of CD95/Apo1/Fas in cell death after myocardial ischemia. Circulation 102: 915-920, 2000.
24. McClintock DS, Santore MT, Lee VY, Brunelle J, Budinger GR, Zong WX, Thompson CB, Hay N and Chandel NS: Bcl-2 family members and functional electron transport chain regulate oxygen deprivation-induced cell death. Mol Cell Biol 22: 94-104, 2002.
25. Bi YM, Wu YT, Chen L, Tan ZB, Fan HJ, Xie LP, Zhang WT, Chen HM, Li J, Liu B and Zhou YC: 3,5-Dicaffeoylquinic acid protects H9C2 cells against oxidative stress-induced apoptosis via activation of the PI3K/Akt signaling pathway. Food Nutr Res 62: 1423, 2018.
26. Maiuri MC, Zalckvar E, Kimchi A and Kroemer G: Self-eating and self-killing: Crosstalk between autophagy and apoptosis. Nat Rev Mol Cell Biol 8: 741-752, 2007.
27. Li L, Tan J, Miao Y, Lei P and Zhang Q: ROS and Autophagy: Interactions and molecular regulatory mechanisms. Cell Mol Neurobiol 35: 615-621, 2015.
28. Kim I, Rodriguez-Enriquez S and Lemasters JJ: Selective degradation of mitochondria by mitophagy. Arch Biochem Biophys 462: 245-253, 2007.
29. Gottlieb RA and Mentzer RM: Autophagy during cardiac stress: Joys and frustrations of autophagy. Annu Rev Physiol 72: 45-59, 2010.
30. Peng S, Xu LW, Che XY, Xiao QQ, Pu J, Shao Q and He B: Atorvastatin inhibits inflammatory response, attenuates lipid deposition, and improves the stability of vulnerable atherosclerotic plaques by modulating autophagy. Front Pharmacol 9: 438, 2018.
31. Guo FX, Wu Q, Li P, Zheng L, Ye S, Dai XY, Kang CM, Lu JB, Xu BM, Xu YJ, *et al*: The role of the LncRNA-FA2H-2-MLKL pathway in atherosclerosis by regulation of autophagy flux and inflammation through mTOR-dependent signaling. Cell Death Differ 26: 1670-1687, 2019.
32. Hassanpour M, Rahbarghazi R, Nouri M, Aghamohammadzadeh N, Safaei N and Ahmadi M: Role of autophagy in atherosclerosis: Foe or friend? J Inflamm (Lond) 16: 8, 2019.
33. Zhang H, Ge S, Ni B, He K, Zhu P, Wu X and Shao Y: Augmenting ATG14 alleviates atherosclerosis and inhibits inflammation via promotion of autophagosome-lysosome fusion in macrophages. Autophagy 17: 4218-4230, 2021.
34. He C and Klionsky DJ: Regulation mechanisms and signaling pathways of autophagy. Annu Rev Genet 43: 67-93, 2009.
35. Nishida K, Yamaguchi O and Otsu K: Crosstalk between autophagy and apoptosis in heart disease. Circ Res 103: 343-351, 2008.
36. Tao H, Nuo M and Min S: Sufentanil protects the rat myocardium against ischemia-reperfusion injury via activation of the ERK1/2 pathway. Cytotechnology 70: 169-176, 2018.
37. Treusch S, Albert FW, Bloom JS, Kotenko IE and Kruglyak L: Genetic mapping of MAPK-mediated complex traits Across S. cerevisiae. PLoS Genet 11: e1004913, 2015.
38. Zheng YH, Tian C, Meng Y, Qin YW, Du YH, Du J and Li HH: Osteopontin stimulates autophagy via integrin/CD44 and p38 MAPK signaling pathways in vascular smooth muscle cells. J Cell Physiol 227: 127-135, 2012.
39. Lv XC and Zhou HY: Resveratrol protects H9c2 embryonic rat heart derived cells from oxidative stress by inducing autophagy: Role of p38 mitogen-activated protein kinase. Can J Physiol Pharmacol 90: 655-662, 2012.
40. Tormos AM, Taléns-Visconti R, Nebreda AR and Sastre J: p38 MAPK: A dual role in hepatocyte proliferation through reactive oxygen species. Free Radic Res 47: 905-916, 2013.
41. Harrison DG, Gongora MC, Guzik TJ and Widder J: Oxidative stress and hypertension. J Am Soc Hypertens 1: 30-44, 2007.
42. Sleight P: The HOPE Study (Heart Outcomes Prevention Evaluation). J Renin Angiotensin Aldosterone Syst 1: 18-20, 2000.
43. Stone NJ: The Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardio (GISSI)-Prevenzione Trial on fish oil and vitamin E supplementation in myocardial infarction survivors. Curr Cardiol Rep 2: 445-451, 2000.
44. Collins R, Peto R and Armitage J: The MRC/BHF heart protection study: Preliminary results. Int J Clin Pract 56: 53-56, 2002.
45. Violi F, Nocella C, Loffredo L, Carnevale R and Pignatelli P: Interventional study with vitamin E in cardiovascular disease and meta-analysis. Free Radic Biol Med 178: 26-41, 2022.
46. Schramm A, Matusik P, Osmenda G and Guzik TJ: Targeting NADPH oxidases in vascular pharmacology. Vascul Pharmacol 56: 216-231, 2012.
47. Takac I, Schröder K and Brandes RP: The Nox family of NADPH oxidases: Friend or foe of the vascular system? Curr Hypertens Rep 14: 70-78, 2012.
48. Cadenas S: ROS and redox signaling in myocardial ischemia-reperfusion injury and cardioprotection. Free Radic Biol Med 117: 76-89, 2018.