Plumbagin Protects H9c2 Cardiomyocytes against TBHP-induced Cytotoxicity via Alleviating ROS-induced Apoptosis and Inducing Autophagy

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Abstract

Plumbagin has been previously reported to alleviate myocardial ischemia/reperfusion injury *in vivo*. In this study, we analyzed the potential role of plumbagin against hydrogen peroxide-induced injury in cardiomyocytes. In the present study, plumbagin (PLB) was used to evaluate its cytoprotective property in H9c2 cardiomyocytes against tertiary butyl hydrogen peroxide (TBHP, 75 μM) induced ROS-mediated oxidative stress and apoptosis. Our results implicate that pretreatment with PLB (5, 10 or 20 μM) notably restored viabilities in TBHP-induced H9c2 cells (p 0.01). Also PLB treatment significantly decreased creatine kinase (CK) (p 0.01) and lactate dehydrogenase (LDH) activity (p 0.01). TBHP induced apoptosis and oxidative stress in cultured cardiomyocyte, whereas PLB pretreatment significantly reduced TBHP-induced apoptosis rate (p 0.01) and ROS level (p 0.01). Furthermore, PLB resulted in decrease in the expressions of cleaved caspase 3, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme 4 (NOX4) and phospho-p38 MAPK in TBHP-induced H9c2 cells. And the active marker of autophagosomes, LC3-II/LC3-I was elevated following treatment with PLB. These findings indicated that PLB may induce autophagy. The present study shows the protective role of PLB against TBHP-induced cardiomyocyte injury via alleviation of ROS-mediated apoptosis and induction of autophagy.

Introduction

According to the **Global** Burden of Diseases Study 2017, stroke and ischaemic heart disease (IHD) were the leading causes of death in China in 2017 [1]. Acute myocardial infarction is the most serious type of cardiovascular disease. There are more than 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 myocardial blood supply and is accompanied by a series of pathophysiological reactions, including peroxidation, inflammation, intracellular calcium overload, and finally irreversible apoptosis and necrosis. This myocardial injury caused by reperfusion is called Reperfusion injury [3]. However, the treatment of reperfusion injury is still a clinical problem to be solved urgently.

During myocardial ischemia-reperfusion, myocardial NOX4 expression is up-regulated and activity is enhanced, producing a large amount of ROS, which is an important mechanism leading 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 mitogen-activated protein kinases (MAPK) signaling pathways play a key role in a large number of cell activities (such as proliferation, differentiation, survival and death) [5]. Inhibition of over-activated p38 MAPK can significantly reduce experimental myocardial ischemia/reperfusion (I/R) injury [6].

Plumbagin (5-hydroxy-2-methyl-naphthalene-1,4-dione) is a major bioactive compound existing in the roots of *Plumbago zeylanica*. Plumbagin (PLB) not only inhibits platelet aggregation induced by ADP in cardiovascular system, but also suppresses the NOX4 expression, which can significantly improve the redox state imbalance of myocardial ischemia and reperfusion [7-9]. The above studies suggest PLB can

alleviate myocardial ischemia-reperfusion injury and has great potential for application research in the treatment of cardiovascular diseases. Therefore, this study intends to verify the protective effect of PLB on the oxidative stress and apoptosis of H9c2 cardiomyocytes induced by tert-butyl hydroperoxide (TBHP).

Materials And Methods

Materials

Plumbagin (S4777) was purchased from Selleck Chemicals (Houston, USA). Anti-LC3-II/LC3-I, anti-NOX4, anti-phospho-p38 MAPK and anti-p38 MAPK were from Bioswamp (Wuhan, China). Anti-cleaved caspase 3 was from Abcam (Cambridge, UK). Anti-GAPDH was provided by Abcam (Cambridge, UK).

Cell culture and treatments

H9c2 cardiomyocytes were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in DMEM (Hyclone, SH30022.01B) requiring supplementation with 10% FBS, 100 μg/mL penicillin and 100 μg/mL streptomycin and maintained at 37 °C in a humidified 5%CO_2 incubator. The confluent cardiomyocytes were cultured in DMEM supplemented with 2%FBS for another 12 h before experiment. For experiments, cells were pre-incubated with PLB (5, 10 or 20 μM) for 24 h and TBHP (75 μM) for another 4 h. In our study, PLB was dissolved in DMSO and then diluted with DMEM with the final concentration of DMSO less than 0.1%. TBHP was also dissolved in DMEM.

Cell viability assays

Cardiomyocytes were seeded in 96-well plates at a density of 5×10^3 cells per well. The cells were pretreated with PLB (5, 10, and 20μM) for 24 h before being exposure to TBHP for another 4 h. The number of viable cells was determined by a CCK-8 kit. Briefly, the DMEM culture medium was discarded and 100 μL CCK-8 reagents (Beyotime Inst, Shanghai, China) were added to a fresh DMEM medium. The 96-well plate was incubated in a carbon dioxide incubator for 2h. The optical density (OD) values were determined at 450 nm wave-length. The cell proliferation rate (%) was calculated as follows: (OD value of experimental well－OD value of control well)/OD value of control well×100%. The CCK-8 assay was repeated three times for consistency.

LDH and CK leakage

Cytotoxicity was evaluated by detecting the extent of plasma membrane damage by using commercially available LDH-estimation kit (Beyotime Inst, Shanghai, China) and CK-estimation kit (Beyotime Inst, Shanghai, China). For LDH and CK leakage assays, H9c2 cells were grown in 24-well plates at a density of 3×10^5 cells/well, and the cells were subjected to further experiment after 24h. The LDH and CK activity were measured after 24h of treatment according to the corresponding detection kits.

Estimation of intracellular ROS production

ROS generation was determined by detecting fluorescence intensity of dichlorofluorescin (DCF) by flow cytometry. Cells were treated with DCFH-diacetate (10 μM) at 37°C in the dark for 20 min. The cells were then collected and suspended in PBS. The fluorescence intensity of DCF was analyzed by using flow cytometry at an excitation wavelength of 488 nm and an emission wavelength of 519 nm. Each assay was performed three times.

Measurement of apoptosis

After the treatment, cardiomyocytes (1.5×10^5-1×10^6) were collected and immobilized in 75% cold ethanol for 12 h at 4˚C. Then the cells were double-stained with Annexin V-FITC (10 μL) and PI (10 μL) in the dark at room temperature for 30 min. The apoptotic rate of cardiomyocytes was analyzed by using flow cytometry (BD Biosciences, Franklin lakes, NJ, USA). Each test was repeated three times.

Western blot analysis

Protein levels were analyzed in whole cardiomyocytes lysates. A total of 30 μg samples were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA, USA). The membranes were blocked in 5% Bovine Serum Albumin for 2h and then incubated with the following primary antibodies overnight at 4°C (NOX4 1:1000, cleaved caspase-3 1:1000, LC3-II/LC3-I 1:1000, p-p38 MAPK 1:1,000, p38 MAPK 1:1000 and GAPDH 1:1000). The next day, membranes were incubated with secondary antibody at room temperature for 1.5 h. The antibody activity was detected by an ECL method (Beyotime institute of Biotechnology, Shanghai, China).

Statistical analysis

Data were presented as the mean±S.D. Statistical analysis of data was implemented by one-way ANOVA with LSD post hoc analysis. Value of p less than 0.05 were regarded statistically significant.

Results

Plumbagin protected H9c2 cells from cell death

Cell viability was measured by using the CCK8 assay as shown in Figure 1A. TBHP significantly reduced cell viability compared with the control group ($p < 0.01$). And PLB (5, 10 or 20 μM) pretreatment obviously promoted cell viability compared with the TBHP group ($p < 0.01$).

On the other hand, the elevated LDH and CK activity by TBHP exposure were reduced by PLB pretreatment ($p < 0.01$, Figure 1B).

Plumbagin alleviated TBHP induced elevation of ROS production

The level of ROS in H9c2 cells was quantified by using DCF-DA staining. TBHP treatment significantly increased the intracellular level of ROS in H9c2 cells. And pretreatment with PLB (5, 10 and 20 μM) decreased the ROS generation ($p < 0.01$, Figure 2 (A-F)).

Plumbagin prevented TBHP induced apoptosis

As shown in Figure 3 (A-F), the apoptotic rate was significantly increased in the TBHP group than in the control group ($p < 0.01$). Pretreatment with PLB (5, 10 or 20 μM) significantly reduced the apoptotic rate as compared with TBHP group ($p < 0.05$).

Plumbagin promoted the autophagy of H9c2 cells

The level of active marker of autophagosomes, LC3-II/LC3-I were analyzed by using western blot (Fig 4). TBHP notably reduced the ratio of LC3-II/LC3-I, whereas the ratio of LC3-II/LC3-I was obviously promoted by PLB pretreatment as compared with TBHP group. The data indicated that PLB might induce autophagy in TBHP-treated H9c2 cells.

Plumbagin suppressed the NOX4/p38 MAPK pathway

NOX4, cleaved caspase 3, phospho-p38/p38 MAPK protein expression in TBHP group was significantly increased as compared with control group ($p < 0.01$). Pretreatment with PLB (5, 10 or 20 μM) notably suppressed NOX4, cleaved caspase 3 and phospho-p38/p38 MAPK protein expression as compared with the TBHP group ($p < 0.05$ and $p < 0.01$, Figure 5).

Discussion

Myocardial ischemia-reperfusion injury is closely related to oxidative stress. Under physiological conditions, a small amount of oxygen free radicals generated in the body can be quickly eliminated. However, when cells are ischemic and hypoxic, intracellular metabolism is disordered and oxygen free radical scavenging capacity is insufficient. When the ischemic tissue suddenly restores blood supply, a large number of oxygen free radicals are produced, which cannot be cleared in time, causing damage to myocardial tissues and surrounding cells. This causes severe myocardial damage [10-12]. Plumbagin (PLB) is a natural naphthoquinone compound. Existing studies have found that PLB can protect the myocardial damage by modulating the cardiac biomarkers, antioxidants, and apoptotic signaling in the doxorubicin-induced cardiotoxicity in rats [13]. PLB can also inhibit NOX4 and regulate redox signals [14]. Thus, we investigated the effect of PLB on H9c2 cardiomyocyte injury induced by oxidative stress. The experimental doses were based on previous *in vitro* study [15].

Tertiary butyl hydrogen peroxide (TBHP) is a kind of pro-oxidant, which leads to membrane permeability, lipid peroxidation, ATP consumption, protein thiol group modification and cytoplasmic calcium ion concentration imbalance by generating tert-butoxy groups [16]. TBHP also causes cytotoxicity through the loss of membrane integrity characterized by the release of cytochrome C, increasing p53 expression and mitochondrial membrane transformation, leading to mitochondrial-mediated apoptosis or necrosis [17]. Therefore, this study selected the classic oxidant TBHP to induce oxidative stress damage in the H9c2 cell line, and explored the cytoprotective effect of plumbagin on TBHP-induced cardiomyocyte injury. Under the mediation of the electron carrier 1-Methoxy PMS, CCK-8 is reduced by dehydrogenase in

cell mitochondria to form a highly water-soluble yellow formazan. The number of formazan produced is directly proportional to the number of living cells. Therefore, the CCK-8 measurement can indirectly reflect the number of living cells. The results of this study showed that TBHP reduced the proliferation rate of H9c2 cells, suggesting that TBHP-induced oxidative stress caused cell viability to decrease. Pretreatment with plumbagin can reduce the negative impact of TBHP on cell proliferation and improve cell viability in a dose-dependent manner. Lactate dehydrogenase (LDH) and creatine kinase (CK) are important indicators for the diagnosis of acute myocardial infarction, angina pectoris, myocarditis and other myocardial injury. After ischemia and reperfusion, the myocardial cell membrane is damaged and the permeability increases, which causes the leakage of intracellular lactate dehydrogenase (LDH) and other enzymes, and the level of LDH in plasma increases. CK is an important enzyme in the body's energy metabolism. When various tissues and organs of the body are diseased, especially myocardial cell membrane damage caused by myocardial ischemia, the intracellular CK leaks, and the vitality of the cells is reduced [18]. Plumbagin pretreatment can reduce LDH and CK activity induced by TBHP, showing that it can improve TBHP-induced cardiomyocyte damage.

The excessively produced ROS during the ischemia-reperfusion period leads to oxidative stress, which is an important pathogenic factor of myocardial ischemia-reperfusion injury [19]. Under physiological conditions, there is a certain amount of ROS in the myocardium. When myocardial ischemia and hypoxia occur, the function of ROS scavenging system decreases and the function of generating system increases. Once the blood oxygen supply is restored, ROS will be produced in large quantities and accumulate sharply, causing acute or chronic cardiomyocytes injury [20]. During myocardial ischemia and reperfusion, ROS can be produced in large quantities through the myocardial cell mitochondria, vascular endothelial cell purine oxidase and other oxidases, neutrophil respiration burst, catecholamines and other pathways to make the myocardial cell membrane and subcellular organelle membrane fluidity and communication. Increased permeability and loss of integrity result in membrane dysfunction of ion transport, a large amount of Ca^{2+} floods into cells, and ROS can directly attack cell structural proteins and nucleic acids. The results of this study showed that TBHP significantly increased the intracellular ROS level of H9c2 cells. However, the production of ROS was reduced after PLB pretreatment.

Apoptosis is one of the main pathogenic mechanisms of ischemia-reperfusion injury [21, 22]. Oxidative stress leads to changes in the metabolic and functional properties of mitochondria in ischemic myocardium, thus activates the mitochondrial apoptotic pathway. ROS causes oxidative damage to membrane proteins and lipids, leading to mitochondrial dysfunction, releasing cytochrome c and ultimately activating caspase, especially caspase-3, all of these can induce apoptosis [23]. In this study, we found that PLB reduced TBHP-induced apoptosis of H9c2 cells by reducing lysed caspase 3, which indicated that PLB inhibited apoptosis by inhibiting the intrinsic apoptotic pathway mediated by mitochondria.

Autophagy is a ubiquitous protein degradation process, and its role is to remove abnormal proteins and organelles, and realize energy recycling [24]. The specific process is divided into: Induction of macroautophagy, Formation of the autophagosome, Autophagosome docking and fusion, and

Autophagic body breakdown [25]. Autophagy occurs at a basic level for 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 [26]. Dramatically, inhibition of autophagy causes adverse effects in cardiomyocytes [27]. Also the dual effect of autophagy in cardiovascular disease has been discussed. An increasing number of investigations have confirmed that autophagy has obvious effects on the stimulation of the inflammatory responses and is responsible for the formation of ceroids in atherosclerosis [28]. Microtubule-associated protein 1 light chain 3 (LC3) plays key role in autophagosome formation during autophagy. Activated LC3-I can conjugate with the target lipid phosphatidylethanolamine (PE) on the outer membrane, forming LC3-II. Finally LC3-II is cleaved to LC3-I and released back to the cytosol or degraded upon autophagosome maturation [29]. Thus, the expression of LC3-II/LC3-I has been regarded as a classic autophagy marker. The fact that PLB induced autophagy in H9c2 cells was confirmed by Western blot analysis, which showed that PLB increased the LC3-II/LC3-I ratio. There is a cross-talk between apoptosis (type 1 cell death) and autophagy (type 2 cell death) in heart injury. When autophagy is promoted, cell death induced by apoptosis will be inhibited [30]. This is in coincidence with our results: TBHP enhanced apoptosis in H9c2 cells, and PLB abolished this when autophagy was induced.

Mitogen-activated protein kinase (MAPK) activation constitutes a pattern of intracellular signaling. Studies have shown that MAPK is involved in myocardial ischemia-reperfusion injury [31]. The p38 mitogen-activated protein kinase (p38 MAPK) pathway regulates the response of cells to growth, apoptosis and stress signals in different cell models. Phosphorylation of threonine and tyrosine residues in p38 leads to conformational changes, thereby increasing the accessibility of the active site and enhancing the catalysis [32]. Some researchers have suggested that autophagy can be induced by activating p38 MAPK, and upregulating autophagy via the p38 MAPK pathway could protect H9c2 cells from oxidative stress [33, 34]. Besides, the downstream role of the p38 MAPK pathway depends on the assembly of Nox subunits into the NAPDH oxidase complex responsible for ROS production [35]. Considering that ROS can activate p38 MAPK phosphorylation involved in cytotoxicity, this study evaluated the effect of PLB treatment on p38 MAPK phosphorylation. Compared with TBHP-only group, PLB treatment significantly reduced TBHP-induced phosphorylation of p38 MAPK. The results showed that PLB reduced p38 MAPK phosphorylation in H9c2 cells induced by TBHP.

Scavenging ROS failed to effectively prevent the progression of cardiovascular disease [36]. Large-scale multi-center clinical studies (HOPE, SECURE, GISSI and HPS) did not confirm the therapeutic value of vitamin E (a free radical scavenger) in slowing atherosclerosis and reducing major cardiovascular events. This may be due to the fact that simply giving antioxidant vitamins failed to eliminate the root cause of a large amount of ROS-oxidase, especially NOX [37]. The NOX family is the homolog of NADPH oxidase, which exists on the plasma membrane of different non-phagocytic cells, and the tissue expression has certain specificity. Among them, Nox1 and Nox5 are mainly expressed in smooth muscle and endothelial cells, respectively. Nox2 is found in endothelial cells and adventitia fibroblasts and vascular smooth muscle cells. Similarly, Nox4 is detected in all the above cell types, which is the main source of intracellular ROS [38]. In addition, the expression and activity of NOX4 in myocardial ischemia and

reperfusion myocardium are up-regulated and the activity is enhanced, producing a large amount of ROS, which is an important mechanism leading to myocardial injury [39]. The results of this study indicated that plumbagin suppressed the expression of NOX4 induced by TBHP in H9c2 cells, which may help reduce the production of ROS.

Overall, we found that plumbagin alleviated TBHP-induced cytotoxicity by reducing ROS-induced apoptosis and inducing autophagy in cardiomyocytes.

Declarations

Funding

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Compliance with ethical standards

Conflicts of interest

All the authors declared that they have no conflict of interest. No human or animal was used in this study.

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Figures

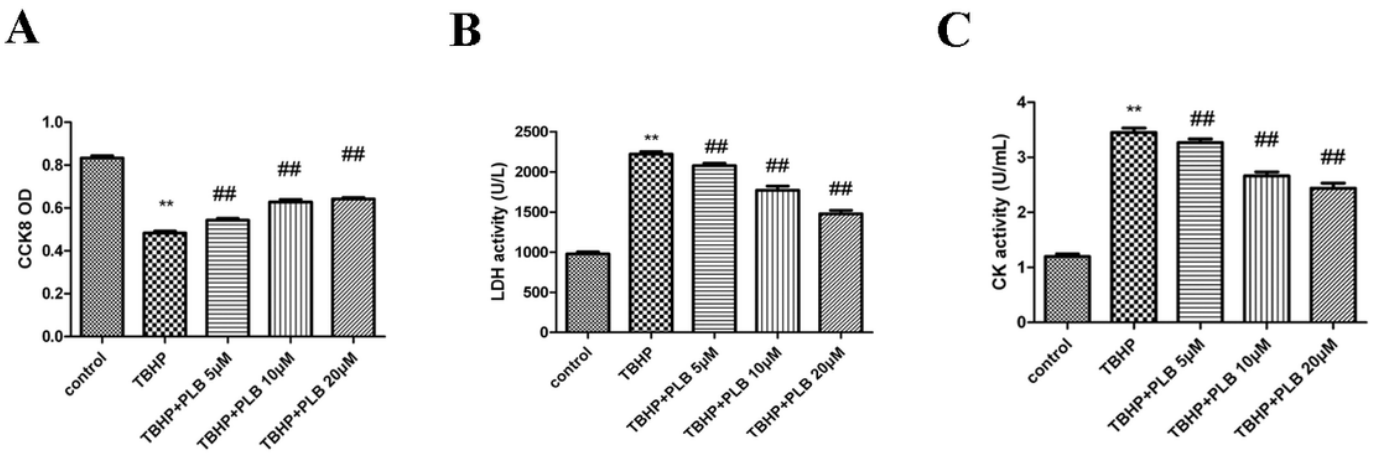

Figure 1

Plumbagin protected H9c2 cells from cell death. (A) Cell viability; (B) LDH activity; (C) CK activity. Data are expressed as mean ±S.D. (n=6). **p < 0.01 vs. the control; ##p < 0.01 vs. the TBHP. (One-way ANOVA with LSD post hoc analysis).

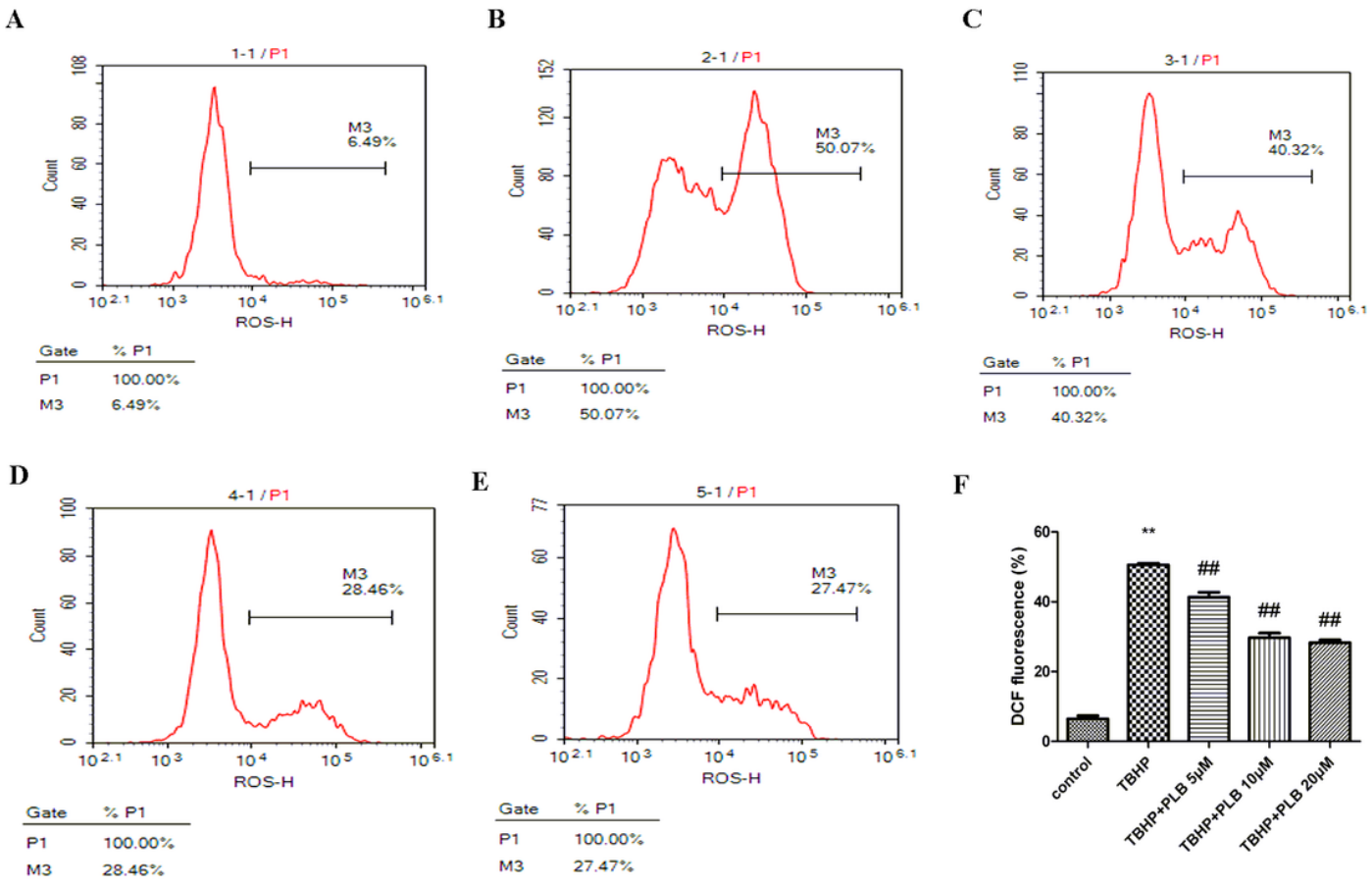

Figure 2

Plumbagin reduced ROS production. ROS level were determined by using DCFH-DA. (A) Control. (B) TBHP. (C) TBHP+PLB (5 μM). (D) TBHP+PLB (10 μM). (E) TBHP+PLB (20 μM). (F) Bar graph represents DCF fluorescence intensity. Data are expressed as mean ±S.D. (n=3). **p < 0.01 vs. the control; ##p < 0.01 vs. the TBHP. (One-way ANOVA with LSD post hoc analysis).

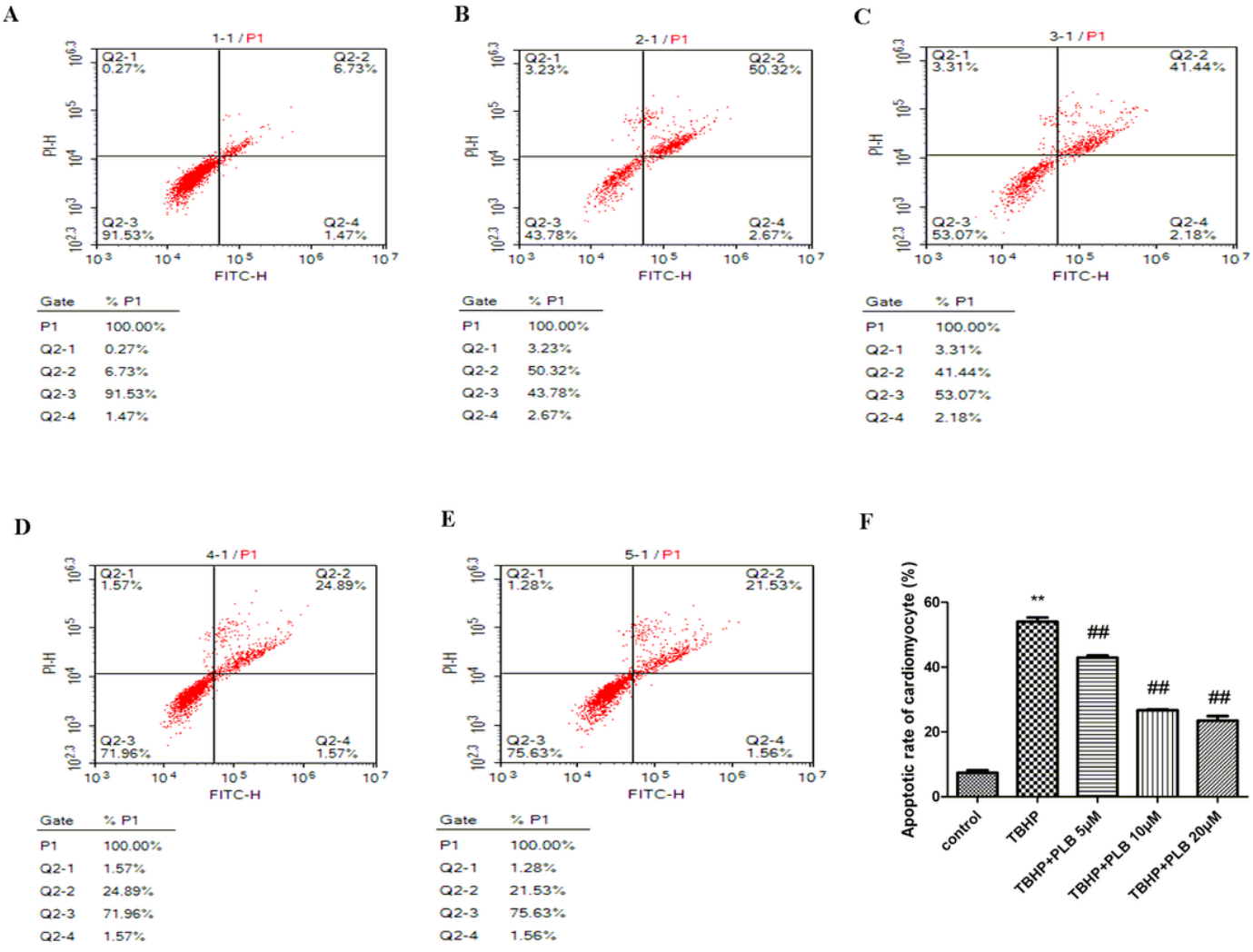

Figure 3

Plumbagin prevented 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). (E) TBHP+PLB (20 μM). (F) Bar graph represents apoptotic rates. Data are expressed as mean ±S.D. (n=3). **p < 0.01 vs. the control; ##p < 0.01 vs. the TBHP. (One-way ANOVA with LSD post hoc analysis).

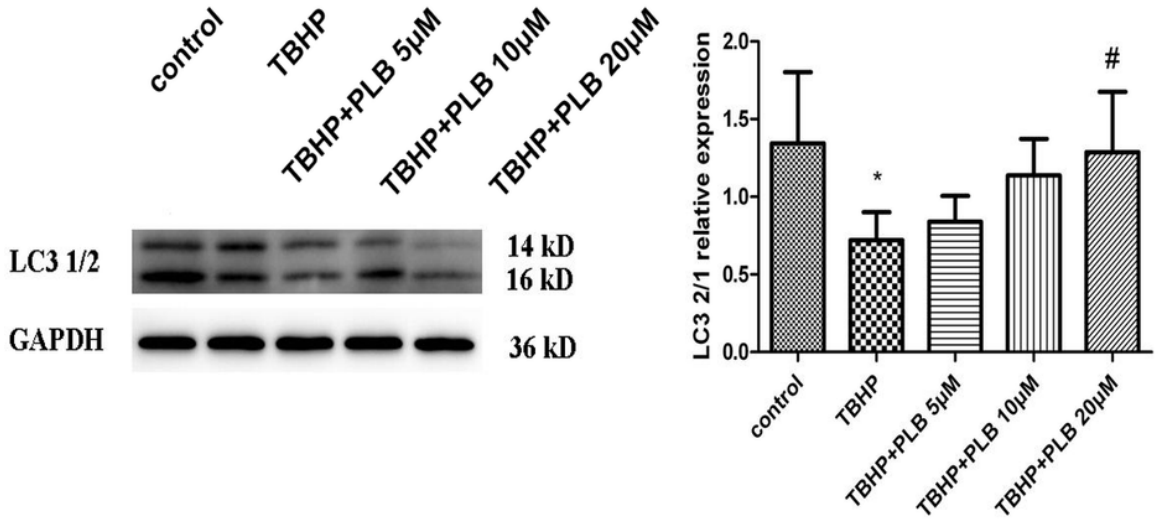

Figure 4

Plumbagin promoted 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 mean ±S.D. (n=3). *p < 0.05 vs. the control; #p < 0.05 vs. the TBHP. (One-way ANOVA with LSD post hoc analysis).

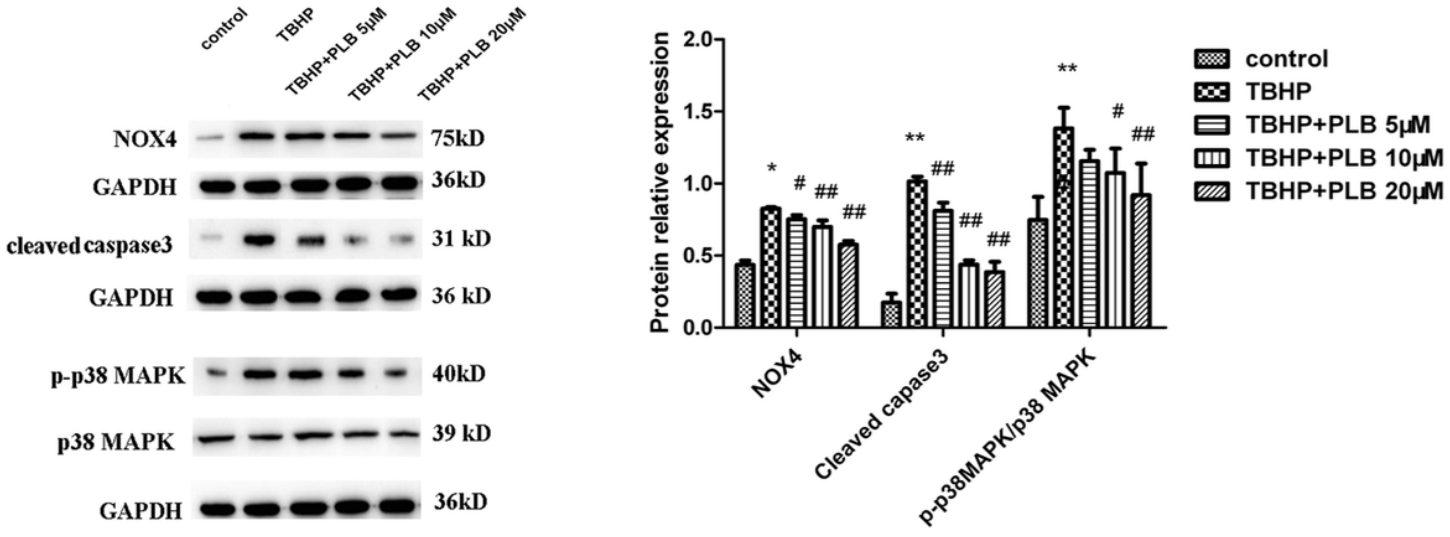

Figure 5

Plumbagin suppressed the NOX4/p38 MAPK pathway. (A) Representative western blots for NOX4, cleaved caspase 3, phospho-p38/p38 MAPK protein; (B) Densitometric analysis for the expression of NOX4, cleaved caspase 3, phospho-p38/p38 MAPK protein. Data are expressed as mean ±S.D. (n=3). *$p < 0.05$ and **$p < 0.01$ vs. the control; #$p < 0.05$ and ##$p < 0.01$ vs. the TBHP. (One-way ANOVA with LSD post hoc analysis).