Research Article

Naoxintong/PPARα Signaling Inhibits H9c2 Cell Apoptosis and Autophagy in Response to Oxidative Stress

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Naoxintong (NXT) is an empirical formula based on the principle of traditional Chinese medicine, which has been approved by China Food and Drug Administration (CFDA, Z20025001) and widely used for treatment of patients with cerebrovascular and cardiovascular diseases in China. The aim of this study is to investigate the protective mechanism of NXT on H9c2 cells (cardiogenic cell line) in response to H2O2. MTT, Western blot, and flow cytometry (FCM) methods were used to identify the protective effect of NXT extract on H2O2-induced H9c2 cells. Here we found that NXT extract significantly increased H9c2 cell viability and reduced H2O2-induced cell apoptosis and autophagy. More importantly, NXT inhibited H2O2-induced H9c2 cell apoptosis and autophagy by increasing PPARα protein levels. In contrast, silenced PPARα terminated NXT protective effect on H2O2-induced H9c2 cells. These findings suggest that NXT/PPARα signaling suppressed H2O2-induced H9c2 cell apoptosis and autophagy.

1. Introduction

Naoxintong (NXT) is an empirical formula based on the principle of traditional Chinese medicine, which has been approved by China Food and Drug Administration (CFDA, Z20025001) and widely used for treatment of patients with cerebrovascular and cardiovascular diseases in China. NXT contains sixteen kinds of traditional Chinese medicines including Astragalus membranaceus (Fish.) Bge. (Huangqi), Radix Paeoniae (Chishao), Salviae miltiorrhizae radix et rhizoma (Danshen), Radix Angelicae Sinensis (Dansgai), Radix Paeoniae Rubra (Chishao), Rhizoma Ligustici Chuanxiong (Chuanxiong), Semen Persicae (Taoren), Achyranthes bidentata (Niuxi), Spatholobus stem (Jixueteng), Mulberry Twig (Sangzhi), Cassia Twig (Guizhi), Boswellia carteri (Ruxiang), and Commiphora myrrha Engl (Moyao) and animal medicines including hirudo nipponica Whitman (Shuizhi), Scorpio (Quanxie), and Pheretima (Dilong). NXT alleviates atherosclerosis involved in inhibition of iNOS expression and dendritic cell maturation [1, 2]. Clinical observation shows that NXT can increase antiplatelet effect and decrease subsequent major adverse cardiovascular events (MACE) in patients with cytochrome P450 2C19* 2 polymorphism undergoing percutaneous coronary intervention (PCI) [3]. Moreover, NXT reduces the development of diabetic retinopathy [4] and cardiomyocyte damage in response to
reactive oxygen species (ROS) [5]; however, the mechanism of NXT on the cardiomyocyte apoptosis and autophagy in response to oxidative stress is still unclear.

Oxidative stress plays an important role in the pathogenesis of cardiovascular diseases. ROS is a main oxidative stress in cardiovascular damage. ROS is a byproduct of the normal metabolism of oxygen and has important roles in the cell signaling and homeostasis. But too much ROS production leads to oxidative stress [6]. The myocardial ischemia–reperfusion pathophysiology produces ROS upon the restoration of blood flow [7]. ROS can be induced by hyperglycemia or \( H_2O_2 \) in vitro. At present, extracellular \( H_2O_2 \)-induced H9c2 cell injury model is well documented [8]. Our previous study shows that the extract of \textit{Angelica sinensis} and \textit{Ligusticum chuanxiong} has protective effect against \( H_2O_2 \)-induced endothelial cell damage [9] and inhibits rat vascular smooth muscle cell proliferation [10]. PPAR\( \alpha \) plays a protective role in cardiomyocyte damage [11], while the mechanism of NXT water extract protecting cardiomyocyte from \( H_2O_2 \)-induced damage is not well known. Here we found that NXT activated PPAR\( \alpha \) signaling decreased \( H_2O_2 \)-induced H9c2 cell apoptosis and autophagy.

2. Materials and Methods

2.1. NXT Water Extract. NXT was kindly provided by Xianyang Buchang Pharmaceutical Co. Ltd. (Shanxi, China). The NXT water extract was isolated from 5 g NXT powder incubating in 15 mL \( H_2O \) 60°C for 6 h, and then the solution was evaporated to 1 mL and filtered. The composition of NXT water extract was identified by UHPLC-Q-TOF Tandem Mass Spectrometry.

2.2. Cell Culture. The rat embryonic-heart derived H9c2 cell line (ATCC, CRL-1446). Cells were cultured at 37°C and 5% CO\(_2\) in 25 mL cell culture flask containing Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10% (v/v) Fetal Bovine Serum (FBS) (Gibco), 100 U/mL penicillin, and 100 \( \mu \)g/mL streptomycin sulfate. Cells were maintained in exponential phase of growth and were subcultured when they reached about 80% confluence at a split ratio of 1:3.

2.3. Cell Viability Assay. MTT assay was used to screen the cytotoxic or protective activity of the water extract compound from NXT. The cell viability was determined with MTT Cell Proliferation and Cytotoxicity Assay Kit (Sangon) according to the manufacturer’s instructions. Cells (1 × 10\(^5\) cells/well) were seeded in 96-well plates. After the corresponding treatment, cells were washed twice with PBS and then incubated with the MTT solution for 1.5 h at 37°C. Cells were then dissolved in 200 \( \mu \)L DMSO in 96-well plates. The absorbance of the reaction solution at 570 nm was measured with an ELISA-plate reader, Multidetection Microplate Reader Synergy H4 (BioTek).

2.4. Western Blot. Cells were seeded in 6-well plates and cultured as mentioned above. Cells were lysed in lysis buffer containing protease inhibitors. The lysate was sonicated (10 s) and then centrifuged at 13,000 \( \times \) g at 4°C for 10 min. Protein concentration in the supernatant was determined by the Pierce BCA Protein Assay Kit (Thermo). Samples were diluted (1:4) in protein loading buffer (67 mM Tris-HCl, pH 6.8, 30% glycerol, 2% SDS, and 0.01% bromophenol blue) and boiled for 5 min. The proteins were separated by SDS-PAGE using 10% acrylamide gels and transferred to a nitrocellulose membrane (PALL). After being blocked with 5% (w/v) nonfat dry milk dissolved in TBST buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, and 0.1% Tween 20), the membranes were incubated with primary antibodies, anti-cas3/9, anti-PARP-1, Bcl-2, Bax, Bad, and beta-actin (Santa Cruz Biotechnology) and LC3b antibody (Novus Biologicals). The membranes were washed three times with TBST buffer at room temperature and then incubated with HRP-conjugated secondary antibody for 1~2 h at room temperature. The secondary antibody was removed by washing three times with TBST buffer. The immunoreactive bands were visualized with ECL reagent.

2.5. Plasmids and Transfection. PPAR\( \alpha \) shRNA plasmids (GV248 vector) were purchased from GeneCHEM (China). Plasmids were transfected by turboFect transfection reagent according to the manufacturer's instructions (Thermo Scientific).

2.6. Flow Cytometry (FCM) Assay. Cells were harvested by trypsinization and centrifuged at 400 \( \times \) g at 4°C for 10 min. For each sample, 10\(^5\) cells were collected. The cells were treated with Annexin V-FITC Apoptosis Detection Kit according to the manufacturer’s instructions (BD Biosciences). The cells flow through the FCM at about 100–1000 cells per second.

2.7. Statistical Analysis. Data are expressed as the mean ± SEM. Statistical comparison was carried out with Student’s \( t \)-test or one-way analysis of variance (ANOVA).

3. Results

3.1. Determination of Composition in NXT Water Extract by UHPLC-Q-TOF. The UHPLC-Q-TOF results show that the main composition of NXT water extract is paenoflorin (80.4%), salvianolic acid B (10.1%), trihydroxybenzoic acid (4.6%), chlorogenic acid (1.8%), and ferulic acid (1.5%). The relative concentration of the main compositions was calculated (Table 1).

3.2. NXT Increases Cell Viability in Response to \( H_2O_2 \). H9c2 cells were treated with \( H_2O_2 \). The results show that \( H_2O_2 \) dose dependently reduced cell viability (Figure 1(a)). Further analysis shows that the NXT extract had no cytotoxicity effect on H9c2 cells (Figure 1(b)). To assay the protective effect of NXT on H9c2 cells, cells were pretreated with different dose (0.1–1.0 \( \mu \)g/mL) of NXT for 12 h. After that cells were induced with \( H_2O_2 \) for 6 h. The results show that NXT significantly increased cell survival in response to \( H_2O_2 \) (Figure 1(c)).

3.3. NXT Increases Antiapoptotic Protein Expression in Response to \( H_2O_2 \). The antiapoptotic MCL-1 protein prevents
Table 1: Diverse composition in NXT water extract identified by UHPLC-Q-TOF.

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Figure 1: NXT extract increases cell viability. (a) H9c2 cells were treated with H<sub>2</sub>O<sub>2</sub> as indicated for 6 h. Cell viability was assayed by MTT. Results are expressed as means ± SEM (n = 5). *P < 0.05 versus no H<sub>2</sub>O<sub>2</sub> treatment. (b) H9c2 cells were treated with NXT extract as indicated for 12 h. Cell viability was assayed by MTT. (c) H9c2 cells were pretreated with or without NXT extract for 12 h, and then cells were treated with 0.5 mM H<sub>2</sub>O<sub>2</sub> for 6 h as indicated. Cell viability was assayed by MTT. Results are expressed as means ± SEM (n = 5). *P < 0.05 versus no NXT treatment.
Figure 2: NXT increases antiapoptotic protein levels. (a) H9c2 cells were pretreated with or without 0.5 μg/mL NXT for 12 h, and then the cells were treated with 200 μM \( H_2O_2 \) for 6 h. Cell lysates were subjected to Western blot. (b) H9c2 cells were pretreated with or without 0.5 μg/mL NXT for 12 h, and then the cells were treated with 200 μM \( H_2O_2 \) for 6 h. Cell lysates were subjected to Western blot as indicated. (c) H9c2 cells were pretreated with or without 0.5 μg/mL NXT for 12 h, and then the cells were treated with 200 μM \( H_2O_2 \) for 6 h. Cell lysates were subjected to Western blot as indicated. (d) H9c2 cells were pretreated with or without 0.5 μg/mL NXT for 12 h, and then the cells were treated with 200 μM \( H_2O_2 \) for 6 h. Cell lysates were subjected to Western blot. Data are triplicates from three independent experiments.
the release of mitochondrial contents against caspase-3 activation [12, 13]. We next detected the effect of NXT on the cell apoptotic signaling. The results show that NXT extract significantly increased antiapoptotic MCL-1 protein levels (Figure 2(a)). In contrast, NXT decreased Bad and Bax proapoptotic protein levels (Figure 2(b)). H₂O₂ induces cell apoptosis through activate caspase-3 [12, 13]. Our results show that NXT extract reversed this apoptotic signaling by inhibiting caspase-3 activation (Figure 2(c)); subsequently, cleaved PARP-1 was decreased in H9c2 cells treated with NXT extract (Figure 2(d)). These findings suggest that NXT inhibited the H9c2 cell apoptosis through inhibiting activation of caspase-3/PARP-1 signaling pathway.

3.4. NXT Decreases Cell Apoptosis. To detect NXT protective effect on H9c2 cells in response to H₂O₂, flow cytometry analysis was performed to detect the effect of NXT on H₂O₂-induced H9c2 cell apoptosis. The results show that H₂O₂ significantly induced cell apoptosis, but NXT reversed this event (Figure 3), suggesting that NXT extract inhibited H₂O₂-induced H9c2 cell apoptosis associated with increased antiapoptotic protein levels.

3.5. NXT Inhibits Cell Apoptosis by Increasing PPARα Expression. Our data have demonstrated that NXT significantly decreased H9c2 cell apoptosis in response to H₂O₂, which was involved in increasing antiapoptotic protein expression. As nuclear receptor, PPAR is a critical regulator of inflammation, adipocyte differentiation, and glucose homeostasis [3, 14–17]. Other reports show that PPARα protects against cardiomyocyte damage [11, 18]. Here we found that H₂O₂ significantly reduced PPARα protein levels (Figure 4(a)), but NXT increased PPARα protein expression levels (Figure 4(b)). More importantly, cells pretreated with NXT did not reduce PPARα protein levels in response to H₂O₂ (Figure 4(c)). Further analysis shows that PPARα silenced H9c2 cells increased proapoptotic protein levels (Bax and Bad) and decreased antiapoptotic protein MCL-1 protein levels (Figure 5(a)). Consistent with this, PPARα silenced H9c2 cells led to increased activation of caspase-3 in response to H₂O₂ (Figure 5(b)). As expected, PPARα silence in H9c2 cells did not increase cell survival in response to H₂O₂ (Figure 5(c)). These findings show that NXT activated PPARα singling leading to inhibition of H₂O₂-induced H9c2 cell damage.

3.6. NXT Reduces Cell Autophagy in Response to H₂O₂. Mammalian cell death has three types: apoptosis, necrosis, and autophagy [12]. We further detected whether H₂O₂ would induce cardiomyocyte autophagy. As expected, H₂O₂ significantly increased processing of LC3-I to LC3-II conversion (Figure 6(a)), suggesting that H₂O₂ induced H9c2 cell autophagy. Further analysis shows that H₂O₂-induced cell autophagy was reduced by NXT treatment (Figure 6(b)), suggesting that NXT decreased H₂O₂-induced H9c2 cell autophagy.
autophagy. As PPARα inhibited H$_2$O$_2$-induced cell apoptosis, further analysis shows that PPARα silence led to increased H9c2 cell autophagy (Figure 6(c)), suggesting that PPARα suppressed cell autophagy in response to H$_2$O$_2$.

4. Discussions

Naoxintong (NXT) is an empirical formula based on the principle of traditional Chinese medicine, which has been approved by China Food and Drug Administration (CFDA) and is widely used for treatment of patients with cerebrovascular and cardiovascular diseases in China. Increasing evidences show that NXT alleviates atherosclerosis involved in reducing expression of iNOS and NO level in the vessel wall [2]. In the mice model of atherosclerosis, NXT suppresses atherosclerosis through lipid-lowering and inhibition of dendritic cell maturation [1]. Clinical observations show that NXT can increase the antiplatelet effect and decrease subsequent major adverse cardiovascular events (MACE) in patients with cytochrome P450 2C19*2 polymorphism undergoing percutaneous coronary intervention (PCI) [3]. In addition to that, NXT reduces the development of diabetic retinopathy involved in inhibition of the expression of CAS3, MMP-2/9, and TNF-α [4]. Moreover, NXT increases the effect on the catalytic activities of drug-metabolising CYP2C19 enzyme [19]. Although ethanol extraction of NXT
reduces cardiomyocyte cell damage [5], the water soluble components of NXT on \( \text{H}_2\text{O}_2 \)-induced cardiomyocyte cell apoptosis are still unclear. Here we found that the NXT water extract has significantly protective effect on H9c2 cells in response to \( \text{H}_2\text{O}_2 \) treatment. To detect the possible component of the NXT water extract, the UHPLC-Q-TOF analysis was performed. Interestingly, the main composition of NXT water extract is paeoniflorin (80.4%), salvianolic acid B (10.1%), trihydroxybenzoic acid (4.6%), chlorogenic acid (1.8%), and ferulic acid (1.5%) (Table 1). These findings suggest that paeoniflorin may be the main or critical effective component for cardiovascular diseases, which needs to be further determined.

MCL-1 is the antiapoptotic proteins, but Bad and Bax are proapoptotic proteins, and MCL-1 inhibits caspase-3-mediated cell apoptotic signaling pathway [20, 21]. It is well known that activated caspase-3 results in cleavage of PARP-1 leading to cell apoptosis [22]. Some natural compounds from traditional medicine increase Bcl-2 family protein levels to inhibit cell apoptosis [23]. Our data show that NXT increased MCL-1 protein levels and decreased activation of caspase-3 and PARP-1 in \( \text{H}_2\text{O}_2 \)-treated H9c2 cells. These results show that NXT protected the cells against apoptosis by reducing caspase-3/PARP-1 signaling pathway. The increased antiapoptotic Bcl-2 family proteins (MCL-1) and decreased proapoptotic proteins (Bad and Bax) suggest that

Figure 5: Silenced PPAR\( \alpha \) decreases NXT protective effect on H9c2 cells. (a) H9c2 cells were transfected with control shRNA or PPAR\( \alpha \) shRNA for 24 h. Cell lysates were subjected to Western blot. Data are triplicates from three independent experiments. (b) H9c2 cells were transfected with control shRNA or PPAR\( \alpha \) shRNA for 24 h. Cells were pretreated with or without 0.5 \( \mu \text{g/mL} \) NXT for 12 h, and then cells were treated with 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for 6 h. Cell lysates were subjected to Western blot. Data are triplicates from three independent experiments. (c) H9c2 cells were transfected with control shRNA or PPAR\( \alpha \) shRNA for 24 h. Cells were pretreated with or without 0.5 \( \mu \text{g/mL} \) NXT for 12 h, and then cells were treated with 200 \( \mu \text{M} \) \( \text{H}_2\text{O}_2 \) for 6 h. Cell viability was assayed by MTT. Results are expressed as means ± SEM (n = 5). ** \( P < 0.05 \) versus control (no treatment); * \( P < 0.05 \) versus only \( \text{H}_2\text{O}_2 \) treatment. # \( P < 0.05 \) versus only \( \text{H}_2\text{O}_2 \) treatment.
Figure 6: NXT/PPARα signaling inhibits cell autophagy. (a) H9c2 cells were treated with 200 μM H2O2 as indicated time. Cell lysates were subjected to Western blot. (b) H9c2 cells were treated with or without 0.5 μg/mL NXT for 12 h. After that, cells were treated with 200 μM H2O2 for 6 h. Cell lysates were subjected to Western blot. (c) H9c2 cells were transfected with control shRNA or PPARα shRNA for 24 h. Cells were pretreated with or without 0.5 μg/mL NXT for 12 h, and then cells were treated with 200 μM H2O2 for 6 h. Cell lysates were subjected to Western blot. Cells were treated with 50 μM bafilomycin A for 1 h before cell lysis. Data are triplicates from three independent experiments.
NXT extract reduced cell apoptosis in response to H$_2$O$_2$. FCM assay further demonstrated that NXT extract decreased H9c2 cell apoptosis. MCL-1 is transmembrane protein in the mitochondria; however, Bax and Bak are two nuclear-encoded proteins that are able to pierce the mitochondrial outer membrane to induce apoptosis [24]. Based on our findings, mitochondria may be one of the targets of NXT water extract. As nuclear receptor, PPARs are the critical regulator of inflammation, adipocyte differentiation, and glucose homeostasis [3, 14–17]. Other reports show that PPAR$\alpha$ protects against cardiomyocyte damage [11, 18]. Here we found that NXT significantly increased PPAR$\alpha$ expression levels in response to H$_2$O$_2$. In contrast, silenced PPAR$\alpha$ terminated the inhibition of NXT on H$_2$O$_2$-induced H9c2 cell damage involved in PPAR$\alpha$ mediated the MCL-1 antiapoptotic protein expression.

Autophagy delivers cytoplasmic materials or organelles into lysosomes for degradation, which is also a progress of nutrient recycling [25]. Especially in response to starvation stress, autophagy increases cell survival; however, long-term periods autophagy without new nutrients replenishment leads to digestion of all available substrates and death (autophagy-associated cell death) [12, 25]. Here we found that H$_2$O$_2$ significantly induced H9c2 cell autophagy. In contrast, NXT reversed this event. More importantly, NXT reduced H9c2 cell autophagy in a PPAR$\alpha$ dependent manner, suggesting that NXT/PPAR$\alpha$ signaling suppressed H9c2 cell autophagy.

5. Conclusion

NXT water extract inhibited H9c2 cell apoptosis and autophagy by increasing PPAR$\alpha$ expression leading to increased H9c2 cell viability.

Competing Interests

The authors declare that they have no competing interests.

Authors’ Contributions

Huimin Xu and Jianhua Jin equally contributed to this work.

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