Naringin protects H9C2 cardiomyocytes from chemical hypoxia-induced injury by promoting the autophagic flux via the activation of the HIF-1α/BNIP3 signaling pathway

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Abstract. Naringin, a natural bioflavonoid, has been shown to exert protective effects in multiple cardiovascular diseases; however, the protective effects of naringin against hypoxic/ischemia-induced myocardial are not yet fully understood. Autophagy is a vital factor involved in the pathogenesis of myocardial injury. The aim of the present study was to investigate the protective effects of naringin on H9c2 cells against chemical hypoxia [cobalt chloride ($CoCl_2$)]-induced injury. The role of autophagy and the hypoxia-inducible factor-1α (HIF-1α)/Bcl-2/BCL2 interacting protein 3 (BNIP3) signaling pathway in the protective effects of naringin were also assessed. The results revealed that naringin pre-treatment significantly attenuated the $CoCl_2$-induced cytotoxicity and apoptosis, and also decreased caspase-3 activity, which had been increased by $CoCl_2$. In addition, $CoCl_2$ increased Beclin-1 expression, enhanced the IL3B-II/IL3B-I ratio and increased p62 expression in the H9C2 cells. Treatment with 3-methyladenine (3-MA), a selective inhibitor of autophagy, also blocked $CoCl_2$-induced cytotoxicity and apoptosis. Notably, treatment with bafilomycin A1 (Baf A1), an inhibitor of the vacuolar H^+ ATPase of lysosomes, resulted in an increase in the upregulation of the LC3B-II/LC3B-I ratio, but did not further increase the LC3B-II/LC3B-I ratio compared with $CoCl_2$ treatment. These results suggested that $CoCl_2$ inhibited the autophagic flux, which resulted in myocardial cell damage. Furthermore, naringin pre-treatment exacerbated Beclin 1 expression and the increased IL3B-II/IL3B-I ratio, and reduced p62 expression in $CoCl_2$-treated H9C2 cells. 3-MA and Baf A1 both reversed the protective effects of naringin against $CoCl_2$-induced injury, indicating that naringin attenuated $CoCl_2$-induced myocardial cell injury by the increasing autophagic flux. Moreover, naringin treatment resulted in upregulated expression levels of HIF-1α and BNIP3 in the $CoCl_2$-treated H9C2 cells. The inhibition of the HIF-1α/BNIP3 signaling pathway using 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (an inhibitor of HIF-1α) prevented the effects of naringin on the autophagic flux and reversed its protective effects against $CoCl_2$-induced injury. Taken together, these results suggest that naringin protects the H9C2 cells against $CoCl_2$-induced injury by enhancing the autophagic flux via the activation of the HIF-1α/BNIP3 signaling pathway.

Introduction

Cardiovascular disorders pose a major health burden and are associated with high morbidity and mortality rates worldwide (1). Hypoxia/ischemia is a major and crucial feature of various cardiac pathologies, including congenital heart disease, myocardial infarction and ischemic heart disease (2-4). Therefore, effective prevention and advances in myocardial protective therapy are necessary for the improvement of myocardial ischemic disease. Naringin is a non-toxic natural bioflavonoid found in citrus fruit peel with multiple pharmacological properties, including anticancer, antioxidant, anti-apoptotic, anti-inflammatory, anti-diabetic, neuroprotective and cardioprotective functions (5-8). Recently, the cardioprotective properties of naringin in various cardiovascular disease models have attracted increasing attention (9,10). Naringin has been shown to protect H9C2 myocardial cells against doxorubicin-induced cardiotoxicity and apoptosis (11). Naringin has also been shown to attenuate anoxia/reoxygenation-induced injury by inhibiting apoptosis and oxidative stress, which is dependent on the Nrf2 signaling pathway (12). However, the cardioprotective effects of naringin on ischemic injury and the underlying mechanisms are not yet fully understood.

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Autophagy, a lysosome-mediated degradation pathway, is involved in major areas of cardiovascular disorders; however, it is still contested whether autophagy exerts a protective effect on cell survival or whether it results in cell death (13-15). Recent studies have found that the dysfunction of autophagy plays a vital role in the development and progression of myocardial damage (16,17). Autophagy is a vital degradation process required to maintain intracellular homeostasis under conditions of hypoxic/ischemic injury (18,19). It has been confirmed that hypoxia enhances the flux of autophagosome formation, and restoring autophagy function is thus a novel promising therapeutic option for managing ischemic injury (13,20). Hypoxia-inducible factor-1α (HIF-1α) is the main regulator of the cellular response to hypoxia (21). HIF-1α is closely associated with the regulation of myocardial survival by activating the downstream protein, BCL2 interacting protein 3 (BNIP3) (22). Notably, studies have demonstrated that BNIP3 is involve in regulating autophagy (23,24). Recently, studies have revealed that the hypoxic/ischemic condition promotes HIF-1α expression, and the HIF-1α/BNIP3 signaling pathway also plays essential roles in hypoxia-induced autophagy (23-25). In addition, studies have confirmed that naringin exerts beneficial effects by regulating autophagy (14,26). Hence, the present study aimed to further investigate whether naringin attenuates ischemic injury via modulating autophagy and the HIF-1α/BNIP3 signaling pathway.

Cobalt chloride ($CoCl_2$), a type of chemical hypoxia mimetic agent (27), is widely used to mimic hypoxic/ischemic conditions, triggering cell damage and stabilizing intracellular HIF-1α expression, and is a key regulator in the adaptation to a low concentration of oxygen and cellular survival (28). H9C2 embryonic rat cardiac cells are derived from rat embryonic hearts and are widely used as *in vitro* models for exploring the molecular mechanisms or protective strategies underlying cardiovascular disorders (29). Therefore, $CoCl_2$-treated H9C2 cells were used as a hypoxic model in *in vitro* in the present study. The present study explored the protective effects of naringin against $CoCl_2$ injury and further investigated the underlying mechanisms, focusing on the autophagic flux and HIF-1α/BNIP3 signaling pathway in H9C2 cells. The findings of the present study may provide novel scientific evidence in favor of the use of naringin for the prevention of hypoxic/ischemic injury, and may highlight potentially novel targets for the management of hypoxic/ischemic injury.

Materials and methods

Cell culture and treatment. Rat H9C2 cardiomyocytes were obtained from the American Tissue Culture Collection (ATCC). Cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% (v/v) FBS (Gibco; Thermo Fisher Scientific, Inc.) and 100 U/ml penicillin-streptomycin (Beyotime Institute of Biotechnology) in an incubator (Thermo Fisher Scientific Inc.) with 5% CO_2 at 37˚C.

To mimic hypoxic conditions, H9C2 cells were treated with various concentrations of $CoCl_2$ (200, 400, 600, 800 or 1,000 μmol/l; Sigma-Aldrich; Merck KGaA) for different periods of time (0, 1.5, 3, 6, 12 and 24 h). To investigate the protective effects of naringin (Sigma-Aldrich; Merck KGaA) on $CoCl_2$ injury, H9C2 cells were pre-treated with naringin (5, 10, 20 and 40 μmol/l) for 1 h prior to treatment with $CoCl_2$ (400 μmol/l) for 12 h. Cells incubated in normoxic medium (DMEM, 10% FBS, and 1% penicillin-streptomycin solution) in an incubator with 5% CO_2 at 37˚C served as the control group.

Treatment with 3-methyladenine (3-MA), bafilomycin A1 (Baf A1) or 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1). 3-MA and Baf A1 were obtained from Santa Cruz Biotechnology, Inc. YC-1 was purchased from Cayman Chemical Co. All reagents were dissolved in DMEM containing 1% DMSO. To inhibit autophagy, H9C2 cells were incubated with 3-MA (5 mM) for 1 h prior to treatment with $CoCl_2$ (400 μmol/l) for 12 h in the presence or absence of naringin (20 μmol/l). To block autophagosome fusion with lysosomes, H9C2 cells were treated with Baf A1 (10 nmol/l) and $CoCl_2$ (400 μmol/l) for 12 h in the presence or absence of naringin (20 μmol/l). To inhibit the HIF-1α/BNIP3 signaling pathway, H9C2 cells were treated with YC-1 (10 μM) for 1 h prior to treatment with naringin (20 μmol/l) for 2 h followed by co-treatment with $CoCl_2$.

Cell Counting kit-8 (CCK-8) assay. Cell viability was assessed using a CCK-8 kit (Beijing Solarbio Science & Technology Co., Ltd.) according to the manufacturer's protocol. Briefly, H9C2 cells were added to 96-well plates ($5x10^3$ cells/well) and cultured. Following treatment as described above, the media from each group were replaced with serum-free DMEM (90 μl) plus CCK-8 regent (10 μl), and incubated for 3 h at 37˚C. The absorbance was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.). Cell viability as a percentage was calculated by comparing the value of the treated group to that of the control group.

Total lactate dehydrogenase (LDH) release assay. Cell injury was assessed based on the amount of total LDH released into the culture supernatant, measured using an LDH kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. Following centrifugation at 1,000 x g, the supernatant was collected and plated in 96-well plates. The absorbance was measured using a microplate reader (Thermo Fisher Scientific, Inc.) at 450 nm.

Annexin V-FITC/PI double staining assay. The apoptosis of H9C2 cells was measured using an Annexin V-FITC/PI apoptosis detection kit (Nanjing Keygen Biotech Co., Ltd.) according to the manufacturer's protocol. H9C2 cells were seeded in 6-well plates ($1x10^6$ cells/well), and treated as described above. After washing with PBS twice, the cells were re-suspended in Annexin V-binding buffer (500 μl) and stained with FITC-labelled Annexin V (5 μl) and PI (10 μl) for 15 min in the dark at 37˚C. The rate of apoptosis was quantified using a flow cytometer (BD LSRFortessa X-20; BD Biosciences).

Caspase-3/caspase-9 activities assay. The H9C2 cells were seeded in 6-well plates at a density of $1x10^6$ per well. Following treatment as described above, the cells were sonicated, centrifuged at 12,000 x g for 10 min at 4˚C, and the supernatant was retained. The concentration of proteins was

determined using a bicinchoninic acid kit (Thermo Fisher Scientific, Inc.). The activities of caspase-3 and caspase-9 was determined using a Caspase-3 Activity Assay kit (Beyotime Institute of Biotechnology) and Caspase-9 Activity Assay kit (Beyotime Institute of Biotechnology) in accordance with the manufacturer's protocol. Caspase-3 and caspase-9 activities were determined by cleavage of the Ac-DEVD-pNA and Ac-LEHD-pNA substrates, respectively, and the absorbance was measured at 405 nm. The data are presented as fold increases over the pre-treatment levels.

Western blot analysis. The treated H9C2 cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) containing 1 mM PMSF (Beyotime Institute of Biotechnology) and centrifuged at 12,000 x g for 10 min at 4˚C. The concentration of proteins was determined using a BCA kit. Equivalent quantities of proteins (40 μg) were loaded on a 10% SDS-gel, resolved using SDS-PAGE and transferred to PVDF membranes (EMD Millipore). After blocking with 5% non-fat milk in TBS containing 0.01% Tween-20 (TBST) overnight at 4˚C, the membranes were incubated with the following primary antibodies all at a dilution of 1:2,000: Rabbit monoclonal anti-Beclin1 (#3495), rabbit polyclonal LC3B-II/I (#3868), rabbit polyclonal anti-p62 (#39749), rabbit polyclonal anti-HIF-1α (#14179), rabbit polyclonal anti-BNIP3 (#44060) and rabbit polyclonal anti-tubulin (#2128) at 4˚C overnight. All antibodies were purchased from Cell Signaling Technology, Inc. After washing twice with TBST, the membranes were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:5,000; #7074; Cell Signaling Technology, Inc.) at room temperature for 2 h. Finally, the bands were visualized using enhanced chemiluminescence reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Densitometric analysis was performed using the Bio-Rad Microscopic Imaging system and Odyssey Image software (LICOR).

Statistical analysis. All data were analyzed using GraphPad Prism version 6 (GraphPad Software Inc.). Data are expressed as the means ± standard deviation. Differences between multiple groups were compared using a one-way ANOVA followed by a post-hoc Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Naringin inhibits $CoCl_2$-induced injury to H9C2 cells. To investigate the toxic effects of $CoCl_2$ on H9C2 cells, the cells were incubated with various concentrations of $CoCl_2$ (200, 400, 600, 800 and 1,000 μmol/l) for different periods of time (1.5, 3, 6, 12 and 24 h). The results of CCK-8 assay revealed that $CoCl_2$ significantly reduced cell viability in a concentration- and time-dependent manner (Fig. 1A). $CoCl_2$ (400 μmol/l) treatment for 12 h significantly decreased cell viability ($P<0.01$) to 40% of the control; thus, this concentration and treatment duration were used in subsequent experiments. Subsequently, the protective effects of naringin on $CoCl_2$-induced toxicity to H9C2 cells were assessed. CCK-8 assay revealed that treatment with various concentrations of naringin (5, 10, 20 or 40 μmol/l) alone for 24 h did not exert any cytotoxic effects on the cells (Fig. 1B), and naringin (10, 20 and 40 μmol/l) pre-treatment reversed the $CoCl_2$-induced decrease in cell viability (Fig. 1C). Pre-treatment with naringin at 20 μmol/l significantly reversed the $CoCl_2$-induced decrease in cell viability ($P<0.01$), and thus this concentration was used in subsequent experiments to explore the protective effects of naringin against $CoCl_2$ injury. These results indicated that naringin protected the H9C2 cells against $CoCl_2$-induced injury.

Naringin reduces the $CoCl_2$-induced apoptosis of H9C2 cells. Subsequently, the effect of naringin on the apoptosis of $CoCl_2$-treated H9C2 cells was assessed. Flow cytometric analysis (Fig. 2A and B) revealed that treatment with $CoCl_2$ resulted in a notable increase in the apoptotic rate ($P<0.01$), and this was prevented by naringin pre-treatment ($P<0.01$). Caspase-3 and caspase-9 are essential for apoptosis during myocardial damage (30). As shown in Fig. 2, the activities of caspase-3 and caspase-9 (Fig. 2C) were increased by $CoCl_2$ ($P<0.01$), and these effects were significantly reversed by naringin ($P<0.05$). Naringin treatment alone had no effect on apoptosis and caspase-3/caspase-9 activity. These results demonstrate that naringin mitigates the $CoCl_2$-induced apoptosis of H9C2 cells.

$CoCl_2$ induces the blockage of the autophagic flux and leads to myocardial cell injury. Autophagy is closely associated with cell death in the hypoxic-ischemic process (31,32). To confirm the role of autophagy in $CoCl_2$-induced injury to H9C2 cells, the expression of autophagy-related markers was determined. Western blot analysis revealed that following treatment with $CoCl_2$, the levels of Beclin 1 expression ($P<0.01$) and the LC3B-II/LC3B-I ratio ($P<0.01$) were both significantly upregulated compared with the control group (Fig. 3A). p62 is a marker of the autophagic flux that is degraded in autophagosomes (33). In the present study, $CoCl_2$ treatment increased the expression of p62 in H9C2 cells (Fig. 3A).

To determine whether the autophagic response following $CoCl_2$ treatment resulted in damage to the H9C2 cells, the $CoCl_2$-treated cells were co-treated with 3-MA, a selective inhibitor of autophagic initiation. The results of CCK-8 assay revealed that cell viability was significantly increased in the 3-MA and $CoCl_2$ co-treatment groups compared with $CoCl_2$ treatment alone (Fig. 3B). 3-MA also attenuated the $CoCl_2$-induced increase in apoptosis (Fig. 3C and D). 3-MA treatment alone did not affect cell viability and apoptosis compared with the control group. The increases in the expression levels of pro-autophagic proteins may be due to either enhanced autophagosome formation or reduced autophagosome degradation. Furthermore, western blot analysis revealed that treatment with Baf A1 (an inhibitor of the vacuolar H^+ ATPase of lysosomes) resulted in the upregulation of the LC3II/LC3I ratio compared with the control group (Fig. 3E), indicating a block on the basal lysosomal-dependent degradation of autophagosome cargo. Notably, the LC3II/LC3I ratio in the $CoCl_2$ group was upregulated compared with the Baf A1 group, and there was no significant difference in the LC3II/LC3I ratio between $CoCl_2$ treatment alone and co-treatment with Baf A1 and $CoCl_2$, indicating that $CoCl_2$ reduced the autophagic flux. Taken together, these data suggest that $CoCl_2$-induced H9C2 cell injury results in the blockage of autophagic clearance rather than in autophagosome formation.

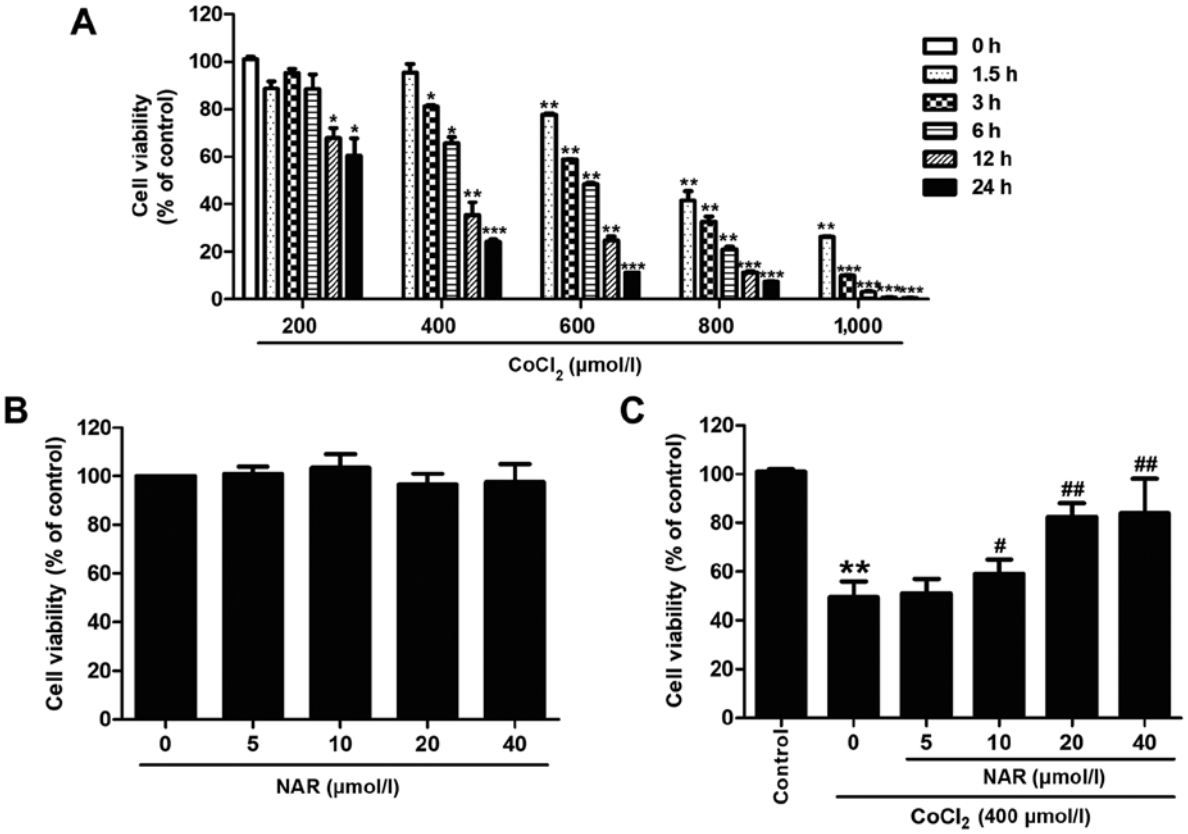

Figure 1. Naringin attenuates $CoCl_2$-induced cytotoxicity. H9C2 cells were incubated with various concentrations of $CoCl_2$ (200, 400, 600, 800 or 1,000 μmol/l) for different periods of time (1.5, 3, 6, 12 or 24 h). (A) Cell viability was determined using a CCK-8 assay. *P<0.05, **P<0.01, ***P<0.001 vs. control group. (B) H9C2 cells were incubated with various concentrations of naringin (5, 10, 20 or 40 μmol/l) and cell viability was measured by CCK-8 assay. H9C2 cells were pre-treated with 20 μmol/l naringin for 2 h followed by co-treatment with 400 μM $CoCl_2$ for 12 h. (C) Cell viability was assessed by CCK-8 assay. Results are presented as the means ± standard deviation; n=3. **P<0.01 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. $CoCl_2$ group. CCK-8, Cell Counting kit-8; $CoCl_2$, cobalt chloride; NAR, naringin.

Naringin protects H9C2 cells against $CoCl_2$-induced injury by restoring the impaired autophagic flux. To further investigate the role of the autophagic flux in the protective effects of naringin against $CoCl_2$-induced H9C2 cell injury, the effects of naringin on the levels of autophagy-related markers were detected. As shown in Fig. 4, western blot analysis revealed that naringin treatment further increased Beclin 1 expression (Fig. 4A and B) and the LC3II/LC3I ratio (Fig. 4A and C) compared with $CoCl_2$ treatment. Naringin reversed the $CoCl_2$-induced upregulation of p62 expression in H9C2 cells (Fig. 4A and D). Compared with the control group, naringin treatment alone had no effect on the expression of these autophagy-related proteins. These results suggested an increase in the autophagic flux induced by naringin pre-treatment. In addition, the inhibitors of autophagy (3-MA and Baf A1) both attenuated the naringin-induced upregulation of cell viability (Fig. 4E) and the downregulation of LDH release (Fig. 4F) in $CoCl_2$-treated H9C2 cells. Flow cytometric analysis also revealed that 3-MA and Baf A1 both mitigated the naringin-induced decrease in the apoptotic rate (Fig. 4G and H), as well as caspase-3 activity (Fig. 4I) and caspase-9 activity (Fig. 4J) in $CoCl_2$-treated H9C2 cells. These results suggest that naringin enhances the autophagic flux, including autophagosome formation and autophagic clearance, and mitigates $CoCl_2$ injury in H9C2 cells.

Naringin attenuates $CoCl_2$-induced injury and promotes the autophagic flux by promoting the activation of the HIF-1α/BNIP3 signaling pathway in H9C2 cells. The HIF-1α/BNIP3 signaling pathway has been shown to play an essential role in hypoxia-induced autophagy (28,29). In the present study, the effects of naringin on the HIF-1α/BNIP3 signaling pathway in $CoCl_2$-treated H9C2 cells was then assessed. The results of western blot analysis revealed that naringin pre-treatment further increased the expression of HIF-1α (Fig. 5A and B) and BNIP3 (Fig. 5A and C) in $CoCl_2$-treated H9C2 cells, indicating that naringin promoted the activity of the HIF-1α/BNIP3 signaling pathway in the $CoCl_2$-treated H9C2 cells. Furthermore, YC-1, an inhibitor of HIF-1α, blocked the naringin-induced increase in the expression of HIF-1α (Fig. 5D and E) and BNIP3 (Fig. 5D and F) in $CoCl_2$-treated H9C2 cells. On these basis, YC-1 also attenuated the naringin-induced upregulation of Beclin 1 expression (Fig. 5G and H) and the ratio of IL3B-II to IL3B-I (Fig. 5G and I), as well as the downregulation of p62 expression (Fig. 5G and J) in $CoCl_2$-treated H9C2 cells. This suggested that HIF-1α/BNIP3 mediated the naringin-induced increase in the autophagic flux. Moreover, treatment with YC-1 eliminated the naringin-induced increase in cell viability (Fig. 5K) and the decrease in LDH release (Fig. 5L). These results indicated that naringin protects H9C2 cells against $CoCl_2$ injury by enhancing autophagic flux via activation of the HIF-1α/BNIP3 signaling pathway.

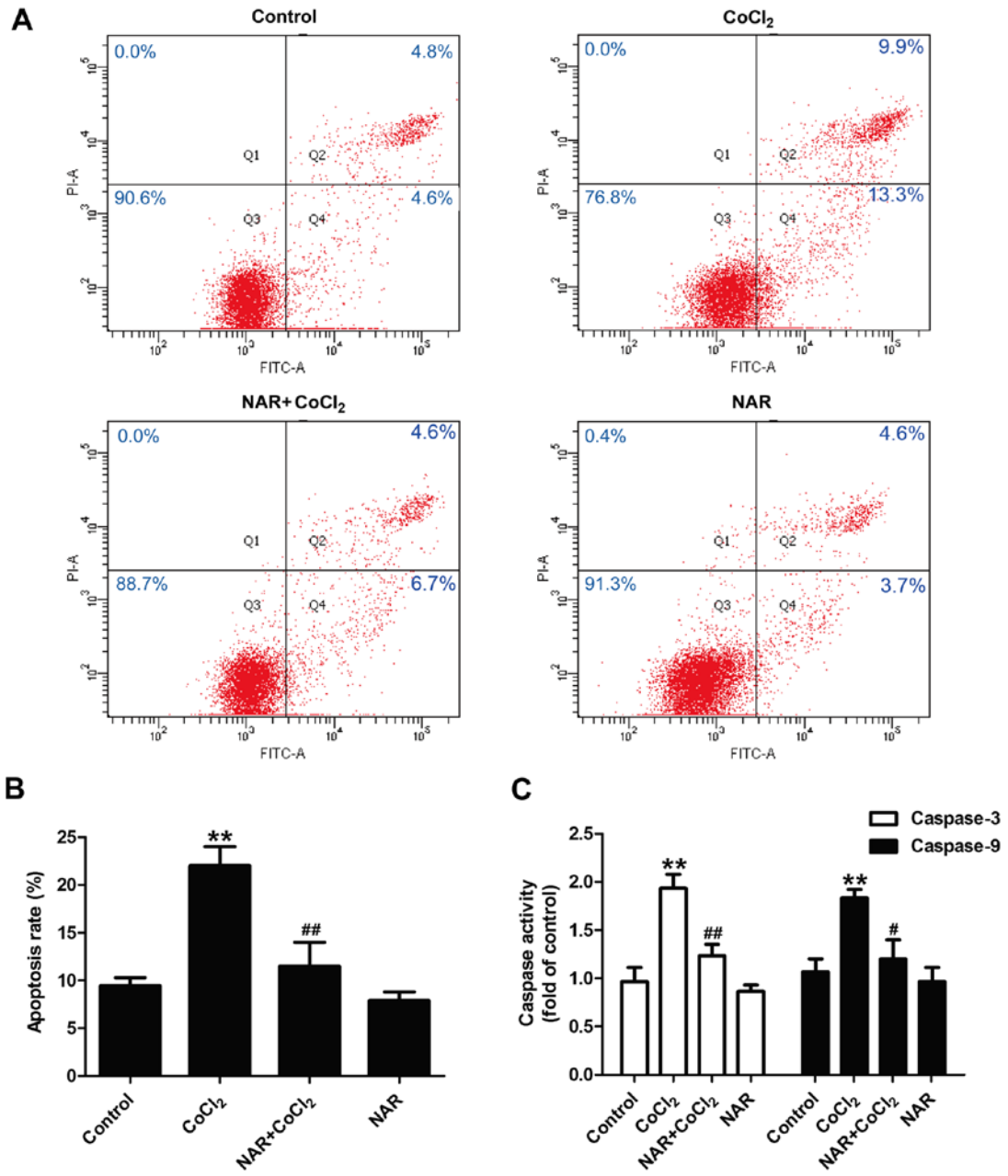

Figure 2. Naringin attenuates the apoptosis of H9C2 cells treated with $CoCl_2$. H9C2 cells were pre-treated with 20 μmol/l naringin for 2 h followed by co-treatment with 400 μmol/l $CoCl_2$ for 12 h. (A) Cell apoptosis was analyzed using Annexin V-FITC/PI double staining. (B) The apoptotic rate was measured and quantified by flow cytometry. (C) Caspase-3 activity and caspase-9 activity was determined using a Caspase-3 Activity Assay kit and a Caspase-9 Activity Assay kit. Results are presented as the means ± standard deviation; n=3. **P<0.01 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. $CoCl_2$ group. $CoCl_2$, cobalt chloride; NAR, naringin.

Discussion

Hypoxia/ischemia can induce sudden cardiomyocyte death, and this may result in further cardiovascular diseases (4,34). Naringin possesses anti-apoptotic, antioxidant and cardioprotective properties in the cardiovascular system (11,12). However, the effects of naringin on hypoxia/ischemia injury remain undetermined. The results of the present study demonstrated that naringin protected H9C2 cells against hypoxia-induced injury by promoting the autophagic flux via activation of the HIF-1α/BNIP3 signaling pathway.

$CoCl_2$ (a chemical hypoxia-mimetic agent)-induced H9C2 cell injury has been shown to be a suitable *in vitro* model of hypoxia-associated damage in cardiomyocytes (35,36), and was thus used to explore the underlying mechanisms in the present study. The mechanisms underlying naringin-induced cardioprotection against hypoxia injury in the $CoCl_2$-treated H9C2 cells were determined. The results revealed that $CoCl_2$ significantly reduced H9C2 cell viability in a concentration- and time-dependent manner, consistent with the findings of previous studies (27,37). Recently, naringin, which is the major active constituent of tomentose pummelo peel, has been found to exert cardioprotective effects in animal and cell damage models (12,38). Naringin has been shown to attenuated the effects of several damaging stimuli, such as doxorubicin (11), hyperglycemia (39) and anoxia/reoxygenation induced myocardial injury (13) via the inhibition of apoptosis and oxidative stress *in vitro* and *in vivo*. Consistent with the findings of

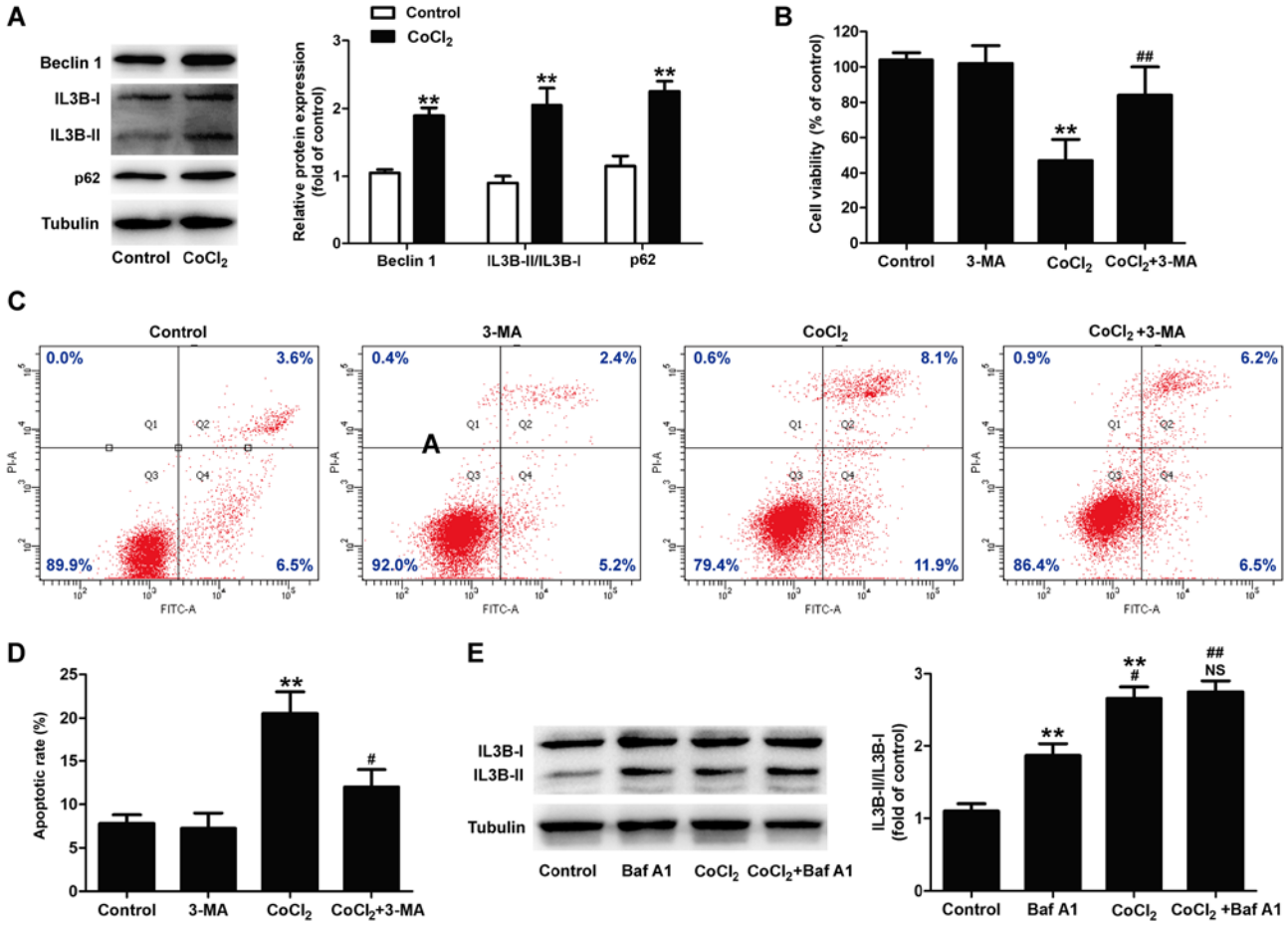

Figure 3. $CoCl_2$ inhibits the autophagic flux, leading to H9C2 cell death. H9C2 cells were incubated with 400 μmol/l $CoCl_2$ for 12 h. (A) Expression of autophagy-related proteins was assessed by western blot analysis. **P<0.01 vs. control group. H9C2 cells were pre-treated with 5 mM 3-MA for 1 h followed by co-treatment with 400 μM $CoCl_2$ for 12 h. (B) Cell viability was determined by CCK-8 assay. (C) Apoptosis was analyzed using Annexin V-FITC/PI double staining. (D) The apoptotic rate was measured and quantified by flow cytometry. **P<0.01 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. $CoCl_2$ group. H9C2 cells were co-treated with 10 nmol/l Baf A1 and 400 μmol/l $CoCl_2$ for 12 h. (E) The expression of IL3B-I and IL3B-II were measured by western blot analysis. **P<0.01 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. Baf A1 group. Results are presented as the means ± standard deviation; n=3. NS, not significant; $CoCl_2$, cobalt chloride; 3-MA, 3-methyladenine; CCK-8, Cell Counting kit-8; Baf A1, bafilomycin A1.

these studies, the results of the present study demonstrated that naringin attenuated the $CoCl_2$-induced decrease in cell viability and the increase in apoptosis. These results indicate that naringin protects H9C2 cells against $CoCl_2$-induced injury.

Autophagy is an evolutionarily controlled lysosome-mediated process that eliminates damaged proteins and intracellular organelle, involved in a variety of physiological processes (40,41). An increasing number of studies have demonstrated that autophagy dysfunction is increased in the heart following hypoxia/ischemia, and restoring impaired autophagic flux may prevent myocardial damage against hypoxia/ischemia (42-44). Recent studies have also indicated that $CoCl_2$ increases the levels of markers associated with the autophagic-lysosomal pathway, including Beclin1 (an indicator of initiation of autophagy), the conversion of LC3B-I to LC3B-II (a surrogate index of autophagosome formation) and p62 (a commonly used indicator of autophagic flux inhibition) (13,45). Consistent with these studies, the results of the present study also indicated that $CoCl_2$ significantly increased Beclin 1 expression and the LC3B-II/LC3B-I ratio, and decreased p62 expression in H9C2 cells. Thereafter, it is was shown that 3-MA attenuated $CoCl_2$-induced cell damage and apoptosis in H9C2 cells, indicating that reducing autophagosome formation contributed to $CoCl_2$-induced myocardial damage. In addition, previous studies have revealed that autophagy also plays important roles in the regulation of cell death, particularly in apoptosis-signaling pathways (46,47). Hence, it was hypothesized that the impaired autophagic flux leads to an increase in the apoptosis of $CoCl_2$-treated H9C2 cells. Increases in the levels of pro-autophagic proteins may be due to either an enhanced autophagosome formation or reduced autophagosome degradation (48,49). To distinguish between these two possibilities, the H9C2 cells were treated with Baf A1 in the present study. The results revealed that Baf A1 treatment alone resulted in the upregulation of the LC3II/LC3I ratio, indicating a block on the basal lysosomal-dependent degradation of autophagosome cargo. Notably, the LC3B-II/LC3B-I ratio in the $CoCl_2$ group was upregulated compared with the Baf A1 group, and there was no significant difference in LC3II/LC3I ratio between $CoCl_2$ and Baf A1 co-treatment group with the $CoCl_2$ group, indicating that $CoCl_2$ inhibited the autophagic flux. Taken together, these data suggest that $CoCl_2$-induced H9C2 cell injury is the result of blockage of autophagic flux,

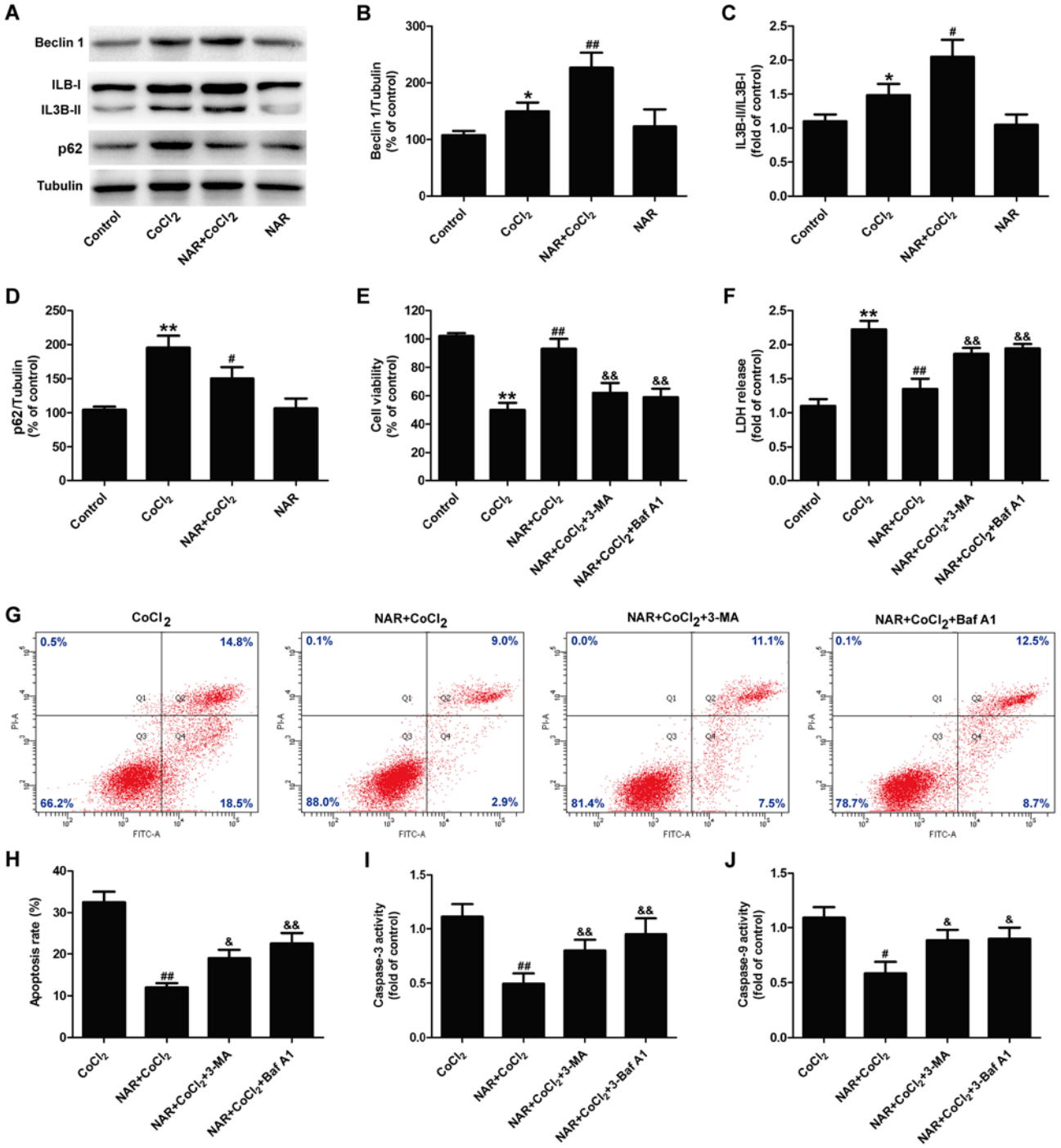

Figure 4. Enhancement of the autophagic flux mediates the protective effects of naringin on $CoCl_2$-induced injury in H9C2 cells. H9C2 cells were pre-treated with 20 μmol/l naringin for 2 h followed by co-treatment with 400 μmol/l $CoCl_2$ for 12 h. (A) Expression of autophagy-related proteins. Quantitative analysis of (B) Beclin 1 expression, (C) the IL3B-II/IL3B-I ratio and (D) p62 expression. *P<0.05, **P<0.01 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. $CoCl_2$ group. H9C2 cells were pre-treated with 5 mM 3-MA for 1 h, followed by treatment with 20 μmol/l naringin for 2 h and then co-treatment with 400 μmol/l $CoCl_2$ for 12 h, or H9C2 cells were treated with naringin for 2 h prior to co-treatment with 10 nmol/l Baf A1 and 400 μmol/l $CoCl_2$ for 12 h. (E) Cell viability was measured by CCK-8 assay. (F) LDH release was measured using a LDH assay kit. (G and H) Apoptosis was analyzed by Annexin V-FITC/PI double staining and the apoptotic rate was measured and quantified by flow cytometry. (I) Caspase-3 activity was determined using a Caspase-3 Activity Assay kit. (J) Caspase-9 activity was determined using a Caspase-9 Activity Assay kit. Results are presented as the means ± standard deviation n=3. **P<0.01 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. $CoCl_2$ group; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. NAR + $CoCl_2$ group. CCK-8, Cell Counting Kit-8; $CoCl_2$, cobalt chloride; NAR, naringin; Baf A1, bafilomycin A1; LDH, lactate dehydrogenase.

as evidenced by the enhancement of autophagosome formation and the reduction of lysosome-mediated autophagosome degradation.

Recently, naringin has been shown to regulate autophagy in several types of diseases (38,50,51). Naringin has been shown to promote the levels of autophagy markers, such as LC3B-II/I and Beclin-1, and autophagy is involved in the protective effect of naringin on oxidative stress-induced apoptosis in nucleus pulposus cells (14). Feng *et al* (50) demonstrated that naringin reduced cerebral apoptotic cell death underlying ischemia-reperfusion via the regulation of the activation of mitophagy. However, the effects of naringin

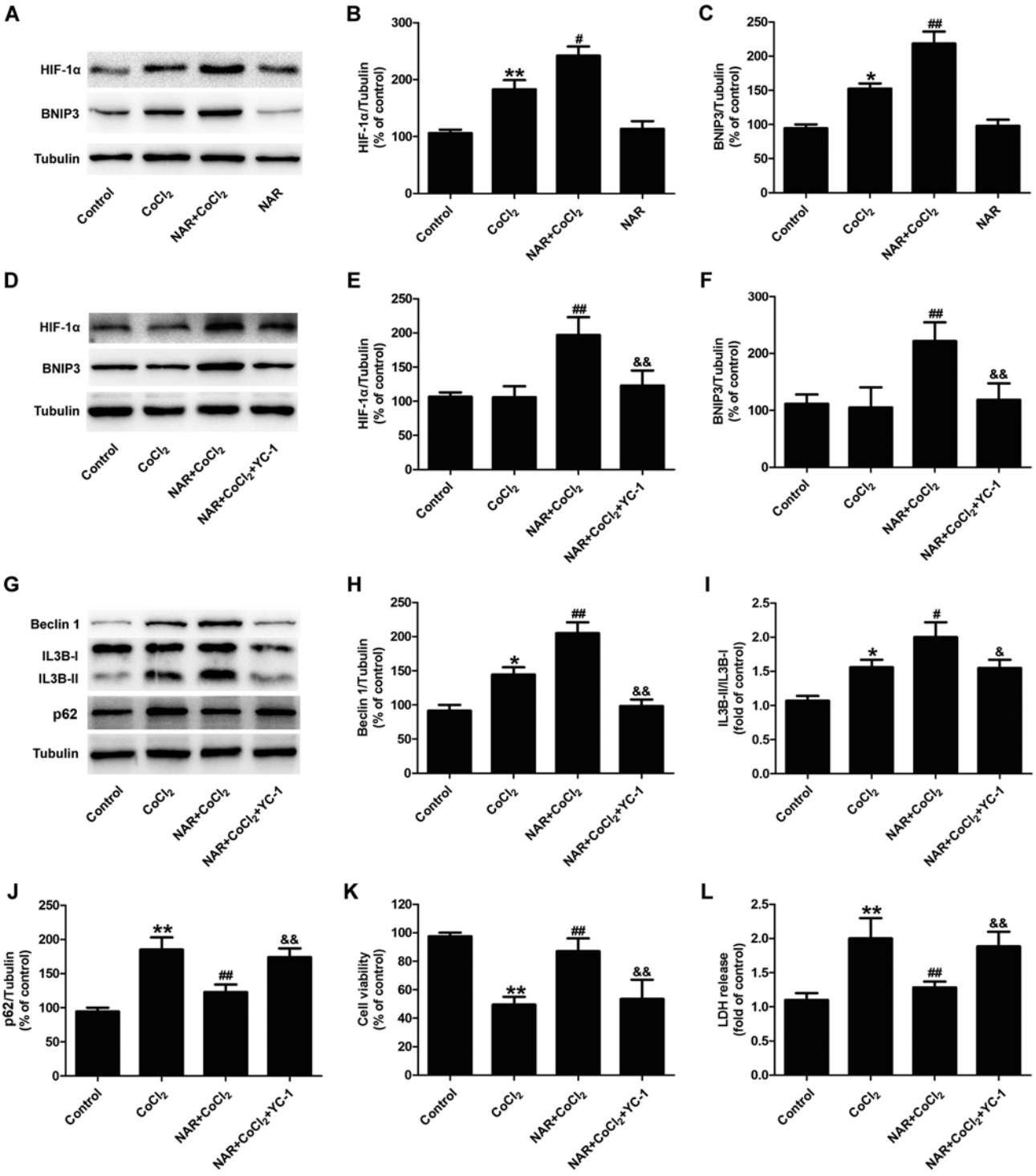

Figure 5. HIF-1α/BNIP3 contributes to the naringin-induced enhancement of the autophagic flux and the cardioprotective effects against $CoCl_2$-induced injury to H9C2 cells. H9C2 cells were treated with 20 μmol/l naringin for 2 h followed by co-treatment with 400 μmol/l $CoCl_2$ for 12 h. (A) HIF-1α and BNIP3 protein expression was determined by western blot analysis. Quantitative analysis of (B) HIF-1α and (C) BNIP3 expression. Results are expressed as the means ± SD; n=3. *P<0.05, **P<0.01 vs. control group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. $CoCl_2$ group. H9C2 cells were pre-treated with 10 μM YC-1 for 1 h, then 20 μmol/l naringin for 2 h, followed by co-treatment with 400 μmol/l $CoCl_2$ for 12 h. (D) Expression of HIF-1α and BNIP3 were measured by western blot analysis. Quantitative analysis of (E) HIF-1α and (F) BNIP3 expression. (G) Expression of autophagy-associated proteins was determined by western blot analysis. (H) Quantitative analysis of Beclin 1 expression. (I) Quantitative analysis of IL3B-II/IL3B-I ratio. (J) Quantitative analysis of p62 expression. (K) Cell viability was measured by CCK-8 assay. (L) LDH release was measured using an LDH assay kit. Results are presented as the means ± standard deviation. n=3. *P<0.05, **P<0.01 vs. control group; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. $CoCl_2$ group; $^{\&}$P<0.05, $^{\&\&}$P<0.01 vs. NAR + $CoCl_2$ group. CCK-8, Cell Counting kit-8; $CoCl_2$, cobalt chloride; LDH, lactate dehydrogenase; NAR, naringin.

on autophagy or the autophagic flux under hypoxia/ischemic conditions have not yet been studied, at least to the best of our knowledge. To the best of our knowledge, the present study was the first to demonstrate that naringin pre-treatment further increased Beclin 1 expression and the IL3B-II/IL3B-I ratio in $CoCl_2$-treated H9C2 cells, and that naringin pretreatment

decreased p62 expression in $CoCl_2$-treated H9C2 cells, suggesting that naringin restores the autophagic flux under hypoxic conditions. In addition, Baf A1 attenuated the protective effects of naringin on cell injury and apoptosis induced by $CoCl_2$ in H9C2 cells. These results suggest that naringin rescues the $CoCl_2$-induced impaired autophagic flux by promoting the initiation of autophagy and degradation, and then protects H9C2 cells against $CoCl_2$-induced injury. Combined with the role of autophagy in $CoCl_2$ injury as discussed above, the ability of naringin to promote lysosome-mediated autophagosome degradation was more potent than the ability to promote autophagosome formation, thus exerting a cardioprotective effect against hypoxia injury.

HIF-1α is an important transcription factor in the response to hypoxia, and BNIP3, a transcriptional target of HIF-1α, is upregulated in response to hypoxia (21,52,53). Notably, previous studies have demonstrated that the HIF-1α/BNIP3 signaling pathway is closely associated with hypoxia-induced autophagy (25,54). Previous studies have also indicated that $CoCl_2$ can disrupt HIF-1α degradation, and increase HIF-1α and BNIP3 expression, thus inducing apoptosis and autophagic cell death (25,55). Consistent with these studies, the expression levels of HIF-1α and BNIP3 in H9C2 cells were also significantly increased following $CoCl_2$ treatment in the present study. Notably, in the present study, it was shown that naringin further increased the HIF-1α and BNIP3 expression levels, activating the HIF-1α/BNIP3 signaling pathway. The HIF-1α inhibitor, YC-1, induced the inhibition of the HIF-1α/BNIP3 signaling pathway and reversed the naringin-induced increase in Beclin 1 expression, in the IL3B-II/IL3B-1 ratio, and the decrease in p62 expression, suggesting that the enhancement of the autophagic flux induced by naringin was mediated by the HIF-1α/BNIP3 signaling pathway. In addition, YC-1 also blocked the protective effects of naringin against $CoCl_2$-induced injury. These results suggest that naringin protects the H9C2 cells against $CoCl_2$-induced injury by enhancing the autophagic flux through the activation of the HIF-1α/BNIP3 signaling pathway.

Limitations still exist in the present study. First, the present study did not detect the change in the autophagic flux with IL-3 fluorescence staining. In addition, drugs were simply used to intervene with the HIF-1 signaling pathway without using gene silencing. Second, as all our assays were performed using cells, in future experiments, the authors aim to introduce tests and to investigate the underlying mechanisms in *in vitro* animal models, such as rats. Other studies have confirmed that myocardial ischemia injury induces the inflammatory response (56,57). Naringin also has been shown to exhibit anti-inflammatory activity (58,59). Hence, it is meaningful to explore the protective mechanism of naringin on myocardial ischemia injury focusing on the inflammatory response in the future studies. In addition, naringin has been shown to exhibit anti-inflammatory activity. In addition, further identification and confirmation of the precise mechanisms underlying naringin and HIF-1α/BNIP3 signaling pathway are required.

In conclusion, the present study demonstrated that naringin attenuated $CoCl_2$-induced injury in H9C2 cells by restoring the autophagic flux via the activation of the HIF-1α/BNIP3 signaling pathway. These findings reinforce the cardioprotective effects of naringin against hypoxic injury and indicate that the HIF-1α/BNIP3 signaling pathway may serve as a novel therapeutic target for the management of hypoxia/ischemia injury.

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Availability of data and materials

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

Authors' contributions

SL and JJ designed the experiments and contributed to the drafting of the manuscript. JF, CH and XL collated and analyzed the data. WL and KW performed the experiments and wrote the manuscript. JJ and CH wrote and revised the manuscript. SL and KW confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

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.

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