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ORIGINAL ARTICLE

Berberine Inhibits Norepinephrine-Induced Apoptosis in Neonatal Rat Cardiomyocytes via Inhibiting ROS-TNF-α-Caspase Signaling Pathway*

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ABSTRACT **Objective**: To determine the effect of berberine (Ber) on norepinephrine (NE)-induced apoptosis in neonatal rat cardiomyocytes. **Methods**: The cultured neonatal rat cardiomyocytes were treated with NE in the presence or absence of Ber. The activity of lactate dehydrogenase (LDH) in the culture medium was examined, and apoptosis of cardiomyocytes was assessed by Hoechst 33258, isothiocyanate (FITC)-conjugated annexin-V, and propidine iodide (PI) staining. In addition, the activities of caspases-2 and-3 were measured by a fluorescent assay kit. The level of secreted tumor necrosis factor α (TNF-α) and production of intracellular reactive oxygen species (ROS) were also determined. **Results**: NE at a concentration of 50 μmol/L induced an obvious increase in the activity of LDH in the culture medium (P<0.05), which was inhibited by coincubation with 0.5, 1.0, or 2.0 μmol/L Ber (P<0.05). Ber also significantly attenuated NE-induced apoptosis in a dose-dependent manner (P<0.01). Moreover, Ber at a dose of 2 μmol/L markedly decreased the ROS and TNF-α productions (P<0.05) and inhibited the activation of caspases-2 and -3 in cardiomyocytes exposed to NE (P<0.05). **Conclusion**: The present study suggested that Ber could reduce NE-induced apoptosis in neonatal rat cardiomyocytes through inhibiting the ROS-TNF-α-caspase signaling pathway.

KEYWORDS apoptosis, cardiomyocytes, berberine, norepinephrine

Enhanced activity of the sympathetic nervous system in the myocardium is one of the important features of heart failure.[1,2] Norepinephrine (NE), the primary transmitter of the sympathetic nervous system, could induce apoptosis in both adult and neonatal rat cardiomyocytes.[3,4] Accumulating evidence showed that the apoptosis of the cardiomyocytes increased in myocardium obtained from patients with heart failure and from various experimental models of myocardial failure.[5-8] Therefore, inhibition of apoptosis provides cardioprotective effects so as to prevent the development of heart failure.[9]

Berberine (Ber) is an isoquinoline alkaloid with multiple pharmacological activities, including positive inotropic, antiarrhythmic, and vasodilator properties.[10] Some researchers found that Ber improved abnormal cardiac function and prevented the development of left ventricular hypertrophy induced by pressure-overload in rats, suggesting that Ber may have therapeutic potential in the treatment of congestive heart failure.[11] Zeng, et al[12] demonstrated that treatment with Ber increased left ventricular ejection fraction, improved quality of life, and decreased mortality in patients with congestive heart failure. Recently, Ber was also found to prevent hyperglycemia-induced endothelial injury and hypoxia/reoxygenation-stimulated cardiomyocyte apoptosis.[13,14] However, it is still unclear whether Ber inhibits NE-induced apoptosis in cardiomyocytes.

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It was well characterized previously that NE induces apoptosis through a reactive oxygen species (ROS)-tumor necrosis factor α (TNF-α)-caspase signaling pathway in neonatal rat cardiomyocytes.[4,15-17] In the present study, we intended to examine the effects of Ber on NE-induced apoptosis in neonatal rat cardiomyocytes with special focus on ROS production, TNF-α secretion, and caspase activation.

METHODS

Animals and Reagents

The neonatal Sprague-Dawley rats were obtained from the Center of Laboratory Animals in Guangdong province of China. The experimental protocol was approved by the Experimental Animal Care and Use Committee in School of Medicine, Jinan University, which conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). NE and neutral sulfate Ber were purchased from Sigma (St. Louis, MO, USA), Dulbecco's modified Eagles' medium (DMEM) was from GIBCO–BRL (USA). Quantikine enzyme-linked immunosorbent assay (ELISA) kits specific for rat TNF-α were products of R&D System (Minneapolis, USA), monoclonal antitroponin Ⅰ (ab19615) antibody and fluorescein isothiocyanate (FITC)-conjugated antimouse IgG antibody were obtained from Abcam plc. (Cambridge, UK) and KPL Inc. (Gaithersburg, MD, USA), respectively. Annexin V-FITC apoptosis detection kit was obtained from Bender MedSystems Inc. (Burlingame, CA, USA), and APO LOGIX carboxyfluorescein caspase detection kit was from Cell Technology Inc. (Mountain View, CA, USA).

Isolation and Culture of Neonatal Rat Cardiomyocytes

Primary cardiomyocytes were isolated from 1 to 2 days old neonatal Sprague-Dawley rat hearts, as described previously.[4] Briefly, the ventricles of the rat heart were cut into approximately 1 mm pieces, and the ventricular cells were dispersed by digestion with 0.125% trypsin. After trypsinization, free cells were collected and resuspended in DMEM containing 10% fetal bovine serum, 100 μg/mL streptomycin, and 100 U/mL penicillin. The cell suspensions were then incubated in the 5% carbon dioxide incubator (Thermo Forma, Co., USA) at 37 ℃ for 3 h, and the nonadherent cells (mostly cardiomyocytes) were removed carefully onto 35 mm plastic culture dishes at a density of 1×10^5 or 5×10^5 cells/mL and cultured in DMEM containing 10% fetal bovine serum and cytosine arabinoside (10 μmol/L, Sigma, St. Louis, MO, USA) to eliminate noncardiomyocytes at 37 ℃ in a humidified atmosphere containing 5% carbon dioxide. The medium was changed every 24 h, and the cardiomyocyte contractile characteristics were observed directly under the microscope. After 48 h in culture, the medium was replaced with serum-free medium, and cardiomyocytes were treated with NE and the indicated reagents for the indicated time.

Immunocytochemistry

In order to identify cardiomyocytes, immunocytochemical staining was performed using a monoclonal antibody against cardiac troponin Ⅰ. Slide cultures of cardiomyocytes were washed with phosphate buffered saline solution (PBS), fixed in 95% ethanol for 10 min at room temperature, and permeabilized in 0.5% Triton-X 100 for an additional 5 min. After rinsing with PBS, coverslips were blocked with 5% normal goat serum for 15 min at room temperature. The cardiomyocytes were then incubated with 1:50 dilution of monoclonal antibody against cardiac troponin Ⅰ at 37 ℃ for 1 h. The excess antibody solution was removed by washing with PBS 3 times, and the coverslips were treated with FITC-conjugated anti-mouse IgG in dark for 30 min at 37 ℃. The coverslips were washed three times with PBS and stained with 50-μg/mL propidium iodide (PI) at room temperature for another 5 min. As shown in Figure 1, the labeled cardiomyocytes with more than 95% purity were visualized using a laser-scanning confocal microscope (LSM510META, Zeiss, Germany)

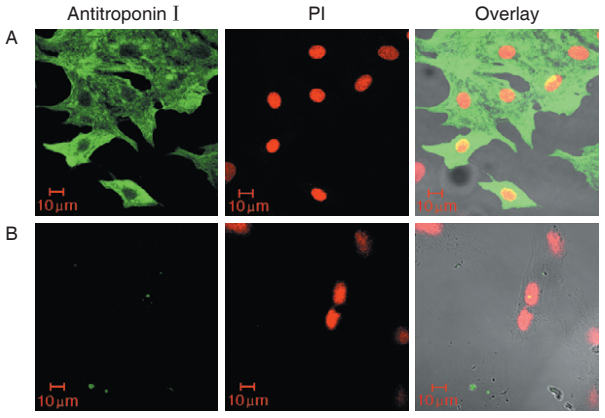

Figure 1. Immunocytochemical lidentification of Cultured Neonatal Rat Cardiomyocytes with Antitroponin Ⅰ Monoclonal Antibody
Notes: A: the negative control; B: antitroponin Ⅰ

with an excitation wavelength of 488 nm and emission wavelength of 525 nm for FITC and an excitation wavelength of 488 nm and emission wavelength of 620 nm for PI.

Lactate Dehydrogenase Activity Determination

In order to evaluate the protective effect of Ber on NE-treated cardiomyocytes, the cytotoxicity of cardiomyocytes was determined by lactate dehydrogenase (LDH) released from damaged cells. Cardiomyocytes at a density of 1×10^5 cells/mL in serum-free medium were exposed to different concentrations of NE or/and various concentrations of Ber for 72 h. After incubation, the supernatants were collected, and the LDH activity was determined using LDH assay kit (Sichuan Maker Biotechnology Co., Chengdu, China, No. Maker-08-03-01-03) by biochemistry autoanalyzer.

Hoechst 33258 Staining

A DNA-specific dye, Hoechst 33258, was used to visualize the chromatin condensation that is a characteristic of apoptosis. The cultured cardiomyocytes at a density of 1×10^5 cells/mL were treated with 50 μmol/L of NE or/and various concentrations of Ber for 48 h. The fixed cells were washed with PBS and stained with PBS containing 10 μmol/L of Hoechst 33258 for 5 min. Then, the stained cardiomyocytes were examined by fluorescence microscopy using an excitation wavelength of 348 nm and emission wavelength of 450 nm.

Annexin V – FITC and PI Staining

Cardiomyocytes (1×10^5 cells/mL) grown on coverslips were cultured as described above for 48 h. After incubation, the coverslips were washed with PBS three times and incubated with FITC-labeled annexin V mixture for 30 min at room temperature. The cardiomyocytes were washed with washing-buffer 3 times and then stained with PI for another 5 min. Finally, the labeled cardiomyocytes were examined by a confocal laser-scanning microscope immediately (LSM510META, Zeiss, Germany) using an excitation wavelength of 488 nm and emission wavelength of 525 nm for FITC and an excitation wavelength of 488 nm and emission wavelength of 620 nm for PI. Green fluorescent sarcolemma was observed in early apoptotic cells. Both green fluorescent sarcolemmal staining and red fluorescent nuclear staining can be observed in late apoptotic or necrotic cells. Apoptosis-positive cells were counted in five randomly selected fields of each of the three independent experiments. The proportion of annexin V and PI-positive cardiomyocytes was expressed as the percentage of the total cells counted.

Flow Cytometry Analysis for Apoptosis

In order to further quantify apoptosis, the cardiomyocytes (5×10^5 cells/mL) were stained with PI, and the DNA fragmentation in sub-G_0/G_1 phase was quantitated by flow cytometry (FACS Aria, BD Biosciences, California, USA). The cardiomyocytes were collected after trypsinization, washed with PBS, and fixed in 70% ethanol at 4 ℃ for 30 min. Then, the cells were rinsed again with PBS and resuspended in 1 mL of PBS with 0.1% Triton X-100, 50 μg/mL RNase A, and 50 μg/mL PI followed by incubation at 37 ℃ for 30 min. The samples were analyzed by flow cytometry with 488 nm excitation and 620 nm emission filters. Apoptotic cells stained with PI show a peak in the hypodiploid region, and the percentage of apoptotic cells was determined.

Caspases-2 and -3 Activity Assay

The activities of caspases-2 and -3 were detected with carboxyfluorescein caspase detection kit according to the manufacturer's protocol. The cardiomyocytes (5×10^5 cells/mL) were treated with 50 μmol/L of NE in the presence or absence of 2.0 μmol/L Ber for 24 h or 36 h. The cultured cardiomyocytes were washed with PBS and incubated with the reaction buffer containing caspases-2 or -3 substrate (FAM-VDVAD-FMK, FAM-DEVD-FMK) for 1 h at 37℃. The cells were harvested after trypsinization, washed with washing buffer, and resuspended in 1× washing buffer. The fluorescence signal was detected by flow cytometry with an excitation wavelength of 488 nm and emission wavelength of 525 nm.

TNF-α Measurement

Cardiomyocytes (1×10^5 cells/mL) were treated with 50 μmol/L NE in the presence or absence of 2.0 μmol/L Ber for 24 h. The supernatant was collected, and the levels of TNF-α were determined using an ELISA assay kit following the instructions provided by the manufacturer.

Measurement of Intracellular ROS

The average level of intracellular ROS in cardiomyocytes was evaluated using a cell-permeable and redox-sensitive probe 2',7'-dichlorofluorescein

diacetate (DCFH-DA).[13] The cardiomyocytes at a density of 5×10^5 cells/mL were treated with 50 μmol/L NE in the presence or absence of 2.0 μmol/L Ber for 12 h. The cells were washed twice in PBS and loaded with DCFH-DA at a final concentration of 5 μmol/L for 20 min. Then, the medium was removed, and the loaded cells were collected and resuspended in PBS. Fluorescence intensities of 500 cells were analyzed by flow cytometry using excitation wavelength of 488 nm and emission wavelength of 525 nm.

Statistical Analysis

Data were analyzed using the statistical software SPSS for Windows 13.0 and presented as mean ± standard diviation. The mean values were compared among groups using One-way ANOVA. Post hoc Tukey's honest significant difference test or Student *t*-test was used when appropriate. Statistical significance was set at *P* values less than 0.05.

RESULTS

Ber Prevents NE-Triggered Damage of Cardiomyocytes

As shown in Figure 2, NE at concentrations of 10 and 50 μmol/L increased the LDH level significantly as compared with the control group ($P<0.05$). More importantly, Ber dramatically inhibited NE-induced LDH release in a dose-dependent manner ($P<0.05$). Treatment with Ber 0.5, 1.0, or 2.0 μmol/L alone had no effect on the LDH level.

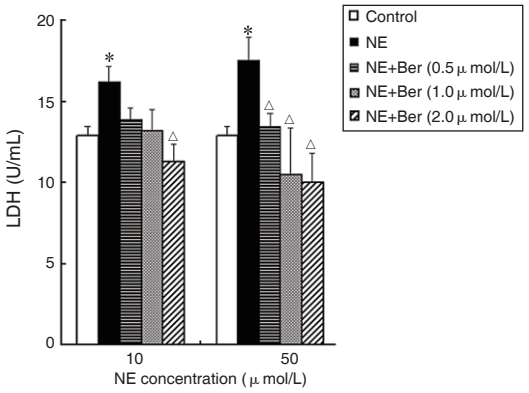

Figure 2. The Effect of Ber on LDH Activity in the Medium of Cultured Neonatal Rat Cardiomyocyes Treated with NE

Notes: $^{*}P<0.05$, compared with the control group; $^{\triangle}P<0.05$, compared with the NE group

Ber Prevents NE-Induced Apoptosis in Neonatal Rat Cardiomyocytes

Previous studies demonstrated that NE could induce apoptosis in rat cardiomyocytes.[3,4] To determine the effect of Ber on NE-induced apoptosis of rat neonatal cardiomyocytes, we treated the cultured cardiomyocytes with 50 μmol/L NE in the absence or presence of various concentrations of Ber for 48 h. The cells were then stained with Hochest 33258 followed by fluorescence microscopy observation. As shown in Figure 3, typical apoptotic morphological changes were found in the nuclei of neonatal rat cardiomyocytes 48 h after 50 μmol/L NE treatment. When the cells were cotreated with Ber of 0.5, 1.0, and 2.0 μmol/L, fewer apoptotic nuclei were observed.

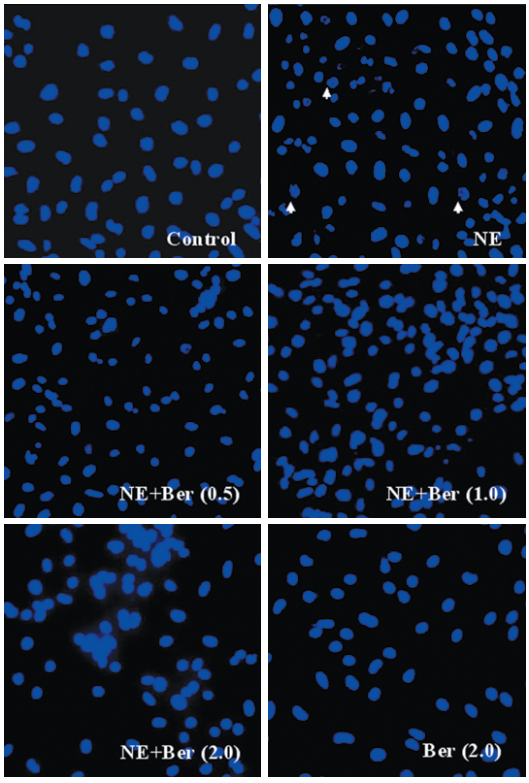

Figure 3. Morphological Changes in Nuclei of Cardiomyocytes

Notes: treated with NE (50 μmol/L) and/or Ber (0.5, 1.0, 2.0 μmol/L); the arrows point to the apoptotic cells

Figure 4 shows that treatment with 50 μmol/L NE significantly increased the amount of early apoptotic cells (annexin V-positive cells) and late apoptotic cells (annexin V- and PI-double positive cells). Specifically, treatment with 50 μmol/L NE induced more than three-fold increase of apoptotic cells compared with the control cells (11.30% ± 0.89% *versus* 42.80% ± 1.03%, $P<0.05$). Ber inhibited NE-induced apoptosis in a dose-dependent manner. With the gradual increase of the Ber concentration of 0.5, 1.0, and 2.0 μmol/L, less apoptotic cells were detected (40.00% ± 1.15%, 27.50% ± 0.83%, and 16.30% ± 0.47%, respectively, $P<0.01$).

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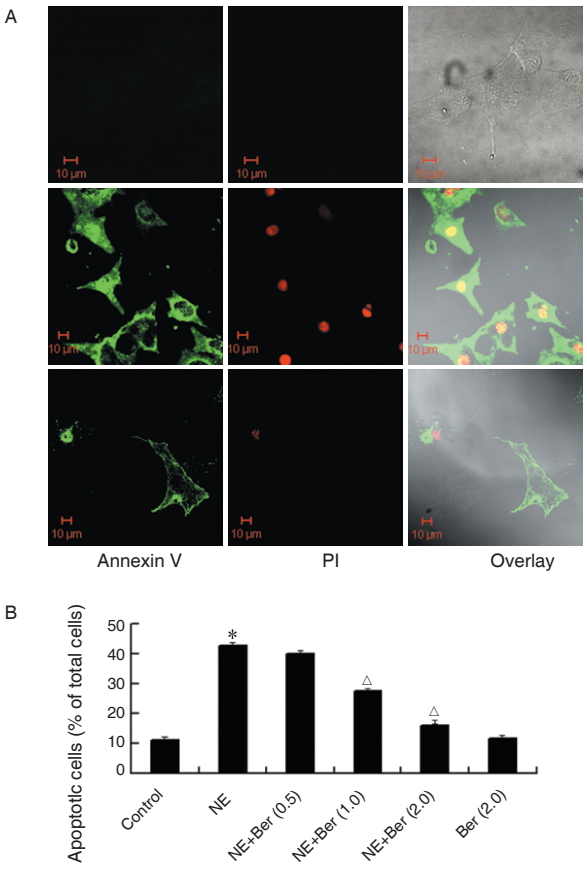

Figure 4. The Neonatal Rat Cardiomyocytes Stained With FITC-conjugated Annexin-V (Green) and PI (Red) 48 h After Treatment

Notes: A: morphology of cardiomyocytes was observed by a laser scanning confocal microscope, the upper panel represents normal cells, the middle panel shows representative cardiomyocytes treated with NE (50 μmol/L), and the lower panel shows cardiomyocytes treated with Ber (1.0 μmol/L)+NE (50 μmol/L). B: Ber at concentrations of 0.5, 1.0 and 2.0 μmol/L inhibited NE-induced apoptosis in a dose-dependent manner. $^{*}P$<0.05, compared with the control group; $^{\triangle}P$<0.01, compared with the NE group

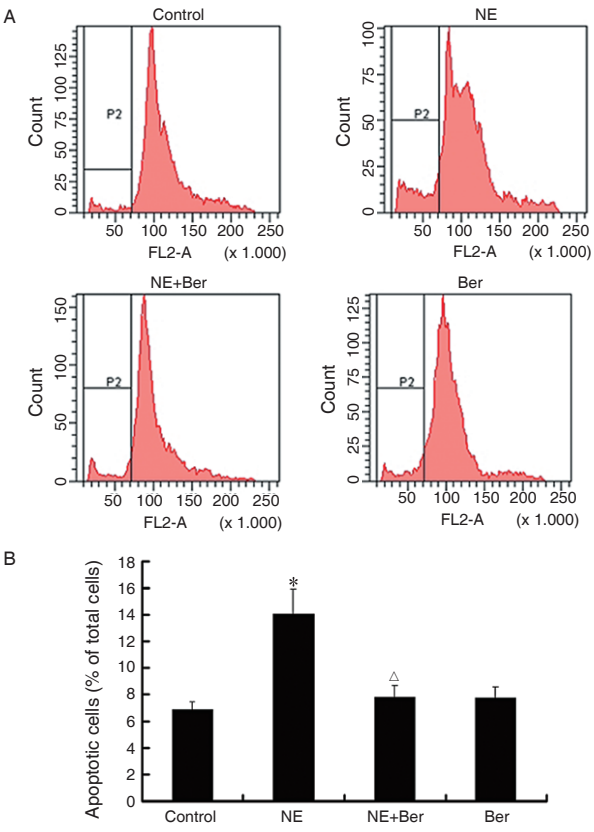

Figure 5. Flow Cytometric Analysis of DNA Content in Neonatal Rat Cardiomyocytes

Notes: $^{*}P$<0.05, compared with the control group; $^{\triangle}P$<0.01, compared with the NE group

The inhibitory effect of Ber on NE-induced apoptosis was further confirmed by flow cytometry analysis, which showed that cardiomyocytes treated with 50 μmol/L of NE exhibited a hypodiploid DNA content that is associated with the late stage of apoptosis (6.88%±0.60 % in control cells and 14.06%±1.90% in NE-treated cells. P<0.05, Figure 5A). This parameter decreased to 7.82%±0.90% in cardiomyocytes treated with 2.0 μmol/L Ber and 50 μmol/L NE (P<0.01). Ber (2.0 μmol/L) alone did not increase apoptosis in cardiomyocytes when compared with the control group (Figure 5B).

Effect of Ber on the Activation of Caspase-2 and -3 in Cardiomyocytes Treated with NE

As shown in Figure 6A, caspase-3 was activated at 24 h and 36 h after stimulation with 50 μmol/L NE (P<0.01). Ber significantly prevented NE-induced caspase-3 activation at 36 h after NE stimulation (P<0.05). Moreover, caspase-2 was activated at 24 h after stimulation with 50 μmol/L NE (P<0.01), and Ber significantly inhibited the caspase-2 activity in cardiomyocytes exposed to NE at 24 h (P<0.05, Figure 6B). Ber alone did not induce caspases-2 and -3 activation in cardiomyocytes (Figure 6).

Ber Inhibits NE-stimulated ROS Production and TNF Secretion in Cardiomyocytes

Figure 6C shows that NE-induced production of TNF-α was significantly attenuated by Ber after 24 h NE treatment (P<0.05). In addition, Ber also decreased NE-stimulated ROS production in cardiomyocytes after 12 h NE treatment (P<0.05, Figure 6D).

DISCUSSION

Apoptosis and hypersympathetic activity play a pivotal role in the development of heart failure. Although Ber was found to effectively prevent congestive heart failure,[12] the effect of Ber on

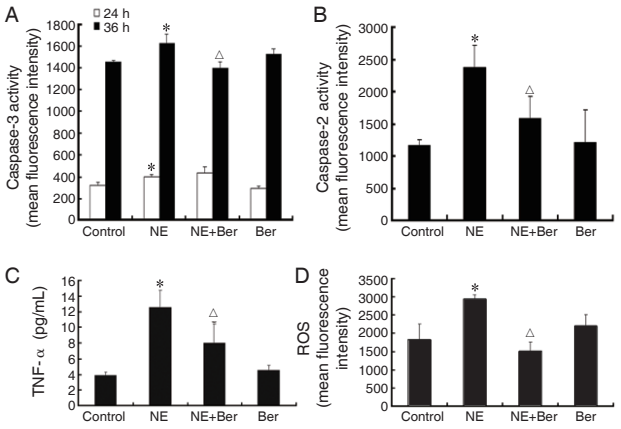

Figure 6. Comparion of Caspase Activation, TNF-α Release, and ROS Production in Neonatal Rat Cardiomyocytes

Notes: *P<0.01, compared with the control group; △P<0.05, compared with the NE group

NE-induced cardiomyocyte apoptosis remains unidentified.

Previous evidence demonstrated that Ber can induce apoptosis in many cancer and noncancer cell lines in a cell-type-dependent manner.[18-21] The concentrations of Ber used in these studies ranged from 10 μmol/L to 200 μmol/L. Recently, Zhou, et al[22] found that at 1–5 μmol/L Ber markedly inhibited apoptosis of PC12 cells induced by oxygen-glucose deprivation, which indicates that the effect of Ber on apoptosis is concentration-dependent and low concentrations of Ber actually inhibits apoptosis. In addition, Zeng, et al[12] found that Ber at concentrations of more than 0.27 μmol/L significantly improved the symptoms of patients with congestive heart failure. In the present study, we observed that Ber at concentrations of 0.5, 1.0, and 2.0 μmol/L protected cardiomyocytes from 50 μmol/L NE-induced injury, as evidenced by decreased LDH release. Therefore, we chose 0.5, 1.0, and 2.0 μmol/L to determine the effect of Ber on apoptosis in cardiomyocytes treated with 50 μmol/L NE.

Hochest 33258 staining demonstrated that treatment with 50 μmol/L NE for 48 h significantly increased apoptosis of cardiomyocytes, which was prevented by treatment with Ber at 0.5, 1.0, or 2.0 μmol/L. Similar results were obtained in annexin-V/ PI double staining experiment. NE at the concentration of 50 μmol/L led to 42.8%±1.03% apoptosis in neonatal rat cardiomyocytes, which is consistent with the previous study.[4] Ber at the concentration of 2.0 μmol/L was able to attenuate NE-induced apoptosis in cardiomyocytes. In order to confirm the above observation, we further performed the flow cytometry analysis of DNA content and demonstrated that 2.0 μmol/L Ber significantly inhibited 50 μmol/L NE-induced apoptosis in cardiomyocytes.

Caspases are critical cysteine-aspartate proteases in the initiation and execution of apoptosis. It has been shown that NE induces the activation of caspases-2 and -3. Inhibition of caspases-2 or -3 activity abrogates NE-induced apoptosis in cardiomyocytes,[4] which suggests that caspases-2 and -3 mediate NE-induced apoptosis in cardiomyocytes. To investigate the mechanisms by which Ber inhibits NE-induced apoptosis, we first determined the activities of caspases-2 and -3. Our results showed that caspase-3 was markedly activated in cardiomyocytes at 24 h and 36 h after exposure to NE. Interestingly, although Ber at concentration of 2.0 μmol/L did not inhibit the activation of caspase-3 in cardiomyocytes treated with 50 μmol/L NE for 24 h, Ber significantly blocked caspase-3 activation in cardiomyocytes exposed to 50 μmol/L NE for 36 h. These results suggested that Ber did not directly inhibit NE-induced caspase-3 activation in cardiomyocytes but may function on the upstream regulators to inhibit NE-induced caspase-3 activation in cardiomyocytes. Robertson, et al[23] demonstrated that caspase-2 could act on the upstream of mitochondria to promote cytochrome c release and activate caspase-3 in lymphocytes. Therefore, we further examined caspase-2 activity at 24 h after NE treatment in cardiomyocytes. The results showed that Ber prevented caspase-2 activation in cardiomyocytes at 24 h after NE treatment. These observations implicate that Ber can reduce NE-stimulated apoptosis in cardiomyocytes at least in part, through the inhibition of caspase-2 activation.

Cardiomyocytes express functional TNF-α type-1 receptors, which can be stimulated by TNF-α *in vitro* to induce apoptosis.[16] NE stimulates TNF-α secretion to induce apoptosis in cardiomyocyte, and this effect can be efficiently blocked by an anti-TNF-α antibody. In addition, NE-induced TNF-α release, caspase-2 activation, and apoptosis in cardiomyocytes can also be abolished in the presence of antioxidants. These findings suggest that NE can stimulate ROS

generation and subsequent TNF-α production, which in turn activates caspase-2 and induces cardiomyocyte apoptosis.[4,15-17] Recently, Wang, et al[13] reported that Ber attenuated high glucose-induced ROS generation and cellular apoptosis in endothelial cells. In our current study, we further examined the effect of Ber on NE-induced ROS and TNF-α generation in cardiomyocytes. The results showed that 50 μmol/L NE induced a significant increase of ROS generation after 12 h and TNF-α secretion after 24 h, which can be inhibited by coincubation with 2.0 μmol/L Ber. These results indicate that Ber can abolish NE-induced cardiomyocyte apoptosis through inhibition of ROS and TNF-α generation. On the other hand, Hong, et al[24] demonstrated that Ber modulated the sympathetic nervous activity and decreased the NE and adrenaline levels both in the plasma and the left ventricular tissue of rats with experimental cardiac hypertrophy. Taken together, our studies further reveal the therapeutic potentials of Ber in patients with heart failure. More recently, it had been demonstrated that Ber can significantly activate adenosine 5'-monophosphate-activated protein kinase (AMPK) pathway in microglia, and AMPK activators can inhibit TNF-α-induced cardiomyocyte apoptosis.[25, 26] In this context, our future study will focus on the role of AMPK activation in the inhibitory effect of Ber on NE-induced cardiomyocyte apoptosis and the exact mechanisms that are responsible for the inhibition of ROS generation by Ber in NE-treated cardiomyocytes.

In summary, the present study provides evidence that Ber at low concentrations inhibits ROS and TNF-α production and subsequent caspase activation to prevent NE-induced apoptosis in cardiomyocytes.

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