Naringin protects cardiomyocytes against hyperglycemia-induced injuries *in vitro* and *in vivo*

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Abstract

We previously reported that naringin (NRG) protects cardiomyocytes against high glucose (HG)-induced injuries by inhibiting the MAPK pathway. The aim of this study was to test the hypothesis that NRG prevents cardiomyocytes from hyperglycemia-induced insult through the inhibition of the nuclear factor kappa B (NF- κ B) pathway and the upregulation of ATP-sensitive K⁺ (K_{ATP}) channels. Our results showed that exposure of cardiomyocytes to HG for 24 h markedly induced injuries, as evidenced by a decrease in cell viability and oxidative stress, and increases in apoptotic cells as well as the dissipation of mitochondrial membrane potential (MMP). These injuries were markedly attenuated by the pretreatment of cells with either NRG or pyrrolidine dithiocarbamate (PDTC) before exposure to HG. Furthermore, in streptozotocin (STZ)-induced diabetic rats and in HG-induced cardiomyocytes, the expression levels of caspase-3, bax and phosphorylated (p)-NF- κ B p65 were increased. The increased protein levels were ameliorated by pretreatment with both NRG and PDTC. However, the expression levels of bcl-2 and K_{ATP} and superoxide dismutase (SOD) activity were decreased by hyperglycemia; the expression level of Nox4 and the ADP/ATP ratio were increased by hyperglycemia. These hyperglycemia-induced indexes were inhibited by the pretreatment of cardiomyocytes with NRG or PDTC. In addition, in STZ-induced diabetic rats, we also observed that NRG or PDTC contributed to protecting mitochondrial injury and myocardium damage. This study demonstrated that NRG protects cardiomyocytes against hyperglycemia-induced injury by upregulating K_{ATP} channels *in vitro* and inhibiting the NF- κ B pathway *in vivo* and *in vitro*.

Key Words

- naringin
- cardiomyocytes
- hyperglycemia
- nuclear factor kappa B pathway
- ATP-sensitive K⁺ channels
- cardioprotection

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Introduction

Currently, statistical analysis shows that diabetes mellitus (DM) affects approximately 387 million people globally and is projected to increase two-fold in the next 25 years

(Huang *et al.* 2009). As a disease of epidemic proportions, DM damages every organ in the human body by causing serious microvascular and macrovascular diseases.

Diabetic cardiomyopathy (DCM), one of the most prevalent cardiovascular complications and a major cause of death of DM, causes congestive heart failure (Lakdawala *et al.* 2013, Camafort 2015) and sudden death in young people (Ellis & Di Salvo 2007). DCM is different from hypertensive cardiomyopathy and is a unique cardiovascular disease due to hyperglycemia-induced oxidative stress (Privratsky *et al.* 2003, Kamalakkannan & Prince 2006, Saandee *et al.* 2009, Murali *et al.* 2013, Peake *et al.* 2013, Umbarkar *et al.* 2015), inflammation (Marfella *et al.* 2003, Di *et al.* 2005, Venkatachalam *et al.* 2008), cardiac fibrosis (Fonarow & Srikanthan 2006), myocardial apoptosis (Fonarow & Srikanthan 2006) and mitochondrial damage (Boudina *et al.* 2007, Ceriello 2008).

The activation of nuclear factor kappa B (NF- κ B), which can control the molecular processes within cardiomyocytes, contributes to many heart diseases, such as myocardial hypertrophy and coronary artery disease. Normally, NF- κ B is an I κ B (inhibitor of NF- κ B)-bound complex in the cytoplasm and consists of homodimers or heterodimers mainly comprising the following subunits: RelA (p65), C-Rel, NF- κ B1 (P50/P105), RelB and NF- κ B2 (P52/P100). The NF- κ B pathway may be activated by various stimuli, including growth factors, lymphokines, pharmacological agents, cytokines and stress. Upon stimulation, NF- κ B is activated and released from its I κ B-bound complex and translocates into the nucleus from the cytoplasm. This molecular process allows NF- κ B (p65) to bind to κ B sequences and alter the expression of various target genes (Werner *et al.* 2005). The same pathophysiologic processes occur in hyperglycemia-induced cardiomyocyte injuries. An increase of NF- κ B activity in high glucose (HG)-induced cardiomyocytes (Min *et al.* 2009, Tsai *et al.* 2012) and in streptozotocin (STZ)-induced diabetic rats (Chen *et al.* 2003, Mariappan *et al.* 2010) has been demonstrated. By binding to κ B sequences and altering the expression of various target genes, the NF- κ B pathway is involved in related processes, including oxidative stress (Valen *et al.* 2001, Fang *et al.* 2004, Eisner *et al.* 2006), inflammation (Fang *et al.* 2004, Westermann *et al.* 2007a,b, Arkan & Greten 2011), mitochondrial damage (Guleria *et al.* 2013), apoptosis (Guleria *et al.* 2013), endothelial dysfunction, hypertrophy and cardiac fibrosis (van Heerebeek *et al.* 2008) in DCM. Thus, NF- κ B plays a key role in the development of DCM. However, the role of NF- κ B in hyperglycemia-induced hypertrophy and cardiac fibrosis in the cardioprotective action of NRG remains unclear.

Another important molecule is the K_{ATP} channel due to its cardioprotection in various types of

cardiovascular diseases (Badalzadeh *et al.* 2014, 2015, Waza *et al.* 2014). In addition, accumulating evidence has revealed that DM has been associated with dysfunction of the cardiovascular K_{ATP} channels (Weintraub 2003). Hyperglycemia and DM impair vasodilation mediated by K_{ATP} channels in human vascular smooth muscle cells (Miura *et al.* 2003, Kinoshita *et al.* 2004, Kinoshita *et al.* 2006). Furthermore, a recent study shows that diabetes reduces mitoK_{ATP} expression and function. These changes in mitoK_{ATP} may provide an opportunity to understand the mechanisms that lead to DCM and the loss of cardioprotective mechanisms in the diabetic heart (Fancher *et al.* 2013). However, the roles of both sarcolemmal K_{ATP} channels and mitochondrial K_{ATP} channels in HG-induced cardiomyocyte injury, particularly the cardioprotective role of NRG, remain unclear.

NRG is recognized by more and more people due to its comprehensive physiological and pharmacological effects, including antioxidant (Jung *et al.* 2003, Jeon *et al.* 2004, Rajadurai & Prince 2007, Bodas *et al.* 2011, Jain & Parmar 2011, Mahmoud *et al.* 2012), antihypercholesterolemic (Jung *et al.* 2003, Kim *et al.* 2004, Jung *et al.* 2006), anti-inflammatory (Jung *et al.* 2003, 2006, Bodas *et al.* 2011, Mahmoud *et al.* 2012), cardioprotection (Huang *et al.* 2013, Chen *et al.* 2014a,b) and anti-apoptotic effects (Jagetia *et al.* 2003, Kanno *et al.* 2003, 2004, Wu *et al.* 2013). Furthermore, Huang *et al.* (2013) and Chen *et al.* (2014a,b) demonstrated that NRG inhibited the MAPK pathway (p38 MAPK, ERK1/2 and JNK) in HG-induced injuries in H9c2 cardiac cells. In recent years, many studies have shown that NRG could regulate glycolipid metabolism. Our earlier report clarified that NRG could protect against cardiomyocyte dysfunction in diabetic hearts by inhibiting NF- κ B (Wu *et al.* 2013). However, the underlying mechanisms are still unclear.

Materials and methods

Chemicals

Naringin (NRG), 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1), Hoechst 33258, STZ and pyrrolidine dithiocarbamate (PDTC, a specific inhibitor of NF- κ B pathway) were purchased from Sigma-Aldrich. Diazoxide (DZ, a mitochondrial K_{ATP} channel opener), pinacidil (Pin, a non-selective K_{ATP} channel opener), 5-hydroxydecanoic acid (5-HD, a mitochondrial K_{ATP} channel blocker) and glibenclamide (Gli, a non-selective K_{ATP} channel blocker) were

purchased from Cayman Chemical. The cell counter kit-8 (CCK-8) was supplied by Dojindo Lab (Kumamoto, Japan). Fetal bovine serum (FBS) and DMEM medium were obtained from Gibco BRL; anti-kir6.1 (R-14) was purchased from Santa Cruz Biotechnology; anti-p-NF- κ B p65 antibody, anti-p65 antibody anticaspace-3 antibody, anti-bax antibody and anti-bcl-2 antibody were purchased from Cell Signaling Technology; HRP-conjugated secondary antibody and BCA protein assay kit were obtained from KangChen Bio-tech, Inc. (Shanghai, China). Enhanced chemiluminescence (ECL) solution was purchased from KeyGen Biotech (Nanjing, China).

Experimental animals and treatment

The experimental protocol was approved by the Institutional Animal Care and Ethical Committee of Guangdong Medical College (No: 01/022/12). Sixty male Sprague–Dawley (SD) rats weighing 200 ± 10 g (7 weeks in age) were used in this experiment. Rats were housed under controlled temperature ($20 \pm 2^\circ\text{C}$) and lighting conditions (07:00 to 19:00 h, 12 h light:12 h darkness), with food and water available *ad libitum*. The high-sugar and high-fat diet comprised 66.6% basic rat chow, 20% sucrose, 10% lard, 3% egg yolk and 0.4% cholesterol. Animals were randomly divided into six groups ($n=10$ in each group): (i) the control group (healthy rats, $n=10$, mortality rate 0%): the rats were given normal saline; (ii) the STZ group (healthy rats, $n=9$, mortality rate 10%): the rats were fed a high-sugar and high-fat diet for 6 weeks and then were administered an intraperitoneal injection of 30 mg/kg/day STZ; (iii) the STZ+low-dose NRG group (STZ+L-NRG, healthy rats, $n=9$, mortality rate 10%, NRG=25 mg/kg): the rats were fed a high-sugar and high-fat diet for 6 weeks and then were administered an intraperitoneal injection of 30 mg/kg/day STZ to establish the diabetic model, followed by an intraperitoneal injection of 25 mg/kg/day NRG; (iv) the STZ+middle dose NRG group (STZ+M-NRG, healthy rats, $n=10$, mortality rate 0%, NRG=50 mg/kg): the rats were fed a high-sugar and high-fat diet for 6 weeks and then were administered an intraperitoneal injection of 30 mg/kg/day STZ to establish the diabetic model, followed by an intraperitoneal injection of 50 mg/kg/day NRG; (v) the STZ+high dose NRG group (STZ+H-NRG, healthy rats, $n=10$, mortality rate 0%, NRG=100 mg/kg): the rats were fed a high-sugar and high-fat diet for 6 weeks and then were administered an intraperitoneal injection of 30 mg/kg/day STZ to establish the diabetic model,

followed by an intraperitoneal injection of 100 mg/kg/day NRG; and (vi) the STZ+PDTC group (healthy rats, $n=10$, mortality rate 0%): the rats were fed a high-sugar and high-fat diet for 6 weeks and then were administered an intraperitoneal injection of 30 mg/kg/day STZ to establish the diabetic model, followed by an intraperitoneal injection of 100 mg/kg/day PDTC. After 6 weeks, the animals were killed (at the end of the experiments, the body weight of the various groups was recorded and the animals were anesthetized by 5% chloral hydrate) and the heart weight index determination of the ventricular myocardium was calculated. Concentration of the PDTC solution was 5 mg/mL; concentration of the NRG solution was 2 $\mu\text{g/mL}$ and concentration of the STZ solution was 10 mg/mL. Our data showed that NRG (doses from 25 to 100 mg/kg/day) and 100 mg/kg/day PDTC were non-poisonous, and the data from the NRG group and PDTC group are not shown in this paper.

H9c2 cardiac cells culture and treatment

The H9c2 cardiac cells, a rat cardiac myoblasts cell line, were obtained from Sun Yat-sen University Experimental Animal Center (Guangzhou, Guangdong, China). The cells were grown in DMEM medium supplemented with 10% FBS under an atmosphere of 5% CO_2 at 37°C and 95% air.

To investigate the role of NF- κ B pathway and K_{ATP} channels in HG-induced cardiomyocyte injury, H9c2 cells were preconditioned with 100 μM PDTC (a specific inhibitor of NF- κ B pathway) or 100 μM DZ or 50 μM Pin for 30 min before exposure to 35 mM glucose for 24 h. To explore the protective effects of NRG on HG-induced injury, the H9c2 cells were preconditioned with 80 μM NRG for 2 h before exposure to 35 mM glucose for 24 h. To further determine whether the protective effects of NRG were associated with the activation of K_{ATP} channels, the cells were preconditioned with 100 μM 5-HD or 1 mM Gli for 30 min before exposure to NRG treatment and 35 mM glucose for 24 h.

Cell viability assay

H9c2 cardiac cells were cultured in 96-well plates at a concentration of $1 \times 10^4/\text{mL}$, the CCK-8 assay was employed to measure the cell viability of H9c2 cells. After the indicated treatments, 10 μL of CCK-8 solution at a 1/10 dilution was added to every well and then the plate was incubated for 3 h in the incubator. Absorbance at

450 nm was measured with a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The means of the optical density (OD) of 4 wells in the indicated groups were used to calculate the percentage of cell viability according to the following formula: cell viability (%)=(OD treatment group/OD control group)×100%. The above experiment was repeated five times.

Hoechst 33258 nuclear staining for assessment of apoptosis

Apoptotic cell death was measured by the Hoechst 33258 staining followed by photofluorography. In brief, H9c2 cardiac cells were plated in 35 mm dishes at a density of 1×10^6 cells/well, the cells were preconditioned with 80 μ M NRG for 2 h or 100 μ M PDTC for 30 min, and subsequently exposed to 35 mM glucose for 24 h. At the end of the different treatments, H9c2 cells were cultured with 4% paraformaldehyde in 0.1 mol/L PBS (pH 7.4) for 10 min. Then the slides were washed three times with PBS; after staining with 5 mg/mL Hoechst 33258 for 30 min, H9c2 cells were washed three times with PBS, PBS was discarded and the cells were air dried. Finally, the cells were visualized under fluorescence microscope (BX50-FLA, Olympus). Viable H9c2 cells displayed a uniform blue fluorescence throughout the nucleus and normal nuclear size; however, apoptotic H9c2 cells showed condensed, fractured or distorted nuclei. The experiment was repeated three times.

Measurement of the mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was obtained using a fluorescent dye, JC-1, a cell-permeable cationic dye that preferentially enters mitochondria based on the highly negative MMP. Depolarization of MMP results in a loss of MMP from the mitochondria and a decrease in green fluorescence. H9c2 cells were cultured in a slide with DMEM at a density of 1×10^6 cells/well. After the indicated treatments, the slides were washed three times with PBS; then the cells were incubated with 1 mg/L Rh123 at 37°C for 30 min in the incubator, washed briefly three times with PBS and air-dried. Then fluorescence was measured over the hold field of vision by using fluorescent microscope connected to an imaging system (BX50-FLA). The mean fluorescence intensity (MFI) of JC-1 from three random fields was analyzed using the ImageJ 1.47i software, the MFI was taken as an index of the levels of MMP. The experiment was repeated three times.

Table 1 Myocardial biometric features of control and STZ-induced diabetic rats.

Group	Control	STZ	STZ + L-NRG	STZ + M-NRG	STZ + H-NRG	STZ + PDTC
Body weight (g)	589.20 ± 15.52	255.80 ± 26.82 ^a	386.10 ± 26.19 ^b	373.10 ± 29.00 ^b	388.10 ± 30.26 ^b	380.80 ± 17.38 ^b
Heart weight index (g)	0.00225 ± 0.00025	0.00283 ± 0.00032 ^a	0.00271 ± 0.00028 ^b	0.00257 ± 0.00026 ^b	0.00239 ± 0.00029 ^b	0.002690 ± 0.00027 ^b
Serum BNP	116.55 ± 19.60	491.28 ± 39.98 ^a	406.02 ± 23.97 ^b	374.14 ± 23.24 ^b	296.22 ± 21.98 ^b	313.01 ± 24.82 ^b
Serum glucose (mmol/L)	5.61 ± 0.99	21.48 ± 2.61 ^a	16.15 ± 4.74 ^b	15.01 ± 5.08 ^b	10.92 ± 4.20 ^b	9.92 ± 2.15 ^b
TGF-β1 (pg/mL)	147.95 ± 10.11	353.76 ± 12.15 ^a	303.10 ± 13.81 ^b	236.36 ± 17.22 ^b	215.37 ± 14.39 ^b	209.47 ± 15.56 ^b
LDH (U/L)	103.63 ± 34.67	584.59 ± 29.36 ^a	468.83 ± 56.03 ^b	403.27 ± 41.28 ^b	238.18 ± 39.82 ^b	285.74 ± 57.36 ^b
CK-MB (U/L)	89.36 ± 44.58	492.17 ± 22.04 ^a	402.46 ± 38.28 ^b	331.25 ± 19.82 ^b	197.26 ± 41.35 ^b	211.37 ± 31.65 ^b

Data are presented as the mean ± S.E.M. ^a $P < 0.01$ vs control group; ^b $P < 0.01$ vs the STZ group.

Figure 1

Naringin reduces hyperglycemia-induced activation of NF-κB p65 in H9c2 cells and in STZ-induced diabetic rats. (A and B) H9c2 cells were exposed to 35 mM glucose for the indicated times (30, 60, 90, 120, 150 and 180 min) to test the effects of HG on NF-κB p65 phosphorylation, and then H9c2 cells were exposed to 35 mM glucose for 24 h in the absence or presence of pretreatment with 80 μM NRG for 2 h before exposure to HG. Cytoplasm and nuclear extracts were extracted. The expression level of NF-κB p65 was also measured in STZ-induced diabetic rats. The expression of p65 was analyzed by Western blotting analysis. (B, C, E, G, H, K and M) Densitometric analysis of the KATP channels expression levels in (A, D, F, J and L) with ImageJ 1.47i software. (C, I and N) The p65 mRNA were tested. Data are shown as the mean ± S.E.M. (n=3). ***P* < 0.01 vs with the control group; ++*P* < 0.01 vs with the HG-treated group and the STZ group, respectively. cyto, cytoplasm; HG, high glucose, 35 mM glucose; NRG, naringin; nuc, nuclear.

Western blot assay for expressions of protein

After the indicated treatments, a small quantity of myocardial tissue of each group of rat was cut into fragments, and then homogenized with radioimmunoprecipitation assay (RIPA) buffer for protein extraction. Following the centrifugation of the lysates (14,000 *g* for 15 min at 4°C), the supernatants were quantified using a bicinchoninic acid protein assay. H9c2 cardiac cells were harvested and lysed with cell lysis solution at 4°C for 30 min. The total proteins were quantified using the BCA protein assay kit. Loading buffer was added to cytosolic extracts; after boiling for about 5 min, the same amounts of supernatant from each sample were fractionated by 10% SDS-PAGE; and then the total proteins were transferred into PVDF membranes. The membranes were blocked with 5% fat-free milk for 60 min in fresh blocking buffer (0.1% Tween 20 in TBS-T, and incubated with anticaspase-3 antibody (1:1000 dilution), anti-bcl-2 antibody (1:1000 dilution), anti-bax antibody (1:1000 dilution), anti-p65 antibody (1:1000 dilution), anti-p-NF-κB antibody (1:1000 dilution) and anti-Nox4 antibody (1:1000 dilution) in freshly prepared TBS-T with 3% free-fat milk overnight with gentle agitation at 4°C. Membranes were washed for three times with TBS-T and successively incubated with horseradish peroxidase (HRP)-conjugated goat antirabbit secondary antibody (1:2500 dilution) in TBS-T with 3% fat-free milk for 90 min at room temperature. Then the membranes were washed three times with TBS-T

for 5 min. The immunoreactive signals were subsequently visualized by using enhanced chemiluminescence (ECL) detection. In order to quantify the protein expression, the X-ray films were scanned and analyzed with ImageJ 1.47i software. The experiment was repeated three times.

Real-time PCR

Real-time PCR was carried out in 200 μL Eppendorf (EP) tubes without bacteria and enzyme. Approximately 2 μg cell total RNA, 1 μL oligo duplicated T, 1 μL diethylnitrophenyl thiophosphate and diethylpyrocarbonate liquid were placed into PCR system for reaction at 65°C for 5 min. Once the reaction ended, the liquid was taken out and put on ice. Then 4 μL 5× first strand buffer and 2 μL DL-dithiothreitol were successively added for reaction at 37°C for 2 min; after that 1 μL reverse transcriptase was added to every tube for reaction at 37°C for 50 min and then at 70°C for 15 min.

Examination of intracellular reactive oxygen species generation

Intracellular reactive oxygen species (ROS) generation was tested by the oxidative conversion of cell-permeable oxidation of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) to fluorescent DCF. H9c2 cells were cultured in a slide. With the different treatments, the slides were washed three times; 10 μM DCFH-DA solution in

Figure 2

NRG and PDTC reduce the hyperglycemia-induced decrease in the expression level of ATP-sensitive potassium (KATP) channel protein *in vitro* and *in vivo*. (A, C and E) KATP channel protein expression levels were semi-quantified by Western blotting analysis. (A, B, C and D). The cells were treated with 35 mM glucose for 24 h with or without preconditioned with 80 μ M NRG for 2 h or 100 μ M PDTC or 100 μ M diazoxide (DZ) or 50 μ M pinacidil (Pin) or 100 μ M 5-hydroxydecanoic acid (5-HD) or 1 mM glibenclamide (Gli) for 30 min. (E and F). The SD rats were treated with or without 30 mg/kg/day STZ or 25, 50 or 100 mg/kg/day NRG or 100 mg/kg/day PDTC. (B, D and F) Densitometric analysis of the KATP channels expression levels in (A) and (C). (G) The ratio of ADP/ATP was assessed using Bradbury method. Data are shown as the mean $\pm$ S.E.M. ($n=3$). ** $P<0.01$ vs control (Con) group; ++ $P<0.01$ vs the HG group or the STZ group, * P vs the HG + NRG group or HG + PDTC group.

serum-free medium was added to the slides, and then H9c2 cells were incubated at 37°C for 30 min in the incubator. The slides were washed five times with PBS, and DCF fluorescence was measured over the entire field of vision using a fluorescence microscope connected to an imaging system (BX50-FLA). The MFI of ROS from five random fields was measured using ImageJ 1.47i software and the MFI was used as an index of the amount of ROS. The experiment was repeated three times.

Measurement of SOD activity

Superoxide dismutase (SOD) activity was tested by using SOD assay kit. After the indicated treatments, cells were washed using PBS and lysed in ice-cold 0.1 M Tris-HCl (pH 7.4) containing 0.5% Triton, 5 mM β -mercaptoethanol and 0.1 mg/mL phenylmethylsulfonyl fluoride. Lysates were clarified by centrifugation at 14,000g at 4°C for 5 min and cell debris was discarded. SOD activity was detected using a commercial 'SOD Assay Kit' according to the manufacturer's protocol (Sigma). Absorbance values

at 450 nm were measured using a microplate reader. The experiment was repeated three times.

Serum cardiac markers

At the end of experimental period, rats were anesthetized with diethyl ether. Blood was collected by retro-orbital puncture and then the serum was separated. Serum levels of creatine kinase-MB isoenzyme (CK-MB) and lactate dehydrogenase (LDH) enzymes were measured by automated chemistry analyzer (Microlab 300, Merck) using reagent kits. Values of LDH and CK-MB were expressed in international units per liter (IU/L).

ELISA for detection of IL-1 β , IL-6 and TNF- α in culture supernatant

H9c2 cells were cultured in 96-well plates. After the different indicated treatments, the levels of interleukin (IL)-1 β , IL-6 and TNF- α in the culture media were tested

Figure 3

Naringin and PDTC alleviate HG-induced cardiomyocyte cytotoxicity in H9c2 cardiac cells. The cells were treated with 35 mM glucose for 24 h with or without preconditioned with 80 μ M NRG or 100 μ M PDTC or 100 μ M diazoxide (DZ) or 50 μ M pinacidil (Pin) or 100 μ M 5-hydroxydecanoic acid (5-HD) or 1 mM glibenclamide (Gli) for 30 min. Cell viability was measured by the cell counter kit (CCK-8) assay. Data are shown as the mean $\pm$ s.e.m. ($n=5$). ** $P<0.01$ vs with the control group; ++ $P<0.01$ vs with the HG group, + $P<0.01$ vs with the HG+NRG group; HG, high glucose, 35 mM glucose; NRG, naringin; PDTC, pyrrolidine dithiocarbamate, a specific inhibitor of NF- κ B pathway.

by ELISA according to the manufacturer's instruction. The experiments were repeated five times.

Assay of ATP and ADP

ATP was measured based on bioluminescence generated from luciferin–luciferase reaction. The following reaction results in the generation of measurable light at a wavelength of 562 nm:

Both intracellular ATP and ADP measurements were performed on H9c2 cardiac cells using the standard curve of ATP. Light emission was measured with a Sirius tube luminometer, Berthold Defection Systems (Germany). After calibration against the ATP standard, the ATP content of the cell lysate was determined. Then, ATP/ADP ratio was calculated.

In brief, the cells were pelleted in a microcentrifuge tube by centrifugation at 3000g for 10 min. The cellular ATP was lysated by adding 0.5 mL water and boiling the cell pellet for 5 min. The sample was then vortexed and centrifuged (3000g for 5 min at 4°C), 12 μ L supernatant was used for bioluminescence measurement. About 12 μ L of the supernatant was added to 25 μ L cocktail (luciferin and Mg) and 10 μ L of Tris buffer; 5 μ L luciferase enzyme was added to the mixture and luminescence was read at 562 nm. Luminescence intensity correlated to ATP level in hepatocytes. For total ATP measurement, 12 μ L supernatant was added to 5 μ L MgCl_2 and 5 μ L phosphoenolpyruvate and 12 μ L phosphokinase. Phosphokinase changed the ADP to ATP. The mixture was incubated for 2 min and then 25 μ L cocktail (luciferin and Mg) and 5 μ L luciferase were added to the mixture and consequently luminescence was read at 562 nm. ADP level was calculated by calculating the difference between total ATP and primary ATP.

Histopathology

The tissues were from the left ventricle. After killing the rats, their hearts were fixed in 4% formaldehyde, embedded in paraffin and sectioned. Sections were stained with hematoxylin and eosin (H&E) and examined with microscope, and photographic images were captured.

Transmission electron microscopy

The tissues were from the left ventricle. Following the commencement of the experiment, selected samples of heart tissues from rats of each group were perfused with 0.9% NaCl at 37°C, and mixed with paraformaldehyde and 4% glutaraldehyde at 4°C. Then the samples were minced into small pieces (~2 mm) and successively fixed in 2.5% glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.3) for 2 h. The specimens were then rinsed in buffer, postfixed in cacodylate-buffered 2% OsO_4 , stained in uranyl acetate, dehydrated gradiently in ethanol and embedded in epoxy resin by standard procedures. Finally, 50–70 nm superthin slices were obtained. The thin sections were stained with uranyl acetate and lead citrate and examined with a Philips TECNA10 electron microscope, and the photographic images were captured.

Statistical analysis

All data are presented as mean $\pm$ s.e.m. Differences between groups were analyzed by one-way (ANOVA) by using

Figure 4

Role of KATP channels and NF- κ B pathway in the protective effect of NRG against the hyperglycemia-induced cardiomyocyte apoptosis *in vitro* and *in vivo*. (A) Hoechst 33258 nuclear staining followed by fluorescence imaging was performed to test cell apoptosis. (B) The expression levels of cleaved caspase-3, bax and bcl-2 were semi-quantified by Western blotting assay. (A-a) Control group. (A-b) H9c2 cells were exposed to 35 mM glucose for 24 h. (A-c) H9c2 cells precondition with 80 μ M NRG for 2 h before exposure to HG for 24 h. (A-d) H9c2 cells precondition with 100 μ M PDTC for 30 min before exposure to HG for 24 h. (A-e) H9c2 cells precondition with 80 μ M NRG for 2 h. (A-f) H9c2 cells pre-treated with 100 μ M PDTC for 30 min. (A-g) The apoptotic rate was analyzed with a cell counter of the Image J 1.47i software. (B-b, c, d, f, g and h) The data in (B-a and e) was quantified by densitometric analysis with Image J 1.47i software. Data are shown as the mean $\pm$ S.E.M. ($n=3$). ** $P<0.01$ vs with the control group; + $P<0.05$, ++ $P<0.01$ vs with the HG group. HG, high glucose, 35 mmol/L glucose; NRG, naringin; PDTC, pyrrolidine dithiocarbamate, a specific inhibitor of NF- κ B pathway. White arrow: the abbreviation for nucleus.

SPSS 13.0 (SPSS) software, and followed by LSD *post hoc* comparison test. A value of $P<0.05$ was considered to be significant.

Results

General features of control and diabetic rats

Diabetic rats displayed a significantly reduced body weight and heart weight index associated with hyperglycemia compared with control rats. Diabetes also exerted an effect on the serum B-type natriuretic peptide (BNP) and transforming growth factor (TGF)- β 1 levels.

The fasting blood glucose level was markedly elevated in STZ-induced diabetic rats. The diabetes-induced changes in biometrics were significantly changed by NRG and PDTC treatment. All these results are shown in Table 1.

NRG attenuates the hyperglycemia-induced expression of NF- κ B p65 *in vitro* and *in vivo*

First, we observed the effects of HG (35 mM glucose) on NF- κ B p65 phosphorylation (p) *in vitro*. H9c2 cells were exposed to HG for the indicated times (30, 60, 90, 120, 150 and 180 min), and the expression level of p-NF- κ B p65 (p-p65) was significantly upregulated,

Figure 5

Naringin and PDTC alleviate HG-induced increased ROS generation and decrease HG-induced decreased SOD activity in H9c2 cells. A-a to b: Amplification factor: 100 $\times$. (A) DCFH-DA staining followed by photofluorography to measure intracellular ROS level. (B) SOD activity was tested by using superoxide dismutase (SOD) assay kit. (Aa) Control group. (Ab) H9c2 cells were exposed to 35 mM glucose for 24 h. (Ac) H9c2 cells were preconditioned with 80 μ M NRG for 2 h before HG for 24 h. (Ad) H9c2 cells were preconditioned with 100 μ M PDTC for 30 min before exposure to HG for 24 h. (Ae) H9c2 cells were preconditioned with 80 μ M NRG for 2 h. (Af) H9c2 cells were pre-treated with 100 μ M PDTC for 30 min. (Ag) Quantitative analysis for the mean fluorescence intensity (MFI) of DCFH-DA in (Aa to e) with the imageJ 1.47i software. Data are shown as the mean $\pm$ s.e.m. ($n=3$). ** $P<0.01$ vs with the control group; ++ $P<0.01$ vs with the HG group. HG, high glucose; NRG, naringin; PDTC, pyrrolidine dithiocarbamate, a specific inhibitor of NF- κ B pathway.

reaching a peak at 90 min (Fig. 1A and B), while the total (t)-p65 expression was unchanged. The HG-induced p65 mRNA level was increased. Then, we tested the nuclear translocation of t-p65, as shown in Fig. 1E, F and G, and the expression level of total t-p65 in the cell nucleus was greater than in the cell cytoplasm, which suggested that NF- κ B was activated and translocated to the nucleus from the cytoplasm in HG-induced cardiomyocytes.

Second, we tested the influence of NRG on the HG-induced activation of the NF- κ B pathway. H9c2 cardiac cells were pretreated with 80 μ M NRG for 2 h before exposure to HG for 24 h. As shown in Fig. 1C and D, the increased expression of p-p65 was significantly reduced by NRG pretreatment. Alone, NRG did not alter the basal expression level of p-p65 in H9c2 cells. Subsequently, we explored the effect of NRG on the nuclear translocation of the NF- κ B p65 subunit. NRG pretreatment significantly ameliorated the HG-induced nuclear translocation (Fig. 1E and G), with increasing amounts of NF- κ B p65 in the cytosol (Fig. 1E and F). Furthermore, we found that NRG inhibited the p65 mRNA level. These results suggested that NRG inhibited NF- κ B activation by blocking the HG-induced phosphorylation and nuclear translocation of p65 subunit.

In STZ-induced diabetic rats, the expression levels of p-p65 and p65 mRNA were markedly increased. The treatment of STZ-induced diabetic rats with NRG (L-NRG, M-NRG and H-NRG) or PDTC also markedly suppressed the STZ-induced increased expression level of p-p65 and the p65 mRNA level (Fig. 1H, I, J and K).

NRG and PDTC attenuate the hyperglycemia-induced decreased expression of the K_{ATP} channel protein *in vitro* and *in vivo*

As indicated in Fig. 2A, B, C and D, H9c2 cells were exposed to 35 mM glucose for 12 h, and the expression level of the K_{ATP} channel protein was obviously reduced. Importantly, the decreased expression of the K_{ATP} channel was ameliorated by the pretreatment of the cells with 80 μ M NRG for 2 h or 100 μ M PDTC for 30 min before exposure to HG for 12 h. However, the basal expression level of the K_{ATP} channel was not changed by separate treatment with 80 μ M NRG for 2 h or 100 μ M PDTC for 30 min. It was shown that the blockade of K_{ATP} channels with 5-HD or Glibenclamide markedly reversed the increased effect of NRG and PDTC against

Figure 6
Naringin and PDTC block HG-induced dissipation of MMP in H9c2 cells. (A, B, C, D, E and F) After the indicated treatments, MMP was tested by Rh123 staining followed by photofluorography. (A) Control group. (B) H9c2 cells were exposed to 35 mmol/L glucose for 24 h. (C) H9c2 cells precondition with 80 μM naringin for 2 h before HG for 24 h. (D) H9c2 cells precondition with 100 μM PDTC for 30 min before exposure to HG for 24 h. (E) H9c2 cells precondition with 80 μM naringin for 2 h. (F) H9c2 cells pre-treated with 100 μM PDTC for 30 min. (G) Quantitative analysis for the mean fluorescence (MFI) of Rh123 in (A, B, C, D, E and F) with the imageJ 1.47i software. Data are shown as the mean ± s.e.m. (n = 3). **P < 0.01 vs with the control group; ++P < 0.01 vs with the HG-treated group. NRG, naringin; PDTC, pyrrolidine dithiocarbamate, a specific inhibitor of NF-κB pathway.

the HG-induced decreased expression of K_{ATP} channel proteins (Fig. 2B). Similarly, compared with the control group, the expression level of the K_{ATP} channel protein was obviously reduced in STZ-induced diabetic rats. However, the STZ-induced decreased expression level of K_{ATP} channel proteins increased to different

degrees in the STZ + L-NRG group, STZ + M-NRG group, STZ + H-NRG group and STZ + PDTC group. These data indicated that NRG and PDTC alleviated the decrease in the expression level of K_{ATP} channels induced by HG in H9c2 cardiac cells.

Furthermore, we observed the effects of HG (35 mM glucose) on the ADP/ATP ratio *in vitro*. H9c2 cells were exposed to HG for 2 h, and the ADP/ATP ratio was significantly upregulated (Fig. 2G). However, the upregulated ADP/ATP ratio was markedly repressed by pretreatment with 80 μM NRG for 2 h or 100 μM PDTC for 30 min before exposure to HG. Alone, NRG or PDTC did not affect the ADP/ATP ratio of H9c2 cardiac cells.

The NF-κB pathway and K_{ATP} channels are involved in the protective effect of NRG against HG-induced cardiomyocyte cytotoxicity in H9c2 cardiac cells

As shown in Fig. 3A, the exposure of H9c2 cells to HG for 24 h obviously induced cytotoxicity, which led to a decrease in cell viability. However, the decreased cell viability was markedly repressed by pretreatment with 80 μM NRG for 2 h or 100 μM PDTC for 30 min before exposure to HG. Alone, NRG or PDTC did not affect the viability of H9c2 cardiac cells.

To explore the role of K_{ATP} channels in HG-induced cytotoxicity, the cells were treated with 100 μM DZ or 50 μM Pin for 30 min before exposure to HG. As shown in Fig. 3B, pretreatment of H9c2 cardiac cells with DZ or Pin considerably mitigated the HG-induced cytotoxicity, as evidenced by an increase in cell viability. To further investigate the role of K_{ATP} channels in the protective effect of NRG against HG-induced cytotoxicity, the cells were treated with 100 μM 5-HD or 1 mM Gli for 30 min before exposure to NRG and HG. It was shown that the blockade of K_{ATP} channels with 5-HD or Gli markedly reversed the protective effect of NRG against the HG-induced cytotoxicity, which resulted in a decrease in cell viability (Fig. 3B). Alone, 5-HD or Gli did not significantly alter the cell viability. These data suggested that K_{ATP} channels mediated the anticytotoxicity of NRG in the HG-treated H9c2 cardiac cells.

NRG and PDTC suppress hyperglycemia-induced cardiomyocyte apoptosis *in vitro* and *in vivo*

In Fig. 4A-b and g, exposure of H9c2 cells to 35 mM glucose for 24 h induced typical apoptosis, which was manifested

Figure 7

Naringin and PDTC alleviate hyperglycemia-induced production of interleukin (IL)-1 β , IL-6 and tumor necrosis factor- α (TNF- α) *in vitro* and *in vivo*. The cells were treated with 35 mM glucose for 24 h with or without preconditioned with 80 μ M NRG for 2 h or 100 μ M PDTC. The SD rats were treated with or without 30 mg/kg/day STZ or 25, 50 or 100 mg/kg/day NRG or 100 mg/kg/day PDTC. Enzyme-linked immunosorbent assay (ELISA) was performed to determine the levels of (A and B) IL-1 β , (C and D) IL-6, and (E and F) TNF- α in cell supernatants. Data are shown as the mean $\pm$ s.e.m. ($n=5$). ** $P<0.01$ vs the control (Con) group; ++ $P<0.01$ vs the HG-treated group.

as the nuclear condensation and fragmentation condensation of chromatin and the shrinkage of nuclei and apoptotic bodies. However, pretreatment of the cells with 80 μ M NRG for 2 h of HG exposure obviously mitigated the HG-induced increase in the number of cells that underwent apoptosis (Fig. 4A-c and d). In addition, preconditioning of the cells with 100 μ M

PDTC for 30 min before exposure to HG also ameliorated HG-induced apoptosis of cardiac cells (Fig. 4A-d and g). Alone, NRG (Fig. 4A-e and g) or PDTC (Fig. 4A-f and g) did not significantly change the number of apoptotic H9c2 cells. Similarly, both in HG-induced H9c2 cardiac cells and in STZ-induced rats, the expression levels of cleaved caspase-3 and bax were markedly enhanced, and

200 X

400 X

Figure 8

The effects of naringin and PDTC on heart tissues changes of STZ-induced diabetic rats. (200x: A→F, 400x: G→L). A and G: Control group; B and H: STZ-induced diabetic rats group; C and I: STZ+L-NRG group, STZ-induced diabetic rats treatment with low-dose NRG; D and J: STZ+M-NRG group, STZ-induced diabetic rats treatment with middle-dose NRG; E and K: STZ+H-NRG group, STZ-induced diabetic rats treatment with high-dose NRG; F and L: STZ+PDTC group, STZ-induced diabetic rats treatment with PDTC. STZ, streptozotocin; NRG, naringin; PDTC, pyrrolidine dithiocarbamate, a specific inhibitor of NF- κ B pathway.

the expression level of bcl-2 was decreased. However, the increased expression levels of cleaved caspase-3 and bax were attenuated by pretreatment with 80 μ M NRG for 2 h or 100 μ M PDTC for 30 min (STZ+NRG treatment rats or PDTC treatment rats, respectively), and the decreased expression level of bcl-2 was upregulated. Alone, NRG or PDTC did not significantly affect the basal expression level of cleaved caspase-3, bax and bcl-2.

NRG and PDTC diminish HG-induced increased oxidative stress in H9c2 cardiac cells

This study demonstrated that oxidative stress contributed to HG-induced cardiomyocyte injury

(Chen *et al.* 2014a,b). As shown in Fig. 5, exposure of H9c2 cells to 35 mM glucose for 24 h markedly induced oxidative stress, as evidenced by an increase in the generation of ROS (Fig. 5A-b and g), a decrease in the SOD activity (Fig. 5B) and an increased expression level of Nox4 (Fig. 5C). However, pretreatment of the cells with 80 μ M NRG for 2 h before HG exposure obviously mitigated the HG-induced increase in ROS generation (Fig. 5A-c and g), increased the HG-induced decrease in SOD activity (Fig. 5B) and decreased the expression level of Nox4 (Fig. 5C). To test whether the NF- κ B signaling pathway is involved in HG-induced oxidative stress, H9c2 cells were pretreated with 100 μ M PDTC for 30 min before exposure to HG. Our data showed that pretreatment with PDTC

x5800

x13,500

Figure 9

The effects of NRG and PDTC on ultrastructure changes of STZ-induced diabetic rats heart tissues (5800 $\times$: A $\rightarrow$ F, 13,500 $\times$: G $\rightarrow$ L). A and G: Control group; B and H: STZ-induced diabetic rats group; C and I: STZ+L-NRG group, STZ-induced diabetic rats treatment with low-dose NRG; D and J: STZ+M-NRG group, STZ-induced diabetic rats treatment with middle-dose NRG; E and K: STZ+H-NRG group, STZ-induced diabetic rats treatment with high-dose NRG; F and L: STZ+PDTC group, STZ-induced diabetic rats treatment with PDTC. STZ, streptozotocin; NRG, naringin; PDTC, pyrrolidine dithiocarbamate, a specific inhibitor of NF- κ B pathway. Black arrow: swollen mitochondria, white arrow: vacuolar mitochondria.

considerably diminished the generation of ROS (Fig. 5A-d and g), upregulated SOD activity (Fig. 5B) and inhibited Nox4 expression (Fig. 5C). NRG or PDTC treatment alone did not change the basal level of ROS, SOD activity and Nox4 expression in H9c2 cells.

NRG and PDTC block the HG-induced dissipation of MMP in H9c2 cells

As with oxidative stress, mitochondrial damage has been shown to be involved in HG-induced cardiomyocyte

injury (Chen *et al.* 2014a,b, Xu *et al.* 2015). It was shown that exposure of the cells to 35 mM glucose for 24 h elicited mitochondrial damage, as manifested by the dissipation of MMP (Fig. 6B and G). The dissipation of MMP was reduced by pretreatment of the cells with 80 μ M NRG for 2 h before exposure to HG (Fig. 6C and G), which showed that NRG protected H9c2 cells against HG-induced mitochondrial damage. Similarly, the pretreatment of cardiomyocytes with 100 μ M PDTC for 30 min before exposure to HG similarly attenuated the HG-induced dissipation of MMP (Fig. 6D and G).

NRG and PDTC suppress the hyperglycemia-induced production of pro-inflammatory cytokines by inhibiting the NF- κ B pathway in H9c2 cardiac cells and in STZ-induced diabetic rats

As shown in Fig. 7, the levels of IL-1 β (Fig. 7A and B), IL-6 (Fig. 7C and D) and TNF- α (Fig. 7E and F) were markedly increased in hyperglycemia-induced H9c2 cardiac cells and in STZ-induced diabetic rats compared with the control group ($P < 0.01$). However, these increased levels of IL-1 β , IL-6 and TNF- α were significantly suppressed by treatment with NRG, which suggested an inhibitory effect of NRG on the production of pro-inflammatory cytokines, including IL-1 β , IL-6 and TNF- α , induced by hyperglycemia. Similarly, the treatment of H9c2 cells with PDTC, a specific inhibitor of the NF- κ B pathway, depressed the enhanced production of IL-1 β , IL-6 and TNF- α .

The effects of NRG and PDTC on structural changes of the heart tissues of STZ-induced diabetic rats by H&E staining

H&E staining of the heart tissues showed that compared with the control group, diabetic hearts displayed structural abnormalities, such as the degeneration of cardiac myofibrils, a marked separation of myocardial fibers from each other, and hemorrhagic areas (Fig. 8B and H). These structural abnormalities in the heart of STZ diabetic animals were at least partially prevented by L-NRG (Fig. 8C and I), M-NRG (Fig. 8D and J), H-NRG (Fig. 8E and K) or PDTC (Fig. 8F and L) treatment.

The effects of NRG and PDTC on ultrastructural changes in the heart tissues of STZ-induced diabetic rats by transmission electron microscopy analysis

The myocardial structure was examined by transmission electron microscopy. In the control group, well-organized, typical symmetric myofibrils and packed mitochondria beside the fibers were observed, the mitochondria were oval in shape, the ridge was closely spaced with the intact membrane, and the gap junctions were visible and dense. The STZ-induced diabetic rat heart tissues showed loss and damage of myofibrils compared with the control group, along with a significantly decreased volume of myofibrils. As shown in Fig. 9B, the mitochondria were swollen, fragmented, distended and clumped, and the cristae in the mitochondria were distorted or completely lysed. In addition, some disks were distorted and ruptured. After treatment with 25 mg/kg/day NRG

(low-dose NRG) (Fig. 9C and L), 50 mg/kg/day NRG (middle-dose NRG) (Fig. 9D and J), 100 mg/kg/day NRG (high-dose NRG) (Fig. 9E and K) or 100 mg/kg/day PDTC (Fig. 9D), the STZ-induced diabetic rats exhibited improved cardiac structure. The damage to myofibrils was reversed, and the volume percentage of myofibrils was increased. The swelling, fragmentation, distension and clumping of mitochondria were suppressed, and the number of STZ-induced injuries in mitochondria was also decreased. There was limited rupture or dissolution of the fibrin in the sarcomere, and the cross-connections of the intercalated disk were also clear.

Discussion

In this study, we demonstrate that the NF- κ B pathway, which regulates cardiac cell apoptosis, oxidative stress, mitochondrial damage, the production of pro-inflammatory cytokines, myocardial hypertrophy, myocardial remodeling and cardiac fibrosis in DM, is a crucial regulatory mechanism for the development of DCM. Otherwise, K_{ATP} has an important role in the HG-induced injury of H9c2 cardiac cells. More importantly, we provide new insight into the mechanisms responsible for the cardioprotection of NRG against hyperglycemia-induced cardiac cell injuries by inhibiting the activation of NF- κ B pathway *in vitro* and *in vivo* and upregulating K_{ATP} channels *in vitro*.

In this study, p65 was our target to test the effect of the NF- κ B pathway in STZ-induced diabetic rats and in HG-induced injuries to H9c2 cardiac cells. Similar to previous studies (Valen *et al.* 2001, Fang *et al.* 2004, Eisner *et al.* 2006, Westermann *et al.* 2007a,b, Mariappan *et al.* 2010, Arkan & Greten 2011), our findings showed that hyperglycemia could increase the expression level of p-NF- κ B p65 (Fig. 1). Furthermore, NF- κ B was activated and dissociated from I κ B and released and translocated into the nucleus from the cytoplasm, which led to an increase in the expression level of nuclear (nuc) p65 (Fig. 1). This allowed NF- κ B (p65) to bind to κ B sequences in DNA and alter the expression of various target genes (Rajadurai & Prince 2009). These results strongly indicated that the NF- κ B pathway was activated and might be involved in the pathogenesis of DCM. In our present research, we found that the NF- κ B pathway was involved in hyperglycemia-induced cytotoxicity (Fig. 3A), apoptosis (Fig. 4), oxidative stress (Fig. 5), dissipation of MMP (Fig. 6), inflammation (Fig. 7) and myocardial remodeling (Figs 8 and 9). These findings demonstrated that the activation of

NF- κ B plays a crucial role in the development of DCM in diabetic patients by controlling different set of genes and participating in the related processes. Moreover, cardiac K_{ATP} channels are very important for the metabolic status of cardiomyocytes as key sensors and effectors, and their roles in the HG-induced cardiomyocyte injury have conspicuously attracted attention. First, in this study, our data showed that exposure of the cardiac cells to HG prominently reduced the expression level of K_{ATP} channels, which suggested that K_{ATP} channels may be involved in the progress of HG-induced cardiac cell injuries. A recent study indicated that a reduction in number of sarcolemmal K_{ATP} channels affects cardiac action potentials under hypoxia (Zhu *et al.* 2011); therefore, we hypothesized that the inhibition of cardiac K_{ATP} channels might be a pivotal mechanism underlying HG-induced cardiomyocyte injury. To confirm our hypothesis, we secondarily observed the influence of K_{ATP} channel activation on HG-induced injury. In agreement with our recent studies (Zhu *et al.* 2011, Chen *et al.* 2014a,b), the findings of this study showed that treatment of H9c2 cardiac cells with HG induced considerable injuries, including a decrease in cell viability. However, pretreatment of H9c2 cardiac cells with DZ or Pin markedly attenuated the HG-induced injuries, as evidenced by an increase in cell viability. The above results suggest that HG impairs the function of cardiac K_{ATP} channels, which contributes to HG-induced injuries. Thus, the above results strongly suggested that NF- κ B and K_{ATP} channels might play critical roles in the pathogenesis of DCM.

An important finding of this study relates to the cardioprotection of NRG in hyperglycemia-induced insults *in vitro* and *in vivo*. First, consistent with the previously reported results (Huang *et al.* 2013), we found that NRG markedly suppressed HG-triggered cytotoxicity and apoptosis, and it was suggested that NRG contributed to cardioprotection by attenuating mitochondrial dysfunction and inhibiting the activation of the p38 MAPK pathway. Our results are supported by a series of previous studies (Jagetiya *et al.* 2003, Kanno *et al.* 2003, 2004, Wu *et al.* 2013). Second, we observed that NRG could exert remarkable antihyperglycemic effects (Table 1) in STZ-induced diabetic rats. Its mechanism has multiple components: (i) NRG activates glutathione (GSH) synthesis through a novel antioxidant defense mechanism against excessive ROS production, which contributes to the prevention of oxidative damage in addition to its effect on glycemic control (Dhanya *et al.* 2015); (ii) NRG plays important roles in preventing the progression of hyperglycemia, partly by binding to starch by increasing hepatic glycolysis and the glycogen concentration and

lowering hepatic gluconeogenesis (Shen *et al.* 2012); (iii) NRG exhibits antidiabetic effects in a rat model of type 2 diabetes mellitus (T2DM) by potentiating the antioxidant defense system and suppressing pro-inflammatory cytokine production (Mahmoud *et al.* 2012). Third, this study demonstrates the inhibitory effect of NRG on myocardial remodeling and cardiac fibrosis in STZ-induced diabetic rats by inhibiting the NF- κ B pathway. Fourth, our study shows the inhibitory effect of NRG on hyperglycemia-induced NF- κ B activation. Currently, the effects of NRG against hyperglycemia-induced cardiac injury in DM have attracted considerable attention due to its cardioprotective effect (Kanno *et al.* 2004, Rajadurai & Prince 2007, 2009). Our more recent study showed that NRG could protect cardiomyocytes against HG-induced injuries by inhibiting the activation of MAPKs (Jung *et al.* 2003) and the leptin-p38 MAPK (Chen *et al.* 2014a,b) pathway in H9c2 cells. However, the mechanisms underlying the effect of NRG on the NF- κ B pathway remain unclear. Thus, this study explored the effect of NRG on the activation of the NF- κ B pathway in HG-treated H9c2 cardiac cells and in STZ-induced diabetic rats. As in our previous study (Wu *et al.* 2013), NRG considerably suppressed the expression levels of p-p65, which suggested that NRG could inhibit the hyperglycemia-induced activation of NF- κ B *in vitro* and *in vivo*. These results suggested that the NF- κ B pathway might play a critical role in the cardioprotection of NRG.

However, the protective mechanisms of NRG are complicated, and other factors may also be involved in the cardioprotection of NRG. Interestingly, the results of this study relate to the role of the activation of K_{ATP} channels in the cardioprotective effects of NRG against HG-induced cardiac injuries. Our results demonstrated that NRG markedly reduced the downregulation of K_{ATP} channel expression by HG and that both 5-HD and Glibenclamide significantly blocked the cardioprotective effect of NRG mentioned above. These results suggested that K_{ATP} channels, particularly the mitochondrial K_{ATP} channels, might play a critical role in the cardioprotection of NRG.

In this study, we also investigated the relationship between the NF- κ B pathway and cardiac K_{ATP} channel activation. As mentioned above, PDTC, an inhibitor of the NF- κ B pathway, markedly blocked a decrease in the expression of K_{ATP} channel proteins induced by HG. Collectively, these results revealed that K_{ATP} channels might be a downstream molecule of the NF- κ B pathway in HG-treated cardiac cells. Elucidating the molecular mechanism underlying this interaction may be significant in the treatment and prevention of DM-related DCM.

Exposing H9c2 cells to HG for 12h markedly decreased the expression level of K_{ATP} channel proteins and increased the ADP/ATP ratio. An increase in the ATP/ADP ratio closes K_{ATP} channels (which leads to depolarization), whereas a decrease in the ATP/ADP ratio opens K_{ATP} channels (which leads to hyperpolarization) (Sun & Feng 2013). These changes suggest that HG both opened and inhibited the expression level of K_{ATP} channels. It is difficult to explain this phenomenon. Its potential mechanism requires further research.

Therefore, the above findings showed that the activation of NF- κ B is involved in hyperglycemia-induced cytotoxicity, apoptosis, ROS production, the dissipation of MMP, myocardial remodeling and myocardial fibrosis *in vitro* and *in vivo*. HG impairs the function of cardiac K_{ATP} channels, which contribute to HG-induced injuries. Our results revealed that the inhibitory effect of NRG on the activation of NF- κ B might be one of the important mechanisms responsible for its protection against hyperglycemia-induced injuries in H9c2 cardiac cells and in STZ-induced type 2 diabetic rats.

Conclusions

In conclusion, our study demonstrated that NRG, which could protect cardiomyocytes against hyperglycemia-induced injury *in vivo* and *in vitro*, had antimitochondrial, anti-oxidant, anti-apoptosis, anti-inflammatory and antifibrosis activities at least partially by inhibiting the NF- κ B pathway and upregulating K_{ATP} channels. These results also raise the possibility that NRG may be a potential candidate in the prevention and treatment of diabetic vascular complications at an appropriate dosage.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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