Naringin inhibits high glucose-induced cardiomyocyte apoptosis by attenuating mitochondrial dysfunction and modulating the activation of the p38 signaling pathway

HAILI HUANG1*, KENG WU2*, QIONG YOU2, RUINA HUANG2, SHANGHAI LI2 and KENG WU2

1Clinical Research Center and 2Department of Cardiology, Affiliated Hospital of Guangdong Medical College, Zhanjiang, Guangdong 524001, P.R. China

Received January 11, 2013; Accepted March 7, 2013

DOI: 10.3892/ijmm.2013.1403

Abstract. Recently, naringin (NAR; 4',5,7-trihydroxyflavanone-7-rhamnoglucoside) has been shown to have cardioprotective properties. However, the specific mechanisms underlying its cardioprotective effects remain unclear. In this study, we aimed to investigate the cardioprotective effects of NAR and the possible underlying molecular mechanisms in cardiomyocytes using high glucose (HG) to induce apoptosis in H9c2 cells. The effect of NAR on apoptosis was assessed by Annexin V and propidium iodide staining, and by determining the levels of active caspase-3, -8 and -9. The effect of NAR on mitochondrial dysfunction was assessed by the loss of mitochondrial membrane potential (MMP). Our results demonstrated that exposure to HG induced apoptosis and mitochondrial dysfunction in cardiomyocytes. Treatment with NAR significantly increased MMP and inhibited the activation of caspase-3, -8 and -9. NAR attenuated the HG-induced p38 and p53 phosphorylation, decreased mitochondrial Bax and Bak expression, prevented the release of cytochrome c and increased Bcl-2 expression. Pre-treatment with SB203580, a p38 inhibitor, also suppressed p53 phosphorylation and prevented the loss of MMP, as well as apoptosis in the HG-treated H9c2 cells. Taken together, these data demonstrate that NAR inhibits HG-induced apoptosis by attenuating mitochondrial dysfunction and modulating the activation of the p38 signaling pathway.

Introduction

Over the last few years, studies have demonstrated that apoptosis is an early event involved in cardiomyopathy associated with diabetes mellitus (1). A key pathological consequence of sustained hyperglycemia is the induction of cardiomyocyte apoptosis in diabetic patients and animal models of diabetes (2,3). Several clinical studies have demonstrated that elevated blood glucose levels are a risk factor for the development of diabetic cardiomyopathy (4-7). Sustained hyperglycemia can produce a large number of oxygen free radicals, which can induce the apoptosis of cardiomyocytes and lead to oxidative myocardial injury. Cardiomyocyte apoptosis causes a loss of contractile units which reduces organ function and provokes cardiac remodeling, which is associated with the hypertrophy of viable cardiomyocytes (8,9). As such, should myocardial apoptosis be inhibited, one would expect to prevent or slow the development of heart failure. Yet, the means by which hyperglycemia induces apoptosis in cardiomyocytes have not yet been fully elucidated.

Apoptosis is a major mechanism of cell death, characterized by a series of tightly regulated processes involved in the activation of a cascade of molecular events leading to cell death. Cells undergoing apoptosis have been shown to have elevated levels of cytochrome c in the cytosol and a corresponding decrease in mitochondrial potential (10). Bcl-2 exerts a pro-survival effect in response to a wide range of apoptotic stimuli through the inhibition of mitochondrial cytochrome c release (11,12). Bak is a pro-apoptotic member of the Bcl-2 family. This protein is located on the outer membrane of the mitochondria and is an essential component for the transduction of apoptotic signals through the mitochondrial pathway (13-15). Bak is a key component for cellular-induced apoptosis through mitochondrial stress. Upon apoptotic stimulation, Bax forms oligomers and translocates from the cytosol to the mitochondrial membrane. Through interactions with pore proteins on the mitochondrial membrane, Bax increases the permeability of the membrane, which leads to the release of cytochrome c from the mitochondria, the activation of caspase-9 and the initiation of the caspase activation pathway for apoptosis (13-15).

Cardiomyocyte apoptosis is a multifactorial process involving the activation of several signal transduction pathways, including the p38 mitogen-activated protein kinase (MAPK) pathway. It has been reported that p38 activation induces p53-dependent apoptosis (16). The inhibition of p38 phosphorylation prevents the activation of p53 (Ser15 phosphorylation...
and expression), suppresses Bax expression and inhibits the cleavage of caspase-3 and cardiomyocyte apoptosis.

The flavonoid naringin (NAR; 4',5,7-trihydroxyflavanone-7-rhamnoglucoside) is a polyphenolic compound that naturally occurs in citrus fruit. NAR has previously been shown to have anti-inflammatory (17,18), antioxidant (19,20), anticancer (21), antibacterial (22), antimutagenic (23), antiulcer, antitussive (24,25), neuroprotective (26-28), as well as anti-hypertensive properties (29), and can reduce blood cholesterol levels, preventing thrombosis (30). In recent years, it has been reported that NAR inhibits the apoptosis of hydrogen peroxide-induced p388 cells (31) and Rotenone-induced human neuroblastoma SH-SY5Y cells (32). However, whether NAR can inhibit high glucose (HG)-induced cardiomyocyte apoptosis has not yet been reported. Therefore, this study aimed to investigate the effects of NAR on HG-induced cardiomyocyte apoptosis at the cellular and molecular level. We hypothesized that NAR can inhibit cardiomyocyte apoptosis by attenuating mitochondrial dysfunction through the mitochondrial signaling pathway and inhibiting p38 activation.

Materials and methods

Reagents. All reagents were purchased from Sigma Inc. (St. Louis, MO, USA) unless otherwise stated. JC-1 was obtained from Beyotime Biotechnology, Inc. (Nantong, China).

Cell culture and treatments. H9c2 cells were obtained from the Sun Yat-sen University Experimental Animal Centre (Guangzhou, China). The H9c2 cell line, a subclone of the original clonal cell line, was derived from embryonic rat heart tissue. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with normal glucose (5.5 mM) and 15% FBS at 37°C under an atmosphere of 5% CO₂ and 95% air.

H9c2 cells were incubated in DMEM with HG (16.7 mM) for 6 h to induce apoptosis. NAR (5 µM) was added into the above medium (DMEM) to examine its cytoprotective effects. The selective p38 inhibitor, SB203580 (10 µM), was administered to the H9c2 cells for 1 h before treatment with HG (16.7 mM) for 6 h in the presence or absence of NAR (5 µM).

Caspase-3, -8 and -9 activity assessment. To measure caspase-3, -8 and -9 enzymatic activity, the H9c2 cells were cultured for 24 h followed by treatment with HG (16.7 mM) for 6 h in the presence or absence of NAR (5 µM). APO LOGIX Carboxyfluorescein Caspase Detection kits (Cell Technology, Inc., Mountain View, CA, USA) were used to detect active caspase-3, -8 and -9 according to the manufacturer's instructions.

Detection of mitochondrial membrane potential (MMP). The MMP detection kit (JC-1; Beyotime Biotechnology Research Institute) was used to detect MMPs. Cells were washed once with PBS and then 1 ml cell culture medium containing serum and phenol red was added. JC-1 dye working solution (1 ml) was then added followed by mixing. The cells were subsequently incubated in a cell incubator at 37°C for 20 min, and then washed with JC-1 staining buffer (1X) twice. Cell culture medium (2 ml) was then added and the cells were observed under fluorescence or confocal microscope. The aggregate JC-1 (red fluorescence) was detected at an emission wavelength of 590 nm, and the monomeric JC-1 (green fluorescence) was monitored at 529 nm. The ratio of aggregated and monomeric JC-1 was used to quantify changes in MMP, and a decreased JC-1 ratio represented the depolarization of the mitochondria, indicating a decrease in MMP.

Western blot analysis. Following treatment, the H9c2 cells were harvested and lysed with ice-cold cell lysis solution and the homogenate was centrifuged at 10,000 x g for 15 min at 4°C. Total protein in the supernatant was quantified using a BCA protein assay kit. Total protein (20 µg) from each sample was separated by 12% SDS-PAGE and transferred onto a PVDF membrane, which was then exposed to X-ray film, and processed using an AlphaImager HP fluorescence/visible light gel imaging analyzer. Image analysis software were used to analyze the gray value.

Detection of apoptosis. As stated in the KGI Annexin V-FITC apoptosis detection kit instruction manual, the cells were digested with trypsin, and centrifuged at 1,000 rpm for 5 min. After collection, the cells were washed twice with PBS, and centrifuged at 1,000 rpm for 5 min. A total of 5x10⁶ cells were collected and suspended in 500 µl binding buffer. Subsequently, 5 µl Annexin V-FITC and 5 µl propidium iodide (PI) were added followed by mixing at room temperature, in the dark for 15 min. Within 1 h, the apoptotic cells were detected by flow cytometry. The excitation wavelength was 488 nm and the emission wavelength was 530 nm. Green fluorescence of Annexin V-FITC was detected using the FITC channel (FL1); PI red fluorescence was detected using the PI channel (FL2 or FL3). For fluorescent compensation adjustment, normal cells not treated with HG to induce apoptosis were used as the controls for fluorescence compensation settings adjustment.

Statistical analysis. The results of each experimental condition were determined from the mean of triplicate trials. Data are expressed as the means ± SEM (n=6 unless otherwise stated). A two-tailed Student's t-test was used to assess the significance of differences between two groups. Analysis of variance was used when comparing more than two groups; differences between two groups within the set were analyzed by a Fisher's protected least-significant differences test. P-values <0.05 were considered to indicate statistically significant differences.

Results

NAR decreases HG-induced H9c2 cell apoptosis. To investigate the effect of NAR on HG-induced cardiomyocyte apoptosis, Annexin V and PI staining was performed in the H9c2 cells treated with HG (16.7 mM) in the absence or presence of NAR. Exposure to HG induced apoptosis in H9c2 cells and the apoptotic rate was 46.5±8.5 in the HG group (Fig. 1). NAR decreased...
the apoptotic rate to 25.6±5.9 in the HG + NAR group. No apoptosis was observed in the cells treated with NAR alone.

Caspase-3, -8 and -9 activity was measured to evaluate the effect of NAR on HG-induced cardiomyocyte apoptosis. Caspase-3 activity was significantly higher 6 h following exposure to HG in the HG group compared with the control group (Fig. 1). Following the addition of 5 µM NAR, caspase-3 activity markedly decreased in the HG + NAR group compared with the HG group. Caspase-9 activity showed a significant increase 6 h following exposure to 16.7 mM glucose, which was almost completely attenuated in the presence of 5 µM NAR. However, no evident changes were observed in the activity of caspase-8 6 h following stimulation with HG and/or NAR.

NAR prevents HG-induced MMP loss in H9c2 cells. The MMP of H9c2 cells was measured by JC-1, an indicator of mitochondrial function, in the H9c2 cells treated with glucose (16.7 mM) in the absence or presence of NAR (5 µM) for 6 h. Red fluorescence represents the mitochondrial aggregate JC-1 and green fluorescence indicates the monomeric JC-1. Quantification of the fluorescence intensity is shown in the histograms. The results from different treatment groups were normalized by a random setting for the control value as 100%. The graph represents the ratio of aggregated and monomeric JC-1, indicating changes in MMP (n=4). **P<0.01 compared with control group. *P<0.05, compared with glucose group.

Mitochondrial pathway is involved in the inhibitory effect of NAR on HG-induced H9c2 cell apoptosis. It has been reported that the HG-induced apoptosis of cardiomyocytes
is a multifactorial process. In this study, we examined Bcl-2, cytosolic cytochrome c, mitochondrial Bax and Bak levels in H9c2 cells. Following exposure to 16.7 mM glucose for 6 h, cytosolic cytochrome c, mitochondrial Bax and Bak levels significantly increased compared with the controls (Fig. 3). By contrast, cytosolic cytochrome c, mitochondrial Bax and Bak levels decreased following treatment with NAR in the cardiomyocytes exposed to HG. HG (16.7 mM) reduced Bcl-2 expression in the H9c2 cells, which was significantly elevated following treatment with NAR. These results suggest that the mitochondrial pathway is involved in the inhibitory effects of NAR on HG-induced H9c2 cell apoptosis.

p38 signaling pathway is involved in the inhibitory effects of NAR on HG-induced H9c2 cell apoptosis. Previous studies have demonstrated that p38 and p53 activation leads to cardiomyocyte apoptosis. Thus, in this study, we measured the protein levels of p-p53 and p53, as well as those of p38 and p-p38 were determined by western blot analysis (n=3). β-actin was used as the internal control. **P<0.01 compared with the control group. *P<0.05, **P<0.01 compared with the glucose group.
In order to determine the role of p38 in HG-induced H9c2 cell apoptosis, the H9c2 cells were pre-treated with the p38 inhibitor, SB203580 (10 µM), for 90 min and then exposed to HG (16.7 mM) and/or NAR (5 µM) for 6 h. p38 and p53 phosphorylation were measured by western blot analysis. β-actin was used as the internal control.

The results from western blot analysis demonstrated that SB203580 significantly inhibited p38 phosphorylation and in turn, p53 phosphorylation, but did not alter the inhibitory effects of NAR on p38 and p53 phosphorylation in the HG-challenged H9c2 cells. Moreover, SB203580 inhibited the HG-induced H9c2 MMP loss in the HG + SB group, but did not alter the inhibitory effects of NAR in the HG + NAR + SB group (Fig. 6).
Discussion

Diabetic cardiomyopathy is related directly to hyperglycemia (6). Cell death (apoptosis) plays a critical role in cardiac pathogenesis. Hyperglycemia induces myocardial apoptosis and leads to diabetic cardiomyopathy (6). Cardiomyocyte apoptosis and mitochondrial dysfunction are the pathophysiological basis of diabetic cardiomyopathy, and can significantly increase the incidence of heart failure (6–8). Therefore, it is of great significance to elucidate the molecular mechanisms involved in cardiomyocyte apoptosis, in order to develop novel and effective clinical methods to prevent this event. Although a variety of signaling pathways have been confirmed to participate in the development of cardiomyocyte apoptosis, the inherent mechanisms involved in cardiomyocyte apoptosis and in the reversal of mitochondrial dysfunction remain unclear.

NAR (4’,5,7-trihydroxyflavone-7-rhamnoglucoside) is derived from grapefruit and related citrus species and has been evaluated for a wide spectrum of activities, including antioxidant (19,20,33), anticancer (21,34), anti-inflammatory (35) and cardioprotective activities (30,36,37). It has been reported to lower glucose levels and elevate plasma insulin levels in rats with streptozotocin (STZ)-induced diabetes (33,38). However, the role of NAR in diabetic complications has not yet been elucidated. In this study, we examined the hypothesis that NAR inhibits HG-induced apoptotic cell death in cardiomyocytes by attenuating mitochondrial dysfunction and modulating the activation of the p38 signaling pathway.

The mitochondrial pathway of apoptosis is dependent upon the Bcl-2 family for the release of pro-apoptotic factors (cytochrome c) from the mitochondrial intermembrane space (IMS), via the process of mitochondrial outer-membrane permeabilization (MOMP) (13,15). The Bcl-2 family of proteins are important regulators of the mitochondrial pathway of apoptosis (14,39). The multi-BH-domain-containing pro-apoptotic members, Bax and Bak, are the essential gateways to MOMP. They directly engage MOMP by creating proteolipid pores responsible for the release of cytochrome c (13,15). This release of cytochrome c in turn activates caspase-9, a cysteine protease. Caspase-9 in turn activates caspase-3 and -7, which are responsible for destroying the cell from within (40). In this study, the exposure of cardiomyocytes to HG induced apoptosis and mitochondrial dysfunction. Treatment with NAR significantly decreased the expression of apoptosis-related proteins (mitochondrial Bax and Bak, cytoplasmic cytochrome c), while it increased the expression of the anti-apoptotic protein, Bcl-2. In addition, the HG-induced loss of MMP was attenuated in the H9c2 cells following treatment with NAR. Caspase-3 and -9 activity was also decreased in the H9c2 cells following treatment with NAR.

Previous studies have demonstrated that hyperglycemia-induced cardiomyocyte apoptosis is associated with the activation of p53 (41). Hyperglycemia induces the overexpression of p53 and its phosphorylation on Ser15 (activated). This activation of p53 is linked to the downregulation of Bcl-2 and the upregulation of Bax, thus activating the intrinsic apoptotic pathway (42). Consistent with these findings, we also found that exposure to HG induced the phosphorylation of p53 on Ser15 and increased p53 expression in H9c2 cells. Treatment with NAR abrogated the expression and phosphorylation of p53 induced by the exposure to HG. Therefore, our data indicate that the mechanisms underlying the inhibition of HG-induced cardiomyocyte apoptosis by NAR are associated with the suppression of p53 overexpression and phosphorylation.

Recently, it was reported that p38 activation induced p53-dependent apoptosis. In a previous study, treatment with doxorubicin (DOX) induced p38 (Thr172) phosphorylation in cardiomyocytes (43). The p38 inhibitor, SB203580, inhibited DOX-induced p53 activation (Ser15 phosphorylation and expression), decreased Bax expression, inhibited the cleavage of caspase-3, preventing cardiomyocyte apoptosis. These results indicate that p38 activation contributes to DOX-induced cardiomyocyte apoptosis by inducing the phosphorylation of p53 on Ser15 (43,44). In the present study, we also observed that exposure to HG significantly increased the phosphorylation of p38 and p53 6 h following stimulation with HG in the H9c2 cells. SB203580, an inhibitor of p38 phosphorylation, suppressed the HG-induced p38 and p53 phosphorylation, preventing cardiomyocyte apoptosis. Similar results were observed in the NAR-treated H9c2 cells incubated in culture medium containing high levels of glucose. However, NAR did not completely reverse HG-induced cardiomyocyte apoptosis. Exposure to HG has been reported to induce cardiomyocyte apoptosis through the activation of a variety of signaling pathways associated with oxidative stress (45), calcium (45), transcription factors (46) and MAPKs (47). Thus, the results from the present study suggest that NAR inhibits HG-induced cardiomyocyte apoptosis by inhibiting the activation of p38.

In conclusion, NAR inhibits HG-induced cardiomyocyte apoptosis by attenuating mitochondrial dysfunction and preventing the activation of p38. Therefore, NAR exerts cardioprotective effects, preventing cardiomyocyte apoptosis. This inhibitory effect of NAR on cardiomyocyte apoptosis may provide insight into the investigation of the prevention and development of diabetic cardiomyopathy.

Acknowledgements

The authors are grateful to Dr Qitao Yan and Dr Rui Zhao (Southern Medical University, Guangzhou, China) for providing technical assistance. The present study was supported by grants from the Science and Technology Planning Project of Guangdong in China (no. 2012A080202020) and the Guangdong Natural Science Foundation (no. S201010002620).

References

Naringin, the major flavanone of grapefruit, inhibits doxorubicin-induced toxicity by modulating the expression of Bcl-2-related factors. 


