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

HAILI HUANG<sup>1\*</sup>, KENG WU<sup>2\*</sup>, QIONG YOU<sup>2</sup>, RUINA HUANG<sup>2</sup>, SHANGHAI LI<sup>2</sup> and KENG WU<sup>2</sup>

<sup>1</sup>Clinical Research Center and <sup>2</sup>Department 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). Bax 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

*Correspondence to:* Professor Keng Wu, Department of Cardiology, Affiliated Hospital of Guangdong Medical College, 57 Renmin Avenue South, Xiashan, Zhanjiang, Guangdong 524001, P.R. China E-mail: wukeng1245@hotmail.com

<sup>\*</sup>Contributed equally

*Key words:* naringin, cardiomyocyte apoptosis, mitochondrial dysfunction, p38

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 antihypertensive 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 mitocondrial dysfuntion through he 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%  $CO_2$  and 95% air.

H9c2 cells were incubated in DMEM with HG (16.7 mM) for 6 h to induce apoptosis. NAR (5  $\mu$ M) was added into the above medium (DMEM) to examine its cytoprotective effects. The selective p38 inhibitor, SB203580 (10  $\mu$ M), was administered to the H9c2 cells for 60 min prior to exposure to HG (16.7 mM) and/or treatment with NAR (5  $\mu$ M).

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  $\mu$ g) from each sample was separated by 12% SDS-PAGE and transferred onto a PVDF membrane, the PVDF membrane was then placed in washing buffer containing skimmed milk powder at room temperature and blocked for 2 h, and then washed three times. Bcl-2, Bax, Bak, voltage-dependent anion channel (VDAC), cytochrome c, p38, phosphorylated p38 (p-p38), p53, phosphorylated p53 (p-p53) and  $\beta$ -actin monoclonal antibodies were then added, followed by incubation at 4°C overnight. Horseradish peroxidase-conjugated secondary antibody was then added followed by incubation for 1 h. The membrane 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 5x10<sup>5</sup> cells were collected and suspensed in 500 µl binding buffer. Subsequently, 5  $\mu$ l Annexin V-FITC and 5  $\mu$ l 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.

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  $\mu$ M). 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.

Statistical analysis. The results of each experimental condition were determined from the mean of triplicate trials. Data are expressed as the means  $\pm$  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







Figure 1. Effects of naringin (NAR) on caspase activity and apoptosis in H9c2 cells exposed to high glucose (HG). (A-C) H9c2 cells were treated with glucose (16.7 mM) in the absence or presence of NAR (5  $\mu$ M) for 6 h, caspase-3 (n=4), caspase-8 (n=4) and caspase-9 (n=6) activity were analyzed by flow cytometry. (D) H9c2 cells were treated with glucose (16.7 mM) in the absence or presence of NAR (5  $\mu$ M) for 6 h, and cell apoptosis was analyzed by flow cytometry. \*\*P<0.01 compared with the control group. \*\*P<0.01 compared with the glucose group.

the apoptotic rate to  $25.6\pm5.9$  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  $5 \mu$ MNAR, 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  $5 \mu$ M NAR. However, no evident changes were observed in the activity of caspase-8 6 h following stimulation with HG and/or NAR.

Figure 2. Effects of naringin (NAR) on mitochondrial membrane potential (MMP) in H9c2 cells exposed to high glucose (HG). 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  $\mu$ M) 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.

NAR prevents HG-induced MMP loss in H9c2 cells. The MMP of H9c2 cells was measured by JC-1, an indicator mitochondrial function, in the H9c2 cells treated with glucose (16.7 mM) in the absence or presence of NAR (5  $\mu$ M) for 6 h. Red fluorescence represents the mitochondrial aggregate JC-1 and green fluorescence indicates the monomeric JC-1. The exposure of H9c2 cells to glucose (16.7 mM) for 6 h induced a marked decrease in MMP, and treatment with NAR prevented the HG-induced loss in MMP (Fig. 2).

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



Figure 3. Effect of naringin (NAR) on apoptosis-related proteins in H9c2 cells exposed to high glucose (HG). H9c2 cells were treated with glucose (16.7 mM) in the absence or presence of NAR (5  $\mu$ M) for 6 h, and the levels of cytoplasmic cytochrome *c* (Cyt-c), mitochondrial Bax and Bcl-2 (whole cell homogenate) proteins were determined by western blot analysis (n=3).  $\beta$ -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.



Figure 4. Effect of naringin (NAR) on p53 and p38 phosphorylation in H9c2 cells exposed to high glucose (HG). H9c2 cells were treated with glucose (16.7 mM) in the absence or presence of NAR (5  $\mu$ M) for 6 h, and the protein levels of p53 and p-p53, as well as those of p38 and p-p38 were determined by western blot analysis (n=3).  $\beta$ -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.

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, cytoplasmic cytochrome c, mitochondrial Bax and Bak levels significantly increased compared with the controls (Fig. 3). By contrast, cytoplasmic 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, p53, p38 and p-p38 in H9c2 cells. The p-p53 level was markedly increased in the HG group compared with the control group, but was decreased in the HG + NAR group compared with the HG group 6 h following exposure to HG (Fig. 4). The same tendency in expression was observed for p-p38.

*The p38 inhibitor, SB203580, and NAR inhibit HG-induced H9c2 cell apoptosis and attenuate mitochondrial dysfunction.* 





Figure 5. (A) The p38 inhibitor, SB203580 (SB), and naringin (NAR) inhibited AMP-activated protein kinase (AMPK) and p53 phosphorylation in the cardiomyocytes exposed to high glucose (HG). H9c2 cells were pre-treated with SB203580 (1.0 mM) or the vehicle for 90 min, and then incubated with HG (16.7 mM) and/or NAR (5  $\mu$ M) for 6 h. p38 and p53 phosphorylation were measured by western blot analysis.  $\beta$ -actin was used as the internal control. (B) The p38 inhibitor, SB203580, and NAR prevented HG-induced cardiomyocyte apoptosis. H9c2 cells were pre-treated with SB203580 (1.0 mM) or the vehicle for 90 min, and then exposed to HG (16.7 mM) and/or NAR (5  $\mu$ M) for 6 h. Cell apoptosis was measured using Annexin V and PI double staining (n=4). \*\*P<0.01 compared with the control group. #P<0.05, ##P<0.01 compared with the HG group.

Figure 6. Effects of the p38 inhibitor, SB203580 (SB), and naringin (NAR) on mitochondrial membrane potential (MMP) in H9c2 cells exposed to high glucose (HG). The MMP of H9c2 cells was measured by JC-1, an indicator of mitochondrial function, in the H9c2 cells pre-treated with SB203580 (1.0 mM) or the vehicle for 90 min, and then incubated with HG (16.7 mM) and/or 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 the control group. #P<0.05, compared with the glucose group.

HG+SB

HG+NAR+SB

Merge

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  $\mu$ M), for 90 min and then exposed to HG (16.7 mM) and/or NAR (5  $\mu$ M) for 6 h. Cell apoptosis was measured using Annexin V and PI double staining. SB203580 significantly inhibited the HG-induced H9c2 cell apoptosis in the HG + SB203580 (SB) group, but did not affect the inhibitory effect of NAR in the HG + SB + NAR group (Fig. 5). 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-trihydroxyflavanone-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 diabetis (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 hyperglycemiainduced 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. S2011010002620).

#### References

- 1. Cai L and Kang YJ: Cell death and diabetic cardiomyopathy. Cardiovasc Toxicol 3: 219-228, 2003.
- 2. Adeghate E: Molecular and cellular basis of the aetiology and management of diabetic cardiomyopathy: a short review. Mol Cell Biochem 261: 187-191, 2004.
- Lakshmanan AP, Harima M, Suzuki K, *et al*: The hyperglycemia stimulated myocardial endoplasmic reticulum (ER) stress contributes to diabetic cardiomyopathy in the transgenic non-obese type 2 diabetic rats: a differential role of unfolded protein response (UPR) signaling proteins. Int J Biochem Cell Biol 45: 438-447, 2013.
- 4. Jankyova S, Kmecova J, Cernecka H, et al: Glucose and blood pressure lowering effects of Pycnogenol<sup>®</sup> are inefficient to prevent prolongation of QT interval in experimental diabetic cardiomyopathy. Pathol Res Pract 208: 452-457, 2012.
- Cai L: Diabetic cardiomyopathy and its prevention by metallothionein: experimental evidence, possible mechanisms and clinical implications. Curr Med Chem 14: 2193-2203, 2007.



- Miki T, Yuda S, Kouzu H and Miura T: Diabetic cardiomyopathy: pathophysiology and clinical features. Heart Fail Rev: Mar 28, 2012 (Epub ahead of print).
- Regan TJ, Ahmed S, Haider B, Moschos C and Weisse A: Diabetic cardiomyopathy: experimental and clinical observations. N J Med 91: 776-778, 1994.
- Frustaci A, Kajstura J, Chimenti C, et al: Myocardial cell death in human diabetes. Circ Res 87: 1123-1132, 2000.
- 9. Cai L, Wang Y, Zhou G, *et al*: Attenuation by metallothionein of early cardiac cell death via suppression of mitochondrial oxidative stress results in a prevention of diabetic cardiomy-opathy. J Am Coll Cardiol 48: 1688-1697, 2006.
- Green DR and Reed JC: Mitochondria and apoptosis. Science 281: 1309-1312, 1998.
- 11. Condorelli G, Morisco C, Stassi G, *et al*: Increased cardiomyocyte apoptosis and changes in proapoptotic and antiapoptotic genes bax and bcl-2 during left ventricular adaptations to chronic pressure overload in the rat. Circulation 99: 3071-3078, 1999.
- 12. Lv X, Yu X, Wang Y, et al: Berberine inhibits doxorubicintriggered cardiomyocyte apoptosis via attenuating mitochondrial dysfunction and increasing Bcl-2 expression. PLoS One 7: e47351, 2012.
- Ruffolo SC and Shore GC: BCL-2 selectively interacts with the BID-induced open conformer of BAK, inhibiting BAK autooligomerization. J Biol Chem 278: 25039-25045, 2003.
- Youle RJ and Strasser A: The BCL-2 protein family: opposing activities that mediate cell death. Nat Rev Mol Cell Biol 9: 47-59, 2008.
- Yi X, Yin XM and Dong Z: Inhibition of Bid-induced apoptosis by Bcl-2. tBid insertion, Bax translocation, and Bax/Bak oligomerization suppressed. J Biol Chem 278: 16992-16999, 2003.
- 16. Kim SJ, Hwang SG, Shin DY, Kang SS and Chun JS: p38 kinase regulates nitric oxide-induced apoptosis of articular chondrocytes by accumulating p53 via NFkappa B-dependent transcription and stabilization by serine 15 phosphorylation. J Biol Chem 277: 33501-33508, 2002.
- Jain M and Parmar HS: Evaluation of antioxidative and antiinflammatory potential of hesperidin and naringin on the rat air pouch model of inflammation. Inflamm Res 60: 483-491, 2011.
- 18 Kawaguchi K, Maruyama H, Hasunuma R and Kumazawa Y: Suppression of inflammatory responses after onset of collageninduced arthritis in mice by oral administration of the Citrus flavanone naringin. Immunopharmacol Immunotoxicol 33: 723-729, 2011.
- Cavia-Saiz M, Busto MD, Pilar-Izquierdo MC, Ortega N, Perez-Mateos M and Muniz P: Antioxidant properties, radical scavenging activity and biomolecule protection capacity of flavonoid naringenin and its glycoside naringin: a comparative study. J Sci Food Agric 90: 1238-1244, 2010.
- Jagetia GC and Reddy TK: Alleviation of iron induced oxidative stress by the grape fruit flavanone naringin in vitro. Chem Biol Interact 190: 121-128, 2011.
- Camargo CA, Gomes-Marcondes MC, Wutzki NC and Aoyama H: Naringin inhibits tumor growth and reduces interleukin-6 and tumor necrosis factor alpha levels in rats with Walker 256 carcinosarcoma. Anticancer Res 32: 129-133, 2012.
- 22. Celiz G, Daz M and Audisio MC: Antibacterial activity of naringin derivatives against pathogenic strains. J Appl Microbiol 111: 731-738, 2011.
- 23. Carino-Cortes R, Alvarez-Gonzalez I, Martino-Roaro L and Madrigal-Bujaidar E: Effect of naringin on the DNA damage induced by daunorubicin in mouse hepatocytes and cardiocytes. Biol Pharm Bull 33: 697-701, 2010.
- Gao S, Li P, Yang H, Fang S and Su W: Antitussive effect of naringin on experimentally induced cough in Guinea pigs. Planta Med 77: 16-21, 2011.
  Liu M, Zou W, Yang C, Peng W and Su W: Metabolism and
- Liu M, Zou W, Yang C, Peng W and Su W: Metabolism and excretion studies of oral administered naringin, a putative antitussive, in rats and dogs. Biopharm Drug Dispos 33: 123-134, 2012.
- Gopinath K, Prakash D and Sudhandiran G: Neuroprotective effect of naringin, a dietary flavonoid against 3-nitropropionic acidinduced neuronal apoptosis. Neurochem Int 59: 1066-1073, 2011.
- 27. Gopinath K and Sudhandiran G: Naringin modulates oxidative stress and inflammation in 3-nitropropionic acid-induced neurodegeneration through the activation of nuclear factor-erythroid 2-related factor-2 signalling pathway. Neuroscience 227: 134-143, 2012.

- 28. Kandhare AD, Raygude KS, Ghosh P, Ghule AE and Bodhankar SL: Neuroprotective effect of naringin by modulation of endogenous biomarkers in streptozotocin induced painful diabetic neuropathy. Fitterapia 83: 650-659, 2012.
- 29. Ikemura M, Sasaki Y, Giddings JC and Yamamoto J: Preventive effects of hesperidin, glucosyl hesperidin and naringin on hypertension and cerebral thrombosis in stroke-prone spontaneously hypertensive rats. Phytother Res 26: 1272-1277, 2012.
- Chanet A, Milenkovic D, Deval C, *et al*: Naringin, the major grapefruit flavonoid, specifically affects atherosclerosis development in diet-induced hypercholesterolemia in mice. J Nutr Biochem 23: 469-477, 2012.
- Kanno S, Shouji A, Asou K and Ishikawa M: Effects of naringin on hydrogen peroxide-induced cytotoxicity and apoptosis in P388 cells. J Pharmacol Sci 92: 166-170, 2003.
- 32. Kim HJ, Song JY, Park HJ, Park HK, Yun DH and Chung JH: Naringin protects against rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells. Korean J Physiol Pharmacol 13: 281-285, 2009.
- 33. Mahmoud AM, Ashour MB, Abdel-Moneim A and Ahmed OM: Hesperidin and naringin attenuate hyperglycemia-mediated oxidative stress and proinflammatory cytokine production in high fat fed/streptozotocin-induced type 2 diabetic rats. J Diabetes Complications 26: 483-490, 2012.
- 34. Ramesh E and Alshatwi AA: Naringin induces death receptor and mitochondria-mediated apoptosis in human cervical cancer (SiHa) cells. Food Chem Toxicol 51: 97-105, 2013.
- 35. Nie YC, Wu H, Li PB, et al: Anti-inflammatory effects of naringin in chronic pulmonary neutrophilic inflammation in cigarette smoke-exposed rats. J Med Food 15: 894-900, 2012.
- 36. Xulu S and Oroma Owira PM: Naringin ameliorates atherogenic dyslipidemia but not hyperglycemia in rats with type 1 diabetes. J Cardiovasc Pharmacol 59: 133-141, 2012.
- Rajadurai M and Prince PS: Naringin ameliorates mitochondrial lipid peroxides, antioxidants and lipids in isoproterenol-induced myocardial infarction in Wistar rats. Phytother Res 23: 358-362, 2009.
- Punithavathi VR, Anuthama R and Prince PS: Combined treatment with naringin and vitamin C ameliorates streptozotocin-induced diabetes in male Wistar rats. J Appl Toxicol 28: 806-813, 2008.
- Shore GC and Nguyen M: Bcl-2 proteins and apoptosis: choose your partner. Cell 135: 1004-1006, 2008.
- 40. Cai L, Li W, Wang G, Guo L, Jiang Y and Kang YJ: Hyperglycemia-induced apoptosis in mouse myocardium: mitochondrial cytochrome C-mediated caspase-3 activation pathway. Diabetes 51: 1938-1948, 2002.
- 41. Fiordaliso F, Leri A, Cesselli D, *et al*: Hyperglycemia activates p53 and p53-regulated genes leading to myocyte cell death. Diabetes 50: 2363-2375, 2001.
- Miyashita T and Reed JC: Tumor suppressor p53 is a direct transcriptional activator of the human bax gene. Cell 80: 293-299, 1995.
- 43 Zhu W, Soonpaa MH, Chen H, *et al*: Acute doxorubicin cardiotoxicity is associated with p53-induced inhibition of the mammalian target of rapamycin pathway. Circulation 119: 99-106, 2009.
- 44. Childs AC, Phaneuf SL, Dirks AJ, Phillips T and Leeuwenburgh C: Doxorubicin treatment in vivo causes cytochrome C release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2:Bax ratio. Cancer Res 62: 4592-4598, 2002.
- 45. Younce CW, Burmeister MA and Ayala JE: Exendin-4 attenuates high glucose-induced cardiomyocyte apoptosis via inhibition of endoplasmic reticulum stress and activation of SERCA2a. Am J Physiol Cell Physiol 304: C508-C518, 2013.
- 46. Kuo WW, Wang WJ, Tsai CY, Way CL, Hsu HH and Chen LM: Diallyl trisufide (DATS) suppresses high glucose-induced cardiomyocyte apoptosis by inhibiting JNK/NFkappaB signaling via attenuating ROS generation. Int J Cardiol doi: 10.1016/j. ijcard.2012.09.080, 2012.
- 47. Yu XY, Geng YJ, Liang JL, *et al*: High levels of glucose induce apoptosis in cardiomyocyte via epigenetic regulation of the insulinlike growth factor receptor. Exp Cell Res 316: 2903-2909, 2010.