

Inhibition of the leptin-induced activation of the p38 MAPK pathway contributes to the protective effects of naringin against high glucose-induced injury in H9c2 cardiac cells

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Abstract. Leptin, a product of the obese gene, has been reported to contribute to the development of cardiomyocyte hypertrophy in patients with diabetes and to activate the p38 mitogen-activated protein kinase (MAPK) pathway in cardiomyocytes. In this study, we demonstrate that naringin, a citrus flavonone, protects cardiomyoblasts (H9c2 cells) against high glucose (HG)-induced apoptosis by modulating the activation of the p38 MAPK pathway. We investigated the hypothesis that naringin prevents HG-induced injury by inhibiting the leptin-induced activation of the p38 MAPK pathway in H9c2 cells. Our results demonstrated that the exposure of H9c2 cells to HG (35 mmol/l) for a 24 h markedly upregulated the expression levels of both leptin and leptin receptors. However, the increase in the expression levels of leptin and leptin receptors was greatly attenuated by treatment of the H9c2 cells with 80 μ mol/l naringin 2 h prior to exposure to HG. In addition, treatment of the cells with 50 ng/ml leptin antagonist (LA) for 24 h prior to exposure to HG markedly ameliorated the increased expression of phosphorylated (p)-p38 MAPK induced by HG. Of note, pre-treatment of the cells with either 80 μ mol/l naringin or 50 ng/ml LA markedly inhibited the HG-induced injury, leading to an increase in cell viability and a decrease in the total number of apoptotic cells, preventing reactive oxygen species (ROS) generation, as well as the dissipation of mitochondrial membrane potential (MMP). In conclusion, the findings of the present study provide the first evidence that the leptin-induced activation of the p38 MAPK pathway is involved in HG-induced injury, including cytotoxicity, apoptosis, ROS

generation and the dissipation of MMP in H9c2 cardiac cells. Our data demonstrate that naringin protects cardiac cells against HG-induced injury by inhibiting the leptin-induced activation of the p38 MAPK pathway.

Introduction

Naringin (4',5,7-trihydroxyflavonone-7-rhamnoglucoside), is a flavonone found in citrus fruit, such as grapefruit. Accumulating evidence has indicated that naringin has a broad spectrum of pharmacological and therapeutic properties, including antioxidant (1-5), antihypercholesterolemic (5-7), anti-inflammatory (1,4,5,8) and anti-apoptotic effects (9-12). Notably, naringin has antihyperglycemic properties (13,14) and cardioprotective effects (12,15,16). A recent study demonstrated that naringin protects H9c2 cardiac cells against high glucose (HG)-induced apoptosis by inhibiting the activation of the p38 mitogen-activated protein kinase (MAPK) pathway (12). Accordingly, we hypothesized that the molecules which activate the p38 MAPK pathway may contribute to HG-induced cardiomyocyte injury and to the cardioprotective effects of naringin. Thus, in this study, we investigated one of these molecules, leptin.

Leptin, a 16-kDa peptide released from adipocytes and other cells, has been implicated in the regulation of appetite and energy metabolism (17,18). Leptin was initially thought to mainly regulate obesity or body weight. However, increasing evidence has demonstrated the effects of leptin in the regulation of inflammation (19), blood pressure homeostasis and cardiovascular disease (20-23). Leptin receptors have a widespread tissue distribution, including the kidneys, pancreas, lungs and heart (22,24-26), through which leptin exerts its physiological effects, primarily by activating Janus tyrosine kinase (JAK), signal transducers and signal transducers and activators of transcription (STATs) (27). In addition, previous studies have demonstrated that leptin can also activate other signaling cascades, such as extracellular signal-regulated kinase 1/2 (ERK1/2) (28), c-Jun N-terminal kinase/stress-activated protein kinase (JNK) (29) and p38 MAPK (30-33) of the MAPK family in a variety of cell types. A previous study demonstrated that leptin induces the selective translocation of p38 MAPK from the cytoplasmic to the nuclear fraction and is

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dependent on intact caveolae and on the activity of the RhoA pathway (33). In rat vascular smooth muscle cells, leptin has been shown to induce hypertrophy through the activation of the p38 MAPK pathway (31).

Thus, there seems to be a link between leptin and diabetes-induced pathophysiological processes. Leptin has been shown to regulate the diabetes-induced increase in extracellular matrix (ECM) protein production in renal mesangial cells (34). Leptin is also involved in the increased production of fibronectin (FN) induced by diabetes and in cardiomyocyte hypertrophy (22). However, evidence that the leptin-induced activation of the p38 MAPK pathway may be involved in hyperglycemia-induced cardiomyocyte injury, including cytotoxicity, apoptosis, generation of reactive oxygen species (ROS) and the dissipation of mitochondrial membrane potential (MMP), is lacking. Thus, the aim purpose of the present study was to determine whether the leptin-induced activation of the p38 MAPK pathway contributes to hyperglycemia-induced cardiomyocyte injury. We also aimed to determine whether the inhibition of the leptin-induced activation of the p38 MAPK pathway is involved in the protective effects of naringin against HG-induced injury in H9c2 cardiac cells.

Materials and methods

Materials. Naringin with a purity of $\geq 98\%$, was obtained from Sigma-Aldrich, St. Louis, MO, USA, stored at $2-4^{\circ}\text{C}$ and protected from sunlight. Dichlorofluorescein diacetate (DCFH-DH), rhodamine (Rh123) and Hoechst 33258 were also purchased from Sigma-Aldrich. Leptin antagonist (LA) was supplied by Prospec (East Brunswick, NJ, USA). The Cell Counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Fetal bovine serum (FBS) and DMEM-F12 medium were obtained from Gibco-BRL (Grand Island, NY, USA). Anti-p38 MAPK, anti-phospho-p38 MAPK, anti-leptin and anti-leptin receptor antibodies were procured from Cell Signaling Technology (Boston, MA, USA). HRP-conjugated secondary antibody and the BCA protein assay kit were obtained from KangChen Bio-tech, Inc. (Shanghai, China). Enhanced chemiluminescence (ECL) solution was purchased from Nanjing KeyGen Biotech Co., Ltd. (Nanjing, China).

Cell culture and treatments. H9c2 cardiac cells, a rat cardiomyoblast cell line, were supplied by the Sun Yat-sen University Experimental Animal Center (Guangzhou, China). The cells were grown in DMEM-F12 medium supplemented with 10% FBS under an atmosphere of 5% CO_2 at 37°C and 95% air.

The H9c2 cells were preconditioned with $80\text{ }\mu\text{mol/l}$ naringin for 2 h prior to exposure to 35 mmol/l glucose (HG) for 24 h. To further determine whether the protective effects of naringin and the activation of the p38 MAPK pathway induced by HG are associated with leptin, the H9c2 cells were preconditioned with 50 ng/ml LA for 24 h prior to exposure to 35 mmol/l glucose for 24 h.

Measurement of cell viability. The H9c2 cells were seeded in 96-well plates at a concentration of 1×10^4 cells/ml, and incubated at 37°C . The CCK-8 assay was employed to assess the viability of the H9c2 cardiac cells. After the indicated treat-

ments, $10\text{ }\mu\text{l}$ of CCK-8 solution were added to each well at a 1/10 dilution and then the plate was incubated for 1.5 h in an incubator. With the use of a microplate reader (Molecular Devices, Sunnyvale, CA, USA) absorbance was assayed at 450 nm. The mean optical density (OD) of 3 wells in the indicated groups was used to calculate the percentage of cell viability according to the following formula: cell viability (%) = $(\text{OD}_{\text{treatment group}} / \text{OD}_{\text{control group}}) \times 100\%$. The experiment was repeated 5 times.

Hoechst 33258 nuclear staining for the detection of apoptosis. Apoptotic cell death was observed by Hoechst 33258 staining followed by photofluorography. In brief, the H9c2 cells were plated in 35 mm dishes at a density of 1×10^6 cells/well. After the indicated treatments, the H9c2 cells were fixed with 4% paraformaldehyde in 0.1 mol/l phosphate-buffered saline (PBS, pH 7.4) for 10 min. The slides were then washed 5 times with PBS. After staining with 5 mg/ml Hoechst 33258 for 5 min, the H9c2 cells were washed 5 times with PBS and then visualized under a fluorescence microscope (Bx50-FLA; Olympus, Tokyo, Japan). Viable H9c2 cells displayed a uniform blue fluorescence throughout the nucleus and normal nuclear size. However, apoptotic H9c2 cells showed condensed, distorted or fragmented nuclei. The experiment was carried out 3 times.

Measurement of intracellular ROS generation. The generation of intracellular ROS was determined by the oxidative conversion of the cell-permeable oxidation of DCFH-DA into fluorescent DCF. The H9c2 cells were cultured on a slide with DMEM-F12 medium. After the different treatments, the slides were washed twice with PBS. DCFH-DA ($10\text{ }\mu\text{mol/l}$) solution in serum-free medium was then added to the slides, and the H9c2 cells were then incubated in an incubator at 37°C for a further 30 min. The slides were washed 5 times with PBS, and DCF fluorescence was measured over the entire field of vision with the use of a fluorescence microscope connected to an imaging system (BX50-FLA; Olympus). The mean fluorescence intensity (MFI) from 5 random fields was measured with the use of ImageJ 1.47i software and the MFI was used as an index of the amount of ROS. The experiment was carried out 5 times.

Measurement of MMP. MMP was assessed using a fluorescent dye, Rh123, a cell-permeable cationic dye that preferentially enters the mitochondria based on the highly negative MMP. The depolarization of MMP results in a decrease in green fluorescence. The H9c2 cells were cultured on a slide with DMEM-F12. After the indicated treatments, the slides were washed 3 times with PBS. The cells were incubated with 1 mg/l Rh123 at 37°C for 30 min in an incubator and briefly washed 3 times with PBS and air dried. Fluorescence was then measured over the whole field of vision with the use of a fluorescence microscope connected to an imaging system (BX50-FLA; Olympus). The MFI of Rh123 from 5 random fields was analyzed using ImageJ 1.47i software. The MFI was taken as an index of the MMP levels. The experiment was carried out 5 times.

Western blot analysis. After the indicated treatments, the H9c2 cells were harvested and lysed with cell lysis solution at 4°C for 30 min. Total protein was quantified using the

BCA protein assay kit. Loading buffer was added to the cytosolic extracts, and boiled for 5 min; the same amounts of supernatant from each sample were subjected to 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and then the total number of proteins was transferred onto polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 60 min in 5% fat-free milk in fresh blocking buffer [0.1% Tween-20 in Tris-buffered saline (TBS-T)] at room temperature, and incubated with either anti-p38 (1:1,000 dilution), anti-phosphorylated (p)-p38 (1:1,000 dilution), anti-leptin (1:1,000 dilution) or anti-leptin receptor (1:1,000 dilution) antibodies in freshly prepared TBS-T with 3% fat-free milk overnight with gentle agitation at 4°C. The membranes were washed for 15 min with TBS-T and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody (1:2,500 dilution; KangChen Biotech, Inc.) in TBS-T with 3% fat-free milk for 1.5 h at room temperature. The membranes were then washed 3 times with TBS-T for 15 min. The immunoreactive signals were visualized by using an ECL detection. In order to quantify the protein expression levels, the X-ray films were scanned and analyzed using ImageJ 1.47i software. The experiment was carried out 3 times.

Statistical analysis. All data are presented as the means \pm SEM. Differences between groups were analyzed by one-way analysis of variance (ANOVA) followed by the LSD post hoc comparison test. Statistical analyses were performed using SPSS 13.0 statistical software (SPSS, Chicago, IL, USA). Values of $P < 0.05$ were considered to indicate statistically significant differences.

Results

Naringin and LA attenuate the HG-induced increase in leptin expression levels in H9c2 cardiac cells. In order to determine the effects of HG (35 mmol/l glucose) on leptin expression levels in H9c2 cardiac cells, a time-response experiment on leptin expression levels was performed. Following exposure of the H9c2 cardiac cells to 35 mmol/l glucose for 3, 6, 9, 12 and 24 h, the leptin expression levels began to markedly increase at 6 h, reaching a peak at 9 h (Fig. 1A and B). Thus, leptin expression levels were examined 9 h post-exposure to HG in the following experiments. Notably, prior to exposure to 35 mmol/l glucose, the H9c2 cells were treated with 80 μ mol/l naringin for 2 h. This pre-treatment significantly reduced the expression levels of leptin which were increased by HG (Fig. 1C and D).

In addition, the administration of 50 ng/ml LA for 24 h prior to exposure to HG, led to marked decrease in leptin expression levels (Fig. 1C and D). The basal expression levels of leptin (control) were not altered by the separate treatment with 80 μ mol/l naringin or 50 ng/ml LA (Fig. 1C and D).

Naringin attenuates the HG-induced increase in the expression levels of leptin receptors in H9c2 cardiac cells. Western blot analysis (Fig. 2A and B) revealed that the exposure of H9c2 cells to 35 mmol/l glucose for 1, 3, 6, 9, 12 and 24 h induced the overexpression of leptin receptor (Ob-R) protein, peaking at 12 h. The expression levels of Ob-R began to decrease at

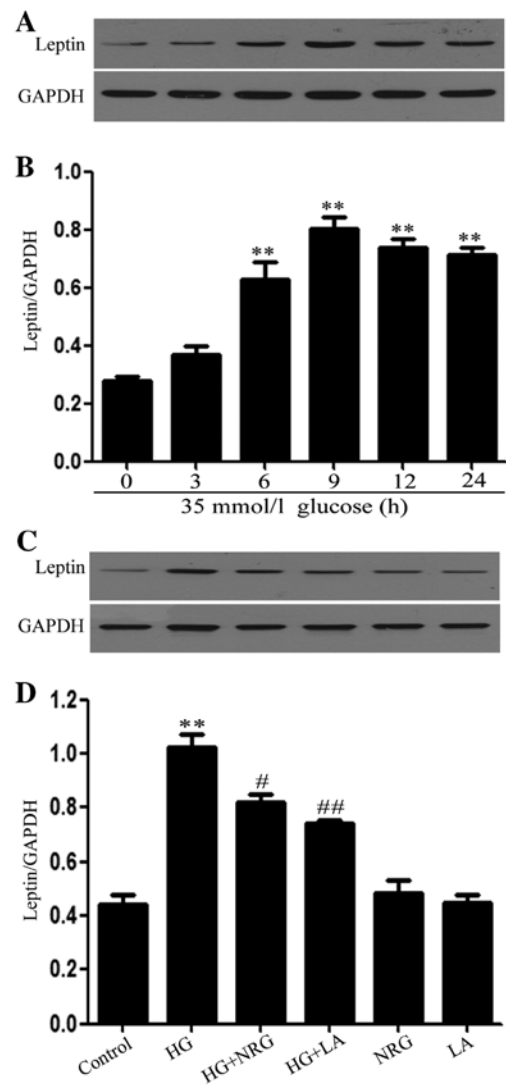


Figure 1. Naringin (NRG) and leptin antagonist (LA) suppress the high glucose (HG)-induced upregulation in leptin expression levels in H9c2 cardiac cells. (A and C) Leptin expression levels were semiquantified by western blot analysis. (A and B) Time course of changes observed in leptin expression levels induced by HG (35 mmol/l glucose) over a 24-h time period. (C and D) H9c2 cardiac cells were exposed to HG for 9 h with or without treatment with 80 μ mol/l NRG for 2 h or with 50 ng/ml LA for 24 h prior to exposure to HG. (B and D) Densitometric analysis of the leptin expression levels in (A and C). Data are presented as the means \pm SE, (n=3). ** $p < 0.01$ vs. the control group; # $p < 0.05$, ## $p < 0.01$ vs. the HG-treated group.

24 h, but remained much higher than those observed at 9 h (Fig. 2A and B).

Additionally, treatment with 80 μ mol/l naringin for 2 h prior to exposure to HG markedly alleviated the increased Ob-R expression levels detected 12 h after exposure to HG ($p < 0.01$) compared with the HG-treated group (Fig. 2C and D). Naringin alone, at 80 μ mol/l, did not alter the basal expression levels of leptin receptors in H9c2 cells.

LA modulates the activation of p38 MAPK induced by HG in H9c2 cardiac cells. Western blot analysis revealed that exposure of the H9c2 cells to HG markedly upregulated the expression levels of p-p38 MAPK (Fig. 3). The increased p-p38 MAPK expression levels were markedly suppressed by treatment with LA (50 ng/ml) for 24 h prior to exposure to

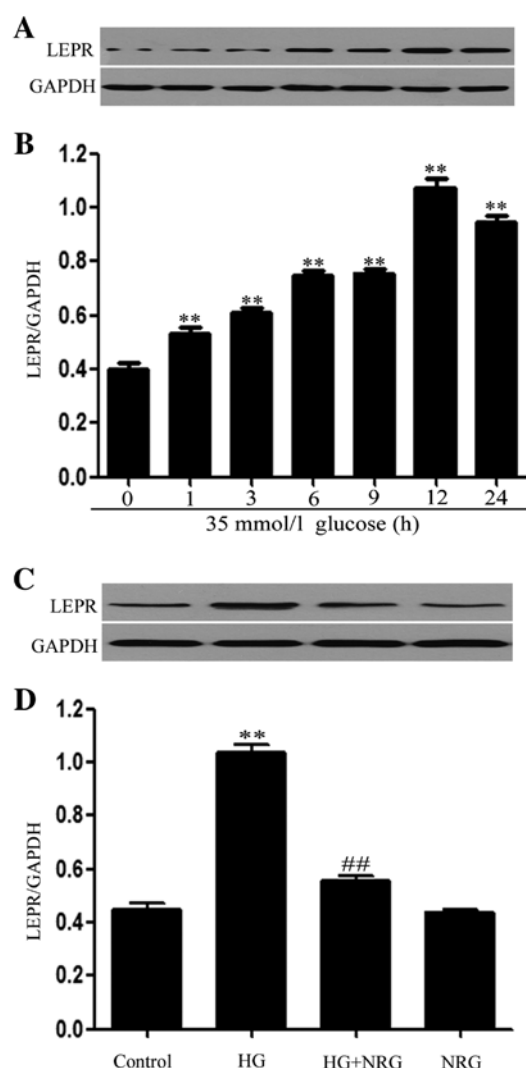


Figure 2. Naringin (NRG) attenuates the increase in the expression levels of leptin receptors induced by high glucose (HG) in H9c2 cardiac cells. (A and C) Leptin receptor expression levels were measured by western blot analysis. (A and B) Time course of changes observed in leptin receptor expression levels induced by HG (35 mmol/l glucose) over a 24-h time period. (C and D) H9c2 cardiac cells were exposed to HG for 12 h with or without treatment with 80 μ mol/l NRG for 2 h prior to exposure to HG. (B and D) Densitometric analysis of the leptin expression levels in (A and C). Data are presented as the means \pm SE, (n=3). **p<0.01 vs. the control group; ##p<0.01 vs. the HG-treated group. LEPR, leptin receptor.

HG, indicating the involvement of leptin in the HG-induced activation of p38 MAPK in H9c2 cardiac cells.

Naringin and LA alleviate HG-induced cytotoxicity in H9c2 cardiac cells. Exposure of the H9c2 cells to HG for 24 h induced marked cytotoxic effects, leading to reduced cell viability (Fig. 4). However, the decreased cell viability was attenuated by treatment with 80 μ mol/l naringin for 2 h or by treatment with 50 ng/ml LA for 24 h prior to exposure to HG. cell viability levels still remained reduced compared to the control group. Naringin or LA alone did not affect the viability of the H9c2 cardiac cells.

Naringin and LA attenuate HG-induced apoptosis in H9c2 cardiac cells. As illustrated in Fig. 5B, exposure of the H9c2 cells to HG for 24 h induced typical characteristics of

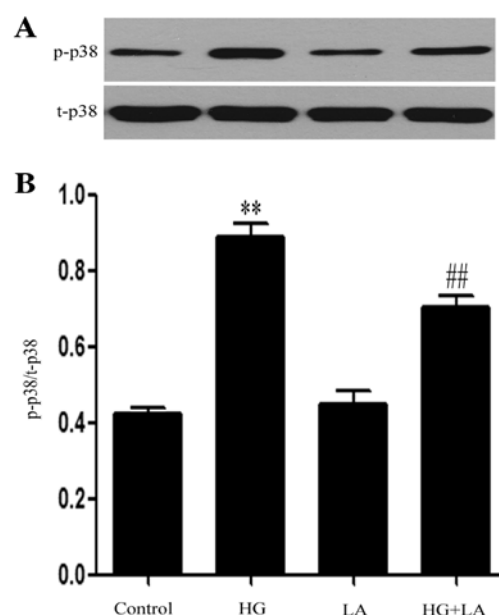


Figure 3. Leptin antagonist (LA) modulates the activation of mitogen-activated protein kinase (MAPK) induced by high glucose (HG) in H9c2 cardiac cells. H9c2 cells were preconditioned with 50 ng/ml LA for 24 h prior to exposure of the cells to HG for 30 min. (A) The expression levels of p38 MAPK were semiquantified by western blot analysis. (B) Densitometric analysis of the data (p-p38 MAPK) shown in (A). Data are presented as the means \pm SE, (n=3). **p<0.01 vs. the control group; ##p<0.01 vs. the HG-treated group. p-p38, phosphorylated p38 MAPK; t-p38, total p38 MAPK.

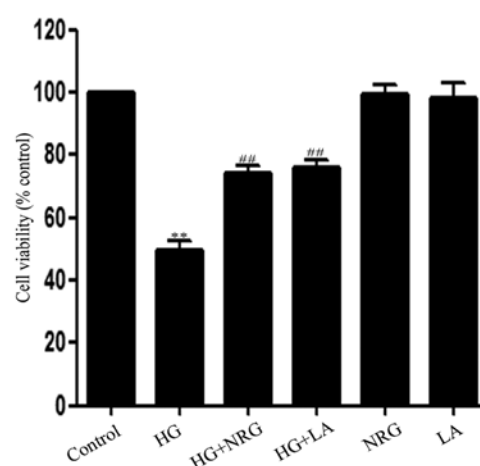


Figure 4. Naringin (NRG) and leptin antagonist (LA) alleviate high glucose (HG)-induced cytotoxicity in H9c2 cardiac cells. H9c2 cells were preconditioned with 80 μ mol/l NRG for 2 h or with 50 ng/ml LA for 24 h prior to exposure to HG. Cell viability was measured by CCK-8 assay. Data are presented as the means \pm SE, (n=5). **p<0.01 vs. the control group; ##p<0.01 vs. the HG-treated group.

apoptosis, as indicated by the condensation of chromatin, the shrinkage of nuclei and the formation of apoptotic bodies. However, treatment of the cells with 80 μ mol/l naringin for 2 h prior to exposure to HG markedly ameliorated the increase in the number of cells which presented nuclear condensation and fragmentation (Fig. 5C). In addition, treatment of the cells with 50 ng/ml LA for 24 h prior to exposure to HG also alleviated HG-induced apoptosis (Fig. 5D). Naringin or LA alone did not significantly alter the morphology or the percentage

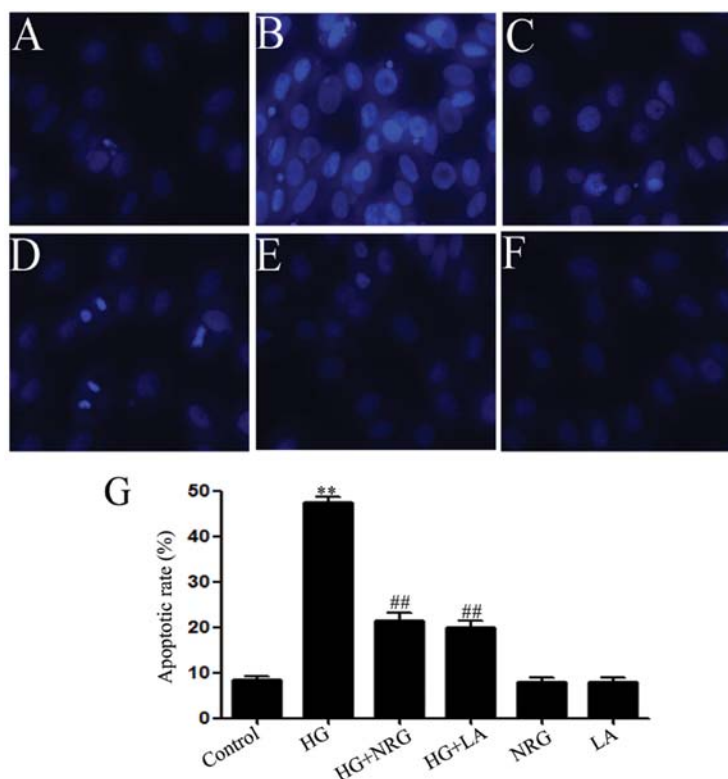


Figure 5. Naringin (NRG) and leptin antagonist (LA) reduce high glucose (HG)-induced apoptosis in H9c2 cardiac cells. (A-F) Hoechst 33258 nuclear staining followed by photofluorography was carried out to observe cell apoptosis. (A) Control group. (B) H9c2 cells exposed to 35 mmol/l glucose (HG) for 24 h. (C) H9c2 cells treated with 80 μ mol/l NRG for 2 h prior to exposure to HG for 24 h. (D) H9c2 cells treated with 50 ng/ml LA for 24 h prior to exposure to HG for 24 h. (E) H9c2 cells treated with 80 μ mol/l NRG for 2 h. (F) H9c2 cells treated with 50 ng/ml LA for 24 h. (G) The apoptotic rate was analyzed using a cell counter and ImageJ 1.47i software. Data are presented as the means \pm SE, (n=3). **p<0.01 vs. the control group; ##p<0.01 vs. the HG-treated group.

of apoptotic H9c2 cells (Fig. 5E-G). These findings suggest that naringin protects the H9c2 cells against HG-induced apoptosis, which is related at least in part, with the increase in leptin expression.

Naringin and LA alleviate the HG-induced increase in ROS generation in H9c2 cells. Previous studies have demonstrated that ROS generation is involved in HG-induced cardiomyocyte injury (35,36). Thus, in this study, we explored the effects of naringin on HG-induced ROS generation in H9c2 cells. The exposure of the H9c2 cells to HG for 24 h markedly enhanced ROS generation (Fig. 6B and G). The increase in ROS generation was suppressed by treatment of the cells with 80 μ mol/l naringin for 2 h prior to exposure to HG (Fig. 6C and G), revealing the inhibitory effects of naringin on HG-induced oxidative stress. In order to determine whether leptin is involved in the HG-induced overproduction of ROS, the H9c2 cells were treated with 50 ng/ml LA for 24 h prior to exposure to HG. Our results revealed that treatment with LA considerably diminished the HG-induced increase in ROS generation (Fig. 6D and G), indicating the involvement of leptin in HG-induced ROS overproduction.

Naringin and LA block the HG-induced dissipation of MMP in H9c2 cells. Since mitochondrial damage has been shown to contribute to HG-induced cardiomyocyte injury (37,38), we investigated whether naringin can prevent the loss of MMP in HG-treated H9c2 cells. Our results revealed that the exposure

of the cells to HG for 24 h induced mitochondrial damage, as indicated by the dissipation of MMP (Fig. 7B and G). Of note, the dissipation of MMP was attenuated by treatment of the cells with 80 μ mol/l naringin for 2 h prior to exposure to HG (Fig. 7C and G), indicating that naringin protected the H9c2 cells against HG-induced mitochondrial damage. Similarly, treatment of the cells with 50 ng/ml LA for 24 h prior to exposure to HG, attenuated the HG-induced dissipation of MMP, suggesting the involvement of leptin in the HG-induced dissipation of MMP (Fig. 7D and G).

Discussion

Hyperglycemia, an important feature of diabetes mellitus (DM), is considered to be the most important factor leading to almost all complications associated with chronic diabetes, including diabetic cardiomyopathy (39,40). However, the mechanisms underlying the deteriorative effects of hyperglycemia on cardiomyocytes are not yet fully understood. A number of factors, such as ROS generation (35,36) mitochondrial insult (37,38) and the activation of MAPKs (12,41-43), have been shown to participate in hyperglycemia-induced injury. Majumdar *et al* (22) reported that the exposure of neonatal rat cardiomyocytes to 25 mmol/l glucose induced increased mRNA and protein expression levels of both leptin and endothelin-1 (ET-1), which are involved in HG-induced cardiomyocyte hypertrophy. Consistent with these findings (22), our results demonstrated that the exposure of H9c2 cardiac cells to HG (35 mmol/l glucose)

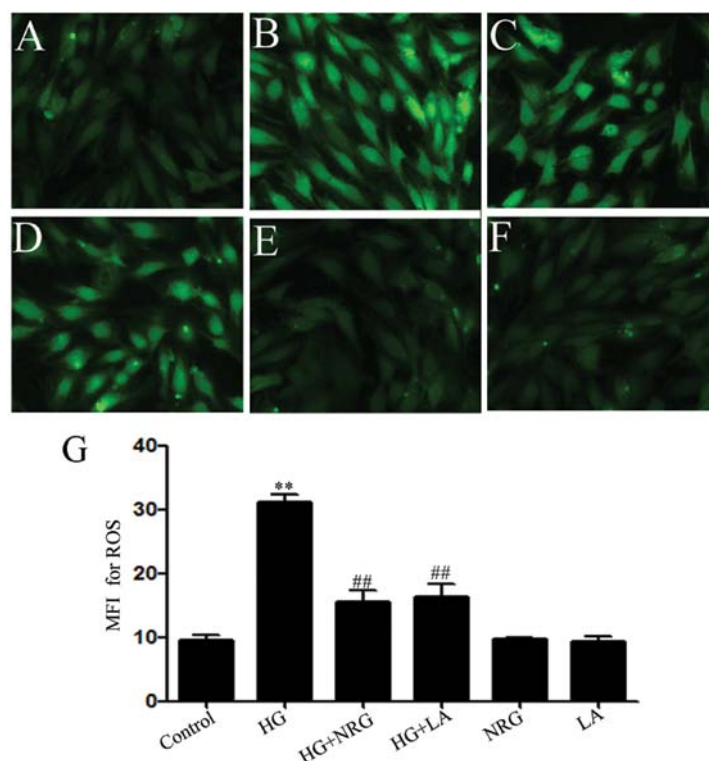


Figure 6. Naringin (NRG) and leptin antagonist (LA) reduce high glucose (HG)-induced ROS generation in H9c2 cells. (A-F) DCF staining followed by photofluorography was carried out in order to observe intracellular ROS levels. (A) Control group. (B) H9c2 cells exposed to 35 mmol/l glucose (HG) for 24 h. (C) H9c2 cells treated with 80 μ mol/l NRG for 2 h prior to exposure to HG for 24 h. (D) H9c2 cells treated with 50 ng/ml LA for 24 h prior to exposure to HG for 24 h. (E) H9c2 cells treated with 80 μ mol/l NRG for 2 h. (F) H9c2 cells treated with 50 ng/ml LA for 24 h. (G) Quantitative analysis of mean fluorescence intensity (MFI) was carried out using ImageJ 1.47i software in each group (A-F). Data are presented as the means \pm SE, (n=3). **p<0.01 vs. the control group; ##p<0.01 vs. the HG-treated group.

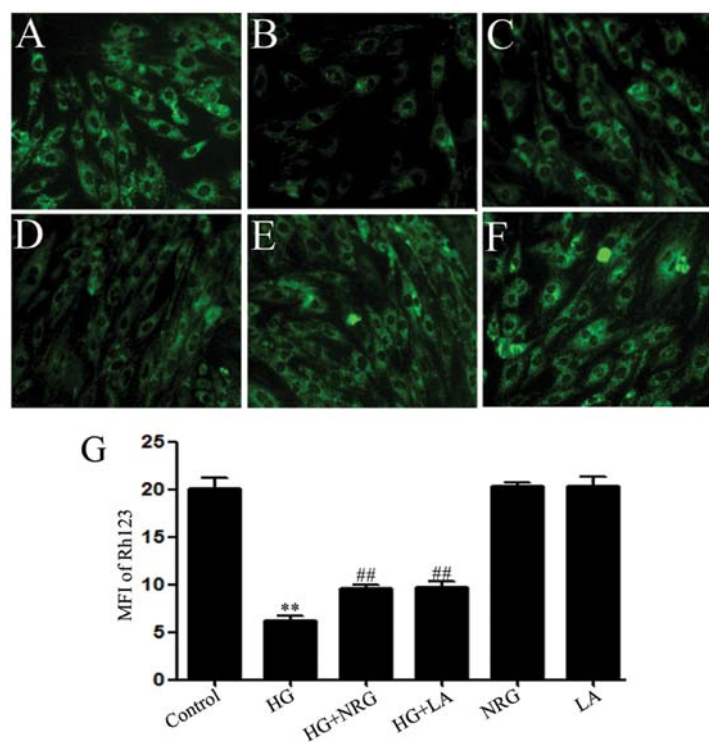


Figure 7. Naringin (NRG) and leptin antagonist (LA) block the high glucose (HG)-induced dissipation of mitochondrial membrane potential (MMP) in H9c2 cells. (A-F) After the indicated treatments, MMP was measured by Rh123 staining followed by photofluorography. (A) Control group. (B) H9c2 cells exposed to 35 mmol/l glucose (HG) for 24 h. (C) H9c2 cells treated with 80 μ mol/l NRG for 2 h prior to exposure to HG for 24 h. (D) H9c2 cells treated with 50 ng/ml LA for 24 h prior to exposure to HG for 24 h. (E) H9c2 cells treated with 80 μ mol/l NRG for 2 h. (F) H9c2 cells treated with 50 ng/ml LA for 24 h. (G) Quantitative analysis of the mean fluorescence intensity (MFI) of Rh123 in (A-G) was carried out using ImageJ 1.47i software. Data are presented as the means \pm SE, (n=3). **p<0.01 vs. the control group; ##p<0.01 vs. the HG-treated group.

markedly upregulated the expression levels of both leptin and leptin receptor. Since leptin has been shown to participate in HG-induced cardiomyocyte hypertrophy (22), we investigated whether leptin contributes to other injuries induced by HG. The findings of this study demonstrated that the exposure of the cells to HG induced marked changes, as shown by the decrease in cell viability, the increase in the apoptotic cell number and ROS generation, as well as in the dissipation of MMP. However, these injuries induced by HG were significantly attenuated by treatment with LA prior to exposure to HG, suggesting that leptin is involved in HG-induced cytotoxicity, apoptosis, ROS generation and mitochondrial damage. Our data confirm the data from a previous study (22), and provide novel evidence of the role of leptin in hyperglycemia-induced cardiomyocyte injury.

Additionally, we observed that the exposure of the cells to HG, induced an increase in p-p38 MAPK expression levels, which is in accordance with the findings of previous studies (12,41-43). Importantly, the high expression levels of p-p38 MAPK were decreased by treatment with LA prior to exposure to HG, indicating that leptin acts upstream of p38 MAPK and contributes to the HG-induced activation of the p38 MAPK pathway in H9c2 cells. It is known that p38 MAPK is activated by a diverse range of physical and chemical stresses, such as hypoxia/ischemia (44,45), drugs (46,47) and oxidative stress (48). To the best of our knowledge, only a few studies to date have addressed the role of p38 MAPK in leptin signaling in different cell types (30-33,49); for example, leptin has been shown to induce hypertrophy through the activation of p38 MAPK in rat vascular smooth muscle cells (31). However, it remains unclear as to whether the leptin-induced activation of the p38 MAPK pathway is involved in HG-induced cardiomyocyte injury. Recently, we (12), as well as others [Xu *et al* (42)] demonstrated the contribution of p38 MAPK activation to HG-induced injury in H9c2 cardiac cells. Thus, the data from the current study (Figs. 3-7), as well as those from previous studies (12,42), provide evidence that the leptin-induced activation of the p38 MAPK pathway may be an important mechanism responsible for HG-induced injury in H9c2 cardiac cells.

Naringin, a citrus flavonone, has been shown to exert protective effects against hyperglycemia-induced cardiac injury in DM and has attracted considerable attention due to its broad spectrum of pharmacological and therapeutic properties (1-16), including its antihyperglycemic (13,14), anti-apoptotic (9-12) and cardioprotective (12,15,16) effects. In a recent study, we demonstrated that naringin protects cardiomyocytes against HG-induced injury by modulating the activation of the p38 MAPK pathway (12). However, the mechanisms underlying the cardioprotective effects of naringin, including the effects of naringin on the leptin-induced activation of the p38 MAPK pathway remain unclear. Thus, in the present study, we explored the effects of naringin on the leptin-induced activation of the p38 MAPK pathway in H9c2 cardiac cells exposed to HG. Our results demonstrated that naringin markedly attenuated the HG-induced increase in the expression levels of both leptin and leptin receptors, indicating the inhibitory effects of naringin on the increase in leptin expression induced by HG. Since we have previously demonstrated that naringin inhibits the HG-induced activation of the p38 MAPK pathway (12), the

findings of the current study support the notion that naringin protects H9c2 cardiac cells against HG-induced injury, at least in part through the inhibition of the leptin-induced activation of the p38 MAPK pathway. This is clearly supported by the following evidence: i) the inhibitory effects of naringin on the increased expression of both leptin and leptin receptor induced by HG; ii) the inhibitory effects of LA on the HG-induced activation of p38 MAPK; iii) the inhibitory effects of naringin on the HG-induced activation of p38 MAPK (12); iv) the protective effects of both naringin and LA against HG-induced cardiomyocyte injury, leading to an increase in cell viability, as well as a decrease in the number of apoptotic cells, ROS generation, and in the attenuation of the loss of MMP; v) the inhibitory effects of SB203580, an inhibitor of p38 MAPK, on HG-induced injury (12,42).

In conclusion, this study provides the first evidence that the leptin-induced activation of the p38 MAPK pathway contributes to HG-induced cardiomyocyte injury, including cytotoxicity, apoptosis, oxidative stress and mitochondrial damage. The understanding of the role of such a pathway is important, as it may lead to the development of novel treatment strategies for diabetic cardiomyopathy. In addition, we provide novel evidence that the inhibition of the leptin-induced activation of the p38 MAPK pathway is involved in the cardioprotective effects of naringin against hyperglycemia-induced cardiomyocyte injury. These findings provide a deeper understanding of the mechanisms responsible for the cytoprotective effects of naringin against cardiovascular complications associated with diabetes and its pharmacological and therapeutic properties.

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