Effects of HIP in protection of HSP70 for stress-induced cardiomyocytes injury and its glucocorticoid receptor pathway

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Abstract Moderate levels of stress can be beneficial to health, while stress overload can cause injury or contribute to diseases. Despite a number of studies of adaptation or stress damage, the mechanisms of adaptation and stress damage remain far from clear. The effect and mechanisms of adaptation on cardiomyocytes damage caused by stress overload are discussed in this study. Data showed that mild repeated stress mitigated stress overload-induced cardiomyocyte injury both in an animal model of restraint stress and in H9C2 cells with GC (glucocorticoid) treatment. HSP70, HIP expression and interaction between HSP70 and HIP increased during adaptation induced by mild stress both in animals and H9C2 cells. Overexpression or inhibition of HSP70 in H9C2 cells with pCDNA-3.1-Hsp70 or KNK437 (HSP70 inhibitor) showed that HSP70 can protect H9C2 cells from GC-induced cell damage. A luciferase assay showed that Hsp70 plays its protective role through inhibition of GR transcription activity dependent on the interaction with HIP. These results indicated that HSP70 may promote adaptation with its interacting protein HIP, and increased levels of HSP70 and its interacting protein HIP during adaptation may play a protective role on stress-overload-induced cardiomyocyte injury.

Keywords HSP70 · HIP · Stress · Cardiomyocyte · Glucocorticoid

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Abbreviations

GC	Glucocorticoid
HSPs	Heat shock proteins
HIP	Hsp70 interacting protein
GR	Glucocorticoid receptor
HPA	Hypothalamic pituitary adrenal
LDH	Lactate dehydrogenase
CK	Creatine kinase
PBS	Phosphate-buffered saline
HSF	Heat shock factors
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
CK-MB	Creatine kinase-MB

Introduction

Organisms are continuously subjected to stress in everyday life. The organismal response to stress promotes survival via adjustments to ongoing physiological processes and behavior. The activation of multiple interacting processes, including the behavioral, autonomic, endocrine, and immune systems, produces an integrated stress response. In essence, chronic stress and mild stress change the rules under which the body regulates homeostasis, requiring new strategies for successful adaptation (Hennessy and Levine 1977; Pfister 1979; Pfister and King 1976). Stress overload, however, can cause pronounced changes in physiology and behavior which have long-term deleterious implications for survival and well-being.

The hypothalamo-pituitary-adrenocortical axis (HPA) plays an important role in promoting homeostasis via adjustments to ongoing physiological processes and behavior during the stress process. In contrast, prolonged

activation of HPA by excess stress can cause pronounced changes in physiology and behavior which have long-term deleterious implications for survival and well-being. Some studies have demonstrated that excess glucocorticoid secretion by stress overload over prolonged periods can impair numerous bodily systems, and an elevated heart rate can lead to cardiovascular disease (Irving et al. 1998; Knardahl and Hendley 1990; McDougall et al. 2000; Esch et al. 2002; D'Angelo et al. 2010; Cohen et al. 2010). Our previous study also demonstrates that glucocorticoids can induce cardiomyocytes apoptosis (Zhao et al. 2007, 2013; Wang et al. 2012).

Although some studies have been carried out on adaptation to stress damage by one stimulus, no discussion has systematically considered the effect and mechanism of the adaptation of HPA activity to mild stress on physiological damage caused by excess stress. We screened the stress neurobiology literature to determine to what degree adaptation of HPA activity to repeated stress meets Thompson and Spencer's criteria for response habituation (De Boer et al. 1990; Natelson et al. 1988; Pitman et al. 1988; Thompson and Spencer 1966). The effect of adaptation on cardiomyocytes damage caused by stress overload was also considered in this study.

Heat shock proteins (HSPs) are a group of phylogenetically conserved proteins found in all prokaryotic and eukaryotic cells. HSP70 also plays an important role in preventing protein aggregation, in degrading unstable and misfolded proteins and in transporting proteins between cellular compartments (Flynn et al. 1989; Beckmann et al. 1990; Hartl and Hayer-Hartl 2002; Murakami et al. 1988; Shi and Thomas 1992). Under conditions of stress, inducible HSPs are highly upregulated by heat shock factors (HSF), which are generated as part of the heat shock response (HSR), to maintain cellular homeostasis and to develop cell survival functions. HIP is an Hsp70-binding cochaperone which was observed as a component of GR complexes and independently identified through its interaction with the ATP binding domain of Hsp70 (Shi and Thomas 1992). In this study, we focused on the role and mechanisms of HSP70 and HIP acting through the GR pathway on adaptation and cardiomyocytes damage.

Materials and methods

Animal treatment procedure

Adult male Wistar rats (180–200 g in weight) were selected to establish the adaptation and stressed animal model using

Galea's restraint stress method (Galea et al. 1997). Thirty rats were divided into different stress level groups; a 0-week group, a 1-week stress group, a 2-week stress group with 4 h/day restraint stress (from 10:00 am to 2:00 pm) and a 3-week stress group with 6 h/day restraint stress (from 9:00 am to 3:00 pm), and a 3-week stress group with 6 h/day restraint stress after 2 week's preconditioning with 4 h/day restraint stress.

Pathological changes detected with Nagar-Olsen staining technique and electron microscopy

To investigate myocardial damage, the myocardial sections were stained according to the Nagar-Olsen staining technique (Li et al. 2009). Nagar-Olsen staining was used to observe the early changes of myocardial injury. The damaged myocardial cells appeared red, normal myocardium appeared yellow or brown yellow, and the nuclei appeared blue. The myocardial sections were deparaffined, hydrated, stained with Harry's hematoxylin for 1 min and then by basic fuchsin for 3 min, and thereafter immersed in acetone-picric acid to enhance the contrast of the stain. For dehydrating and clearing, acetone and dimethylbenzene were used. A microscopic examination (Olympus-BX41 IF, Olympus Co) incorporated with an image analysis software (ImagePro-4, Cold Spring Co.) was employed to determine the area of myocardial ischemia (magnification $\times 200$). The myocardial material was also processed following the protocol of previous study (Zhao et al. 2007). Scanning electron microscopy and high-definition photos (magnification $\times 8,000$) were obtained by the FEI QuantaTM FEG SEM device (FEI Company, Hillsboro, OR, USA).

Detection of plasma corticosterone levels by radioimmunoassay

Corticosterone levels in plasma were determined by established radioimmunoassay. At the end of the experiment, rats were killed by decapitation at 9:00–10:00 in the morning, blood was collected and serum was separated. The levels of serum cortisol were detected by radioimmunoassay as previous study (Leu et al. 2005).

Cell culture and protocol of cell treatment

The embryonic rat heart derived H9C2 cells were purchased from the cell repository of the Chinese Academy of Sciences and cultured in DMEM (Gibco) supplemented with 10 % fetal bovine serum (Gibco), antibiotics (50 U/ml penicillin and 50 μ g/ml streptomycin) (Choi et al. 2008; Will et al. 2008). The cells were seeded in 10^{-8} mol/L GC for 24 h to simulate mild stress, and 10^{-5} mol/L GC treatment for 24 h was used as excess

stress. The cells were treated with 10^{-8} mol/L GC for 24 h before 10^{-5} mol/L GC treatment for 24 h to evaluate the effect of mild stress preconditioning on excess stress-induced cell damage. The cells were seeded at 37°C in a water-saturated atmosphere containing 5 % CO₂ in air. Cells were harvested at various time points after seeding.

Assay for cardiomyocytes injury

Lactate dehydrogenase and creatine kinase-MB activity assay

Cells were seeded at a density of 5×10^4 cells/ml in 200 µl medium per well in 96-well-plates (TPP, Switzerland). Protein samples were diluted with 0.9 % NaCl to a concentration of 45 µmol/L and added to the cells to yield a final concentration of 15 µmol/L. Cells were grown in the presence of 10^{-5} µmol/L GC for 3, 6, 12, and 24 h, and the medium was removed and collected for detection of lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) activity in the supernatants according to the supplier's protocol. Briefly, 200 µl supernatant and 1,000 µl LDH or CK-MB reagent were added to a new 1 cm colorimetric cylinder following 30 min incubation at room temperature in the dark. After incubation, the absorbance was read at a wavelength of 340 nm in a U-2100 spectrophotometer (HITACHI, Japan).

MTT cell viability assay

Cells were treated as described above for the LDH assay. After 24 h, the medium was removed and 200 µl MTT medium (0.5 mg/ml MTT reagent in fresh medium) was added to each well. After incubation for 4 h the MTT reagent was removed and 150 µl dimethyl sulfoxide was added to each well following 10 min gentle shaking. The absorbance was read at a wavelength of 490 nm in a MULTISKAN MK3 (Thermo Electron Corporation, The Netherlands).

Analysis of cardiomyocytes apoptosis with flow cytometry

The detection of cardiomyocytes apoptosis was based on DNA content evaluation with the use of propidium iodide and flow cytometry (Nicoletti et al. 1991). Cardiomyocytes were washed twice with PBS, fixed with 70 % ethanol for 30 min at 4°C, and then, after washing with PBS, were stained with propidium iodide (Sigma, 50 µg/ml) overnight at 4°C. The cardiomyocyte suspensions were analyzed with a FACSCalibur flow cytometer (Becton Dickinson, USA). The percentage of

cells containing subdiploid DNA content, a characteristic of apoptosis, was quantified.

Caspase 3 activity assay

Caspase 3 activity was assessed via a colorimetric assay utilizing specific substrates (Calbiochem). After treatment, the cells were washed once with ice-cold PBS and collected by trypsinization followed by centrifugation. The cellular pellet was resuspended in cell lysis buffer and incubated on ice for 10 min. Lysates were centrifuged for 5 min at 13,000 rpm, and the supernatants were assayed for caspase 3 activity in assay buffer (50 mmol/L HEPES, pH 7.4, 100 mmol/L NaCl, 0.1 % CHAPS, 10 mmol/L dithiothreitol, 0.1 mmol/L EDTA, 10 % glycerol). After addition of DEVD-specific caspase substrate (2 mmol/L), samples were incubated for 60 min at 37 °C and read at 405 nm in an EL-312 Bio-Kinetics microplate reader (Bio-Tek Instruments).

Cell transfection

Transient transfections were performed using LipofectAMINE according to the procedure of the manufacturer (Life Technologies, Inc.). PET-28A-hsp70 was kindly provided by Dr. Zhan Rui. HIP plasmids were kindly provided by Dr. H. Hauser (Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany). Hsp70 and HIP gene were inserted into pCDNA-3.1 vector after polymerase chain reaction amplification resulting in an in-frame fusion of a triple hemagglutinin tag. siRNAs of HIP (sc-40683) and scrambled RNA (sc-37007) were purchased from Santa Cruz and were transfected with oligofectamine (Lifetech) according to the manufacturer's protocol.

Expression knockdown of Hsp70 with KNK437

KNK437, *N*-formyl-3,4-methylenedioxy-benzylidene-*g*-butyrolactam, was purchased from Sigma. KNK437 was administered 24 h before GC treatment. It was used mainly at a concentration of 100 mg/ml (Liu et al. 2012; Koishi et al. 2001).

Western blot

Myocardial tissue was collected and harvested with 1 ml ice-cold RIPA lysis buffer and incubated for 1 h at 4 °C. The insoluble material was removed by centrifugation at 10,000g for 10 min at 4°C. Protein content was determined by Folin-phenol method with BSA as a standard substance. The proteins were then placed in diluted SDS sample buffer and denatured for 5 min at 99 °C before being subjected to 12.5 % SDS-PAGE.

Forty micrograms of total protein were loaded in each lane, subjected to electrophoresis, and subsequently transferred to PVDF membranes (Millipore Corp., Bedford, MA) by electroblotting. The membranes were blocked in TBS buffer (25 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.5) containing 0.05 % Tween-20 and 5 % milk and incubated with a mouse monoclonal antibody against rat HIP (R-19, Santa Cruz), α -Tubulin (BM1452, Boster, china), HSP70 (SC-1060, Santa Cruz) in blocking solution. Membranes were washed in TBS buffer containing 0.05 % Tween-20. Immunoreactive proteins were visualized by enhanced chemiluminescence (ECL) reagents (Santa Cruz, CA) using a horseradish peroxidase-conjugated secondary antibody (1:5,000) (Santa Cruz, CA). Results of representative chemiluminescence were scanned and densitometrically analyzed using ImageMaster VDS system (Amersham, UK) with the help of the Imagequant TL site program.

Co-immunoprecipitation analysis

Myocardial tissue was collected and harvested with 1-ml ice-cold RIPA lysis buffer and incubated for 1 h at 4 °C. After centrifugation at 12,000g for 15 min, the supernatant was mixed with HSP70 antibody (SC-1060, Santa Cruz) or with normal mouse IgG (Santa Cruz). After 16–20 h, 25 μ l of protein G beads (Santa Cruz) were added to the antibody-cell suspension solution for an additional 2 h. Beads were washed with wash buffer (220 mmol/L NaCl, 50 mmol/L Tris-HCl pH 7.5, 1 mmol/L PMSF, 0.5 % *n*-octylglucoside) four times by inverting the tubes three times followed by 5 min incubation on ice and another three washes; 100 μ l of 3 $\times$ sample buffer was added to beads and incubated on ice for 1.5 h. Beads were boiled for 5 min and supernatants were resolved on a 12 % acrylamide gel by electrophoresis at 90 V. Gels were presoaked in transfer buffer (Tris, glycine, 0.015 % SDS, 20 % methanol) for 30 min at 4 °C and then transferred to nitrocellulose membranes (30 V, 16 h) using a transfer cell (Bio-Rad).

Luciferase assay

For a promoter activity assay, cells were cotransfected with Hsp70 or HIP, pRL-SV40 (Promega Corporation) and pGL3 basic-GR promoters. Luciferase activity was measured with a Luciferase Assay System kit (Promega Corporation), as specified by the manufacturer, and normalized to pRL-SV40 expression.

Statistical analyses

All experimental data were replicated a minimum of three times and expressed as mean $\pm$ SEM. Statistical analysis was performed by one way ANOVA using SPSS version 11.0 software, and multiple comparisons were done by Student *t* test. Differences were considered significant at $P\leq 0.05$.

Results

Mild repeated restraint stress mitigated stress-overload-induced cardiomyocyte injury in animal model

The animal adaptation model was made by 4 h/day restraint stress, and the myocardial damage model were made by 6 h/day restraint stress. To investigate myocardial damage, the myocardial sections were stained according to Nagar-Olsen staining technique and electron microscopy. Nagar-Olsen staining could be used to observe the early changes of myocardial injury. The damaged myocardial cells appeared red, normal myocardium appeared yellow or brown yellow, and the nuclei appeared blue. As shown in Fig. 1a, b, the myocardial cells appeared yellow, and this showed that 4 h/day restraint stress did not induce myocardial damage. As shown in Fig. 1c, the myocardial cells appeared red, and this showed that 6 h/day restraint stress could induce myocardial damage. As shown in Fig. 1d, the pathological slice viewed with routine light microscopy showed acid fuchsin-positive cells decreased in the myocardium of excess stress group with mild stress preconditioned rats compared with only excess stress group with 6 h restraint stress per day (Fig. 1c). Also, the specific staining grade became lighter and the stain area became smaller (Fig. 1d), indicating that the severity of myocardium injury decreased. The changes of myocardial ultrastructure in stressed rats observed by electron microscopy also showed that mild repeated stress mitigated stress-overload-induced cardiomyocyte injury (Fig. 1e–g).

Data also showed that chronic repeated mild restraint stress led to mild GC response according to the criteria of habitation identified by Thompson and Spencer compared with the only excess stress group (Fig. 2a). The data also showed that a preconditioning mild stress could mitigate the excess HPA response to severe stress. Further experiments were performed; cardiomyocyte injuries were evaluated by serum LDH activity and CK-MB activity. According to Fig. 2b, c, the serum LDH activity and CK-MB activity of stress group

Fig. 1 Pathological change in myocardium of stressed rats. **a–d** Early pathological change in myocardium of stressed rats by Nagar-Olsen staining. Basic fuchsin-positive cells were defined as those staining bright red, clearly distinct from the yellow staining of the control, with the stained areas and intensity representing the severity of injury (magnification $\times 200$). Pathologic tissue slices showed mild stress treatment can decrease stress-induced cardiac injury. **a** Control; **b** mild stress for 2 weeks with 4 h/day restraint stress; **c** excess stress with 6 h/day restraint stress for 3 weeks; **d** excess stress preconditioned with mild stress. **e–g** Changes of myocardial ultrastructure in stressed rats by electron microscopy. The left

ventricular muscle (1 mm^3) from rats was ultrathin sectioned and stained after routine treatment and then was examined on the electron microscope. Micrographs showed that mitochondria and nuclei were normal in control group (**e**), mitochondrial swelling and nuclear chromatin margination occurred in stress groups with 6 h/day restraint stress for 3 weeks (**f**), and mitochondrial swelling occurred slightly in 6 h/day excess restraint stress groups preconditioned with 4 h/day mild stress (magnification $\times 8,000$). The data showed that preconditioned mild stress treatment can decrease stress-overload-induced cardiac injury

increased significantly. These data showed that cardiac injury was induced by restraint stress. However, when stress rats were pretreated with mild restraint stress, the cardiomyocyte injury caused by restraint stress reduced resulting in reduced release of serum LDH and CK-MB activity compared with the restraint stress group.

Mild GC treatment mitigated high-level GC-induced cell injury in H9C2 cardiomyocyte cells

The animal model had shown that mild repeated restraint stress could mitigate stress-overload-induced cardiomyocyte injury; 10^{-8} mol/L GC treatment was used as mild stress, and 10^{-5} mol/L GC treatment was used as stress overload in H9C2 to observe whether preconditioning mild stress could mitigate stress-overload-induced cardiomyocyte injury in the cell model. The results of LDH, CK-MB, apoptosis rate and caspase 3 activity showed that preconditioning mild stress with

10^{-8} mol/L GC treatment would migrate cell damage with 10^{-5} mol/L GC treatment (Fig. 3a–d).

HSP70, HIP expression, and interaction between HSP70 and HIP increased during mild repeated stress

HSP70 plays an important role in preventing protein aggregation, degrading unstable and misfolded proteins and transporting proteins between cellular compartments (Flynn et al. 1989; Beckmann et al. 1990; Hartl and Hayer-Hartl 2002; Murakami et al. 1988; Shi and Thomas 1992). HIP is also an Hsp70-binding cochaperone. We presumed that HSP70 and HIP may play an important role to improve adaptation to stress. So the expression of HSP70, HIP, and interaction between HSP70 and HIP during mild repeated stress were detected with western blot and co-immunoprecipitation. The results of the western blots showed that the expression of HSP70 and HIP

increased during mild restraint stress and mild GC treatment. The expression changes of HSP70 and HIP were also examined at different treatment time periods with mild stress (Fig. 4a–d). Co-immunoprecipitation (co-IP) experiments were used to determine whether HSP70 and HIP interact during mild stress. The results demonstrate that the interaction between HSP70 and HIP increased during mild restraint stress in animals (Fig. 5a) and 10^{-8} mol/L GC treatment of cultured cells (Fig. 5b).

Hsp70 protected cardiomyocytes from GCs-induced damage

HSP70 expression and interaction with HIP increased during mild repeated stress. So, we presumed that the increase of HSP70 expression may play an important role in the process of mild repeated stress mitigated stress-overload-induced cardiomyocyte injury. To investigate the role of HSP70 in cardiomyocytes during GC treatment, HSP70 expression was increased with pcDNA-3.1-Hsp70 in H9c2 cardiomyocytes (Fig. 6). According to Fig. 6b–e, when cultured H9C2 cardiomyocytes were treated with 10^{-5} M GC for

12 h, cell survival rate assayed by MTT method apparently declined (6C). Meanwhile, the LDH activity in culture media (6B), apoptosis rate (6D), and caspase-3 activity (6E) of the H9C2 cardiomyocytes increased significantly. These data showed that cardiac injury was induced by GC. However, when cardiomyocytes were infected by pcDNA-3.1-Hsp70 to elevate HSP70 content, the cellular injury caused by GC treatment was reduced resulting in increased cell viability and reduced release of LDH to culture media. Therefore, it was confirmed that HSP70 played a protective role for H9C2 cardiomyocytes under GC treatment (Fig. 6b–e).

In order to determine whether HSP70 is necessary for GC-induced cell injury, KNK437, an HSP70 inhibitor was used to mediate the expression of HSP70 in H9C2 cells, a rat cardiac muscle cell line. KNK437 substantially reduced basal HSP70 protein abundance (Fig. 7a). More important, KNK437 treatment could lead to more serious cell damage under GC treatment, as assayed by serum LDH activity (Fig. 7b), cell survival rate (Fig. 7c), apoptosis rate (Fig. 7d), and caspase-3 activity (Fig. 7e), indicating that HSP70 could protect H9C2 cells from GC-induced cell damage.

Fig. 2 Effects of preconditioned mild stress on cardiomyocytes injury induced by excess restraint stress. **a** Blood serum GC levels of each groups were detected by radioimmunoassay (RIA). The results showed that chronic repeated mild restraint stress (4 h/day) led to mild GC response ($p < 0.05$), while excess stress (6 h/day) led to excess GC response ($p < 0.05$), but rats preconditioned with mild restraint stress had mild GC response after stress overload treatment ($p < 0.05$). The data showed that preconditioned mild stress could mitigate the excess HPA

response to severe stress. **b, c** The serum LDH activity and CK-MB activity of stress group increased significantly, the serum LDH activity and CK-MB activity of rats (adaptation+stress group) increased significantly compared with rats of restraint stress group. The data showed that preconditioning mild stress would migrate stress-overload-induced cell damage. The bar graphs represented the mean $\pm$ SEM of results from six replicate experiments (**a, b, c**). * $p < 0.05$ as compared with control. # $p < 0.05$ as compared with stress group

Fig. 3 Effects of preconditioning 10^{-8} M GC treatment on cardiomyocytes injury induced by 10^{-5} M GC. The embryonic rat heart derived H9c2 cells were seeded in 10^{-8} mol/L GC for 24 h to simulate mild stress, and 10^{-5} mol/L GC treatment for 24 h was used as excess stress. The cells were treated with 10^{-8} mol/L GC for 24 h before 10^{-5} mol/L GC treatment for 24 h to evaluate the effect of preconditioning mild stress on excess stress-induced cell damage. The enzyme activity of

LDH (a), the enzyme activity of CK-MB (b), the apoptotic rate (c) and the caspase 3 activity (d) were detected during treatment. Data showed that preconditioning mild stress with 10^{-8} mol/L GC treatment would migrate cell damage with 10^{-5} mol/L GC treatment. The bar graphs represented the mean $\pm$ SEM of results from six replicate experiments (a, b, c, d). * $p < 0.05$ as compared with control. [#] $p < 0.05$ as compared with stress group

Hsp70 interacting with HIP plays its protective role through inhibition GR transcription activity

To more thoroughly understand the protective role about HSP70, HSP70 was overexpressed along with knockdown

of HIP with SiRNA (Fig. 8a). The results showed that HSP70 can protect H9C2 cells from GC-induced cell damage in the presence of HIP, but it cannot protect H9C2 cells from GC-induced cell damage following the knockdown of HIP expression (Fig. 8b–e). This result implied that HSP70's

Fig. 4 HSP70 and HIP expression during mild repeated restraint stress and mild GC treatment. The rats treated with 4 h/day restraint stress were used as mild repeated restraint stress, and the cells were seeded in 10^{-8} mol/L GC for 24 h to simulate mild stress. HSP70 and HIP

expression were detected by western blot. a, b The HSP70 and HIP expression increased with time during 0 (control), 1, and 2 weeks mild restraint stress. c, d The HSP70 and HIP expression also increased with time during 0 (control), 6, 12, and 24 h 10^{-8} mol/L GC treatment

Fig. 5 The interaction between HSP70 and HIP during mild repeated restraint stress and mild GC treatment. The rats treated with 4 h/day restraint stress were used as mild repeated restraint stress, and the cells were seeded in 10^{-8} mol/L GC for 24 h to simulate mild stress. The interaction between HSP70 and HIP was detected by co-

immunoprecipitation. **a** The interaction between HSP70 and HIP increased with time during 0 (control), 1, and 2 weeks mild restraint stress. **b** The interaction between HSP70 and HIP increased with time during 0 (control), 6, 12, and 24 h 10^{-8} mol/L GC treatment

protective role on GC-induced cell damage must depend on its interaction with HIP. A luciferase reporter gene system showed that HIP and HSP70 could decrease the GR

transcription activity. This may be a mechanism of HSP70 and HIP protection of H9C2 cells from GC-induced damage (Fig. 8f).

Fig. 6 Effects of overexpression of Hsp70 in cardiomyocytes on cell injury induced by GC treatment. **a** Overexpression of Hsp70 in cardiomyocytes by transient infection with pCDNA-Hsp70. Cardiomyocytes were infected with pCDNA-Hsp70 for 2 h and cultured for 24 h. Then HSP70 content in cardiomyocytes was detected by western blot method. Alpha (α)-tubulin levels were measured as an internal control. **b–e** Cardiomyocytes were infected with pCDNA-Hsp70 or non-coding constructs for 2 h and maintained in culture media for 24 h. Then, cardiomyocytes were cultured in fetal bovine serum free media for

12 h and treated with 10^{-5} mol/L GC for 24 h. The enzyme activity of LDH (**b**), the survival rate of cardiomyocytes determined by MTT assay (**c**), apoptotic rate determined by flow cytometry (**d**) and caspase 3 activity (**e**) were assayed subsequently. Data showed that the cellular injury caused by GC treatment was reduced by HSP70 overexpression. The bar graphs represented the mean $\pm$ SEM of results from six replicate experiments (**b, c, d, e**). * $p < 0.05$ as compared with control. # $p < 0.05$ as compared with GC+Vector

Fig. 7 Effects of inhibition of Hsp70 expression in cardiomyocytes on cell injury induced by GC treatment. **a** Inhibition of HSP70 expression in cardiomyocytes with KNK437. Then HSP70 content in cardiomyocytes was detected by western blot method. Alpha (α)-tubulin levels were measured as an internal control. **b–e** The enzyme activity of LDH (**b**), the survival rate of cardiomyocytes (**c**), apoptotic rate (**d**), and caspase 3 activity (**e**) were assayed subsequently. Data showed that the cellular injury caused by GC treatment was increased with the decrease of HSP70 expression. The bar graphs represented the mean $\pm$ SEM of results from six replicate experiments (**b, c, d, e**). * $p < 0.05$ as compared with control. # $p < 0.05$ as compared with GC

Discussion

Organisms are subjected to stress in everyday life. Stress is defined as an adaptive physiological response to disruption of homeostasis. Moderate stress load can invoke protection, though stress overload can cause injury or contribute to diseases, such as diabetes, gastric ulcer, obesity, cancer, and Parkinson's disease. To be clear, glucocorticoid responses are required for survival and adaptation. The relationship between glucocorticoid secretion and adaptation (e.g., in terms of appropriate behavioral performance) is often described as an "inverted-U" shaped curve, wherein an optimal level of glucocorticoid signaling is required to produce the most effective organismal response (De Kloet et al. 1998). Consistent with a number of previous studies, we found that chronic and repeated mild stress could mediate the HPA response in this study. This indicated that restraint stress of 4 h per day for 2 weeks could lead to adaptation to stress.

The cardiovascular system is the primary target organ attacked by stress (D'Angelo et al. 2010; Cohen et al. 2010). Many kinds of cardiovascular diseases, such as hypertension, atherosclerosis, coronary artery disease and myocardial infarction are closely related to the trigger of the stress response (Esch et al. 2002; McDougall et al. 2000; Irving et al. 1998; Knardahl and Hendley 1990). Our previous studies also

observed that myocardial injury existed in rats exposed to restraint stress and with increased GC levels for three weeks (Zhao et al. 2013; Wang et al. 2012). The effect of adaptation on cardiomyocytes damage caused by the excess stress was discussed in this study. Interestingly, we found that mild repeated stress mitigated stress-overload-induced cardiomyocyte injury. This may be the result of the decline of GC induced by a preconditioning mild repeated stress and the increase of HSP70 expression during mild repeated stress.

Glucocorticoids (GCs) are effective antileukemic agents because of their ability to induce growth arrest and evoke apoptosis of normal thymocytes, immature peripheral T cells and many leukemic cells. GCs activate the GC receptor (GR), a transcription factor that regulates expression of genes involved in modulating GC-induced actions such as immunosuppression, anti-inflammation and apoptosis (Vakili and Cattini 2012; Shen and Young 2012; Selye and Horava 1953; Pimenta et al. 2012).

HSP70, the most conserved of the HSP families, includes the cytosolic and nuclear constitutive Hsc70 (Hsp73) and the stress-inducible Hsp70 (Hsp72) proteins, the endoplasmic reticulum (ER) Bip (Grp78), and the mitochondrial mt-Hsp70 (Grp75) protein (Hunt and Morimoto 1985; Tavarria et al. 1996; Daugaard et al. 2007). Heat shock protein 70 (Hsp70) works as

Fig. 8 Effect of overexpressed HSP70 with or without HIP expression on cardiomyocytes injury induced by GC treatment. **a** Overexpression of Hsp70 in cardiomyocytes by transient infection with pCDNA-Hsp70, and knockdown of HIP with SiRNA. Then HIP and HSP70 content in cardiomyocytes were detected by western blot method. Alpha (α)-tubulin levels were measured as an internal control. **b–e** Cardiomyocytes were infected for 2 h and maintained in culture media for 24 h. Then, cardiomyocytes were cultured in fetal bovine serum free media for 12 h and treated with 10^{-5} mol/L GC for 24 h. The enzyme activity of LDH (**b**), the survival rate of cardiomyocytes (**c**), apoptotic rate (**d**) and caspase 3 activity (**e**) were assayed subsequently. The data showed that HSP70

can protect H9C2 cells from GC-induced cell damage with HIP overexpression, but it cannot protect H9C2 cells from GC-induced cell damage with the knockdown of HIP expression. The *bar graphs* represented the mean $\pm$ SEM of results from six replicate experiments (**b, c, d, e**). $^*p < 0.05$ as compared with control. $^{\#}p < 0.05$ as compared with HSP70 transfection group. **f** Luciferase assay of GR. H9C2 cells were cotransfected with Hsp70 or HIP, pRL-SV40 (Promega Corporation) and pGL3 basic-GR promoters. Luciferase activity was measured with a Luciferase Assay System kit. The data showed that HIP and HSP70 could decrease the GR transcription activity. $^*p < 0.05$ as compared with control

a molecular chaperone and plays an important role in the adaptive response. A cellular level stress response has been observed in nearly all organisms, and its characteristic feature is the induction of Hsps. Hsps encompass several families of cytoprotective proteins. HSP plays an important role in preventing protein aggregation, degrading unstable and misfolded proteins and transporting proteins between cellular compartments (Flynn et al. 1989; Beckmann et al. 1990; Hartl and Hayer-Hartl 2002; Murakami et al. 1988; Shi and Thomas 1992). This may be one reason for how mild repeated stress mitigates cardiomyocyte injury induced by stress overload. The protective role of Hsp70 was demonstrated in H9C2 cells with over expression and inhibition of Hsp70 expression.

Hsp70 is a member of GR complexes, and HIP is also an Hsp70-binding cochaperone. HIP was confirmed as a component of PR and GR complexes and independently identified through its interaction with the ATP binding domain of Hsp70 (Nelson et al. 2004; Prapapanich et al. 1998.). HIP stabilizes

the ADP-bound conformation of Hsp70, thus promoting Hsp70 binding to and refolding of misfolded model substrates (Velten et al. 2000, 2002; Nollen et al. 2001). In the present study, we found that Hsp70 plays its protective role through inhibition of GR transcription activity when in a complex with HIP.

Taken together, our results demonstrate that adaptation could migrate excess restraint stress and high level GC treatment induced cardiomyocyte injury. This may be the result of a mild GC response preconditioned by mild stress or increase of HSP70 induced by mild stress. Further study showed that Hsp70 plays its protective role through inhibition of GR transcriptional activity when complexed with HIP. These data indicated that HSP70 protected the cardiomyocytes from GC-induced damage, and that HSP70 may constitute a new therapeutic target for stress-induced myocardium injury.

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