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Full-length article

Gene transfer of heat-shock protein 20 protects against ischemia/reperfusion injury in rat hearts[1]

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Key words

reperfusion injury; adenovirus; apoptosis; myocardial infarction; heat-shock proteins

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Abstract

Aim: To explore whether overexpression of HSP20 in the myocardium could protect against ischemia/reperfusion injury in rats. **Methods:** Rat hearts were injected with vector, recombinant adenovirus encoding green fluorescent protein (Ad.GFP) or recombinant adenovirus encoding wild-type HSP20 (Ad.HSP20) in the left ventricle. Four days later, hearts were removed and expression of HSP20 was measured in the left ventricle. Subsets of animals in the vector-, Ad.GFP- , and Ad.HSP20-treated groups were subjected to 20-min ischemia and 120-min reperfusion. Myocardial injury was evaluated by infarct size and level of serum cardiac troponin T and creatine phosphokinase. Apoptosis of cardiomyocytes was determined by TUNEL staining. Cardiac function was evaluated by hemodynamic indexes. **Results:** Infarct size and serum cardiac troponin T and creatine phosphokinase levels were significantly reduced in Ad.HSP20-treated hearts compared with vector- and Ad.GFP-treated hearts. The ratio of TUNEL-positive cardiomyocytes to total number of cardiomyocytes in the Ad.HSP20 group was significantly reduced as compared with the vector and Ad.GFP groups. Left ventricular end systolic pressure, and maximal rate of pressure increase ($+dp/dt_{max}$) and decrease ($-dp/dt_{min}$) values were increased significantly, while left ventricular end diastolic pressure was decreased significantly in Ad.HSP20-treated hearts compared with vector- and Ad.GFP-treated hearts. **Conclusion:** These data indicate that the cardioprotective effects of HSP20 may contribute to the reduction of myocardial necrosis and apoptosis in ischemia/reperfusion injury in rats.

Introduction

Members of the small heat shock protein (sHSP) family, including HSP20, HSP25, HSP27, αB-crystallin, and myotonic dystrophy kinase binding protein, are expressed in muscle tissues and share a homologous sequence of approximately 80–100 amino acids at the C-terminus, known as the α crystallin domain[1,2]. The past decade has witnessed the discovery of new mammalian sHSP, of which HSP20 is the best characterized. HSP20 was co-purified from skeletal muscle with αB-crystallin and HSP27 by affinity chromatography on a column of immobilized antibodies against αB-crystallin[3]. Exposure of rat diaphragm tissue to heat stress *in vitro* results in the redistribution of HSP20, as well as αB-crystallin and HSP27, from the cytosol into insoluble fractions, and enhanced dissociation of the aggregated form to the small form, which is characteristic of stress proteins[4]. Stable overexpression of HSP20 in Chinese hamster ovary cells results in enhanced survival after heat shock, which is similar to results obtained with αB-crystallin[5]. Chu *et al*[6] were the first to identify the *de novo* phosphorylation of cardiac HSP20 in mouse cardiomyocytes after prolonged activation of the β-adrenergic signaling pathway. The adenovirus-mediated overexpression of HSP20 in adult rat cardiomyocytes increases cell contractility, which indicates that HSP20 is involved in the regulation of myocardial contractility[6,7].

Heat shock proteins have been implicated in modulating the cellular response to many stressors, and as molecular

chaperones in suppressing the aggregation or assisting in the refolding of partially denatured proteins. They usually protect against ischemic/reperfusion (I/R) injury *in vitro*[8–11] and *in vivo*[12–14]. However, whether gene transfer of the HSP20 gene into the beating heart produces a myocardial protective effect has not been shown. In the present study, we transferred the HSP20 gene through a recombinant adenovirus encoding HSP20 into the myocardium, and showed that HSP20 protected against I/R injury, probably by reducing myocardial apoptosis and necrosis in rats.

Materials and methods

Animals and experimental protocols Male adult Sprague-Dawley rats (230 g–280 g) received a standard diet and free water. The treatment of the animals and experimental protocols adhered to the guidelines of the Health Sciences Center of Peking University (Beijing, China). The animals were allowed to readjust to the new housing environment for 1 week before the experiments.

Rats were assigned randomly to 4 groups. In the no-vector control group, the chest was opened and injected with saline. The I/R control group was also injected with saline. The third group received the recombinant adenovirus encoding wild-type HSP20 (Ad.HSP20), and the fourth group received the recombinant adenovirus encoding green fluorescent protein (Ad.GFP).

Construction of recombinant adenoviruses The recombinant Ad.HSP20 and Ad.GFP were prepared as described previously[15]. Adenovirus was propagated in 293 cells and purified by 2 rounds of CsCl density ultracentrifugation (4 °C, 13 000×*g* for 105 min and 16 h, respectively). Viral stocks were then desalted through a PD-10 desalting column (Amersham Biosciences, Buckinghamshire, UK) into a Tris-buffered solution (10 mmol/L Tris, pH 8.0, 2 mmol/L $MgCl_2$ and 4% sucrose)[16], plaque-titered, aliquoted, and stored at -80 °C with 4% sucrose until use.

***In vivo* intracoronary delivery of adenoviruses** The surgical procedures were carried out as described previously[17]. Donor rats were anesthetized with sodium pentobarbital (50 mg/kg, ip). Further injections were given as needed throughout the surgical procedure. Animals were placed supine on a thermoregulated table (37 °C) . The surgery was carried out under sterile conditions. The animals were intubated and ventilated on a positive-pressure ventilator. The tidal volume was set at 1.5 mL–2.5 mL, and the respiratory rate was adjusted to within the range of 80 cycles/min to 90 cycles/min to maintain normal arterial p_{aO2}, p_{aCO2}, and pH. The chest was entered through a left intercostal approach. Before virus infusion, adenosine (0.15 mg), lidocaine (0.03 mg), and heparin (50 U) were administered via the jugular vein. With the use of a 26 gauge needle, 200 μL diluted replication-deficient adenovirus (2.2×10^{10} pfu) or 200 μL sterile saline were injected from the apex of the left ventricle into the left ventricular cavity while the aorta and pulmonary arteries were clamped just above the aorta root. The clamp was maintained for 15 s when the heart pumped against a closed system. After injection, the exposed heart was monitored for 5 min for resumption of normal sinus rhythm. Hemodynamic indices were measured and electrocardiography was carried out throughout the experimental period.

Myocardial infarction protocol Four days after the injection of saline or virus, the animals were re-anesthetized and ventilated artificially with room air. The thorax was reopened and the heart were exposed to identify the left anterior descending coronary artery (LAD). A 7-0 silk suture was passed around the LAD with an atraumatic needle just 4 mm inferior to the left auricle, and the artery was occluded by snaring with a vinyl tube through which the ligature had been passed. The coronary artery was occluded by pulling the snare tight and securing it with a hemostat. Ischemia was confirmed by myocardial blanching and electrocardiography evidence of injury. After 20-min ischemia, the ligature was released and the heart was reperfused for 2 h. Reperfusion was identified by an obvious ST segment change.

Measurement of infarction At the end of the infarction protocol, the ligature around the LAD was retightened and 0.1 mL of 10% Evans blue dye was injected as a bolus into the left ventricle (LV) cavity with a 26-gauge needle positioned in the apex of the heart. When the eyes turned blue, the animals were euthanized immediately, the heart was excised and rinsed in water to remove excess dye, the atria and right ventricular free wall were removed, and the remaining LV was frozen. The LV was then cut from apex to base into 4–6 transverse slices of 2 mm thick. Each slice was weighed and then incubated in 4% triphenyltetrazolium chloride solution (TTC) in isotonic pH 7.4 phosphate buffer at 37 °C for 30 min. The slices were subsequently fixed in 10% formalin solution for 24 h. Viable tissue (red-stained by the TTC) was distinguished easily from the infarcted regions (pale or unstained by the TTC) and the risk area (unstained by Evans blue). The total slice area, the infarcted area, and the risk area of each slice were determined by computer-assisted planimetry (Leica Qwin image analysis software; Leica, Cambridge, UK). During planimetry, the operator was blinded as to the type of animal. The ratios of risk area to total slice area, infarct area to total slice area, and infarct area to risk

area were calculated and multiplied by the weight of the slice to determine risk and infarct weight per slice. Infarct size was expressed as a proportion of LV mass or risk area mass.

Hemodynamic studies Hemodynamic measurements were taken at 0 min, 10 min and 20 min ischemia and 30 min, 60 min and 120 min reperfusion. A 1.5 F micronanometer-tipped catheter was advanced into the LV through the right carotid artery. The heart rate, blood pressure, left ventricular end diastolic pressure (LVEDP), left ventricular end systolic pressure (LVESP) and maximal rates of pressure increase ($+dp/dt_{max}$) and decrease ($-dp/dt_{max}$) were recorded on a polygraph (NEC San-ei Instruments, Japan).

Measurement of serum cardiac troponin T (cTnT) and creatine phosphokinase (CK) levels At the end of the myocardial I/R experiment, a 1-mL blood sample was obtained from the carotid cannula, stored at 4 °C for 30 min, and centrifuged at 3000×*g* for 10 min. The serum was stored at -40 °C prior to analysis. The concentration of serum cTnT was determined by the short-turn-around-time (STAT) assay (Roche Diagnostics, Basel, Switzerland), with use of the Roche Elecsys 2010 immunoassay analyzer (Roche Diagnostics). The serum was also analyzed spectrophotometrically for CK activity (Roche Diagnostics).

Western blot analysis Three hearts from each of the experimental groups were used separately for measurement of HSP20 by Western blot analysis. After 4 d of injection of saline or virus, the hearts were removed quickly, and the LV was separated and frozen in liquid nitrogen. The frozen LV tissue was homogenized in protein extraction buffer containing 20 mmol/L Tris-HCl, pH 7.4, 1% Trion X-100, 150 mmol/L NaCl, 1 mmol/L ethylenediaminetetracetic acid, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L NaF, 1 mmol/L Na_3VO_4 and 0.1 mmol/L phenylmethylsulfonyl fluoride. Aliquots were resolved on sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (Schleicher & Schuell, Keene, NH, USA) and incubated with primary polyclonal anti-HSP20 antibodies (1:1000) (presented by Prof Rui-ping XIAO, NIH, USA), which recognized HSP20, at 4 °C overnight. Bound antibodies were detected using a secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Inc, CA,USA) and visualized by use of an enhanced chemiluminescence kit (SuperSignal® West Pico Trial Kit, Pierce Biotechnology, Inc, IL, USA) and exposed to X-ray film for the appropriate time.

Terminal dUTP nick-end labelling staining Hearts were isolated from each group after I/R for analysis using the terminal dUTP nick-end labeling (TUNEL) assay. Tissue samples were fixed in a 4% paraformaldehyde solution, paraffin embedded, and cut transversely into 6-μm sections. The assay was operated according to the manufacturer's instructions (DeadEnd™ Fluorometric TUNEL System; Promega, WI, USA). Stained samples were analyzed using a confocal microscope; at least 500 cells were counted in randomly selected views.

Statistical analysis Data were expressed as mean±SD. Differences were analyzed for significance by one-way repeated-measures ANOVA and further analyzed with the use of the Newman-Keuls test for multiple comparisons between treatment groups. The results were considered significant at $P<0.05$.

Results

Expression of HSP20 Intraventricular injection of Ad.HSP20 *in vivo* resulted in increased HSP20 expression in the LV as compared with vector and Ad.GFP treatments (Figure 1A). The hearts treated with Ad.GFP showed only low-level of HSP20 expression, which indicates that treatment with viral vectors has no significant effect on HSP20 expression in the rat myocardium. LV treated with Ad.HSP20 showed homogenous expression of GFP, whereas those treated with vector showed no background fluorescence (Figure 1B).

Myocardial infarction Ad.HSP20-treated hearts showed a significant reduction in infarct size (39.2%±4.3% risk area) compared with vector- and Ad.GFP-treated hearts (56.3%±2.9% and 54.9%±8.1%, respectively; $P<0.01$; Figure 2A). Infarct size did not differ between vector- and Ad.GFP-treated hearts ($P>0.05$). A similar result was observed when infarct size was expressed as a proportion of LV (Figure 2B). Both results suggest that the reduced infarct size observed in Ad.HSP20-injected hearts is entirely due to the overexpression of HSP20. However, the risk areas (% of LV) were not significantly different among the groups (ie 53.2%±6.5%, 57.9%±7.3%, and 56.4%±7.5% in the vector-, Ad.GFP-, and Ad.HSP20-treated groups, respectively; $P>0.05$; Figure 2C).

HSP20 gene delivery reduced serum cTnT and CK levels Ad.HSP20-treated hearts showed a significant reduction in cTnT release (2.2 μg/L±1.7 μg/L) compared with vector- and Ad.GFP-treated hearts (12.9 μg/L±3.2 μg/L, and 11.8 μg/L±3.1 μg/L, respectively; $P<0.01$; Figure 3A). Similar results were observed for CK release (Figure 3B).

HSP20 gene delivery attenuated apoptosis in the acute ischemia/reperfusion rat model Figure 4A shows representative apoptotic cardiomyocytes identified by TUNEL staining in the I/R-injured region. The ratio of TUNEL-positive

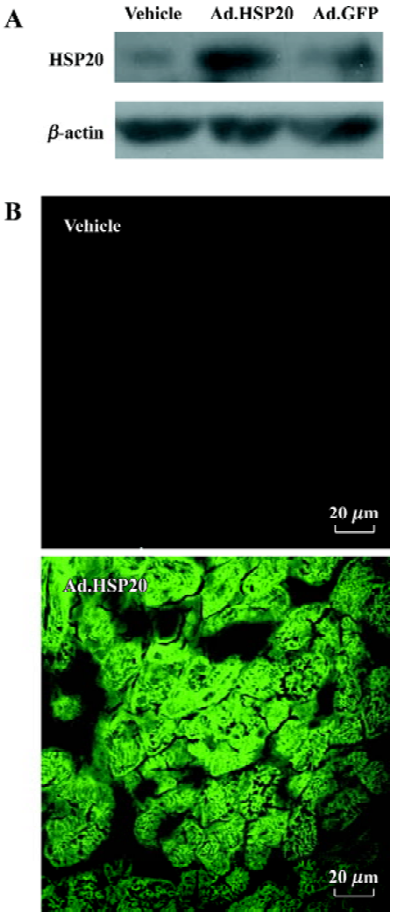

Figure 1. Expression of adenovirus-mediated gene delivery 4 d after intracoronary gene delivery. (A) Western blot showing expression of HSP20 proteins and β-actin after intracoronary delivery of vector, recombinant adenovirus encoding wild-type HSP20 (Ad.HSP20) or recombinant adenovirus encoding green fluorescent protein (Ad.GFP). (B) Representative image of high efficiency of GFP expression in hearts compared with vector after injection of Ad.HSP20.

cardiomyocytes to total number of cardiomyocytes in the Ad.HSP20 group was significantly reduced as compared with the vector and Ad.GFP groups (15.4%±3.2% *vs* 25.7%±4.5% and 27.6%±2.2%, $P<0.01$; Figure 4B).

HSP20 gene delivery improved cardiac function *in vivo* LVEDP, LVESP, $+dp/dt_{max}$ and $-dp/dt_{max}$ values are shown in Figure 5. All parameters were comparable among the 3 groups before and during ischemia and during reperfusion. All parameters, except LVEDP, declined in value after ischemia. LVEDP in HSP20-treated rats was significantly decreased after 60-min reperfusion compared with that in vector- and Ad.GFP-treated rats ($P<0.05$, Figure 5A). LVESP, $+dp/dt_{max}$, and $-dp/dt_{max}$ values in HSP20-treated rats were significantly increased after 60-min reperfusion compared with those in vector- and Ad.GFP-treated rats ($P<0.05$, Figure 5B, 5C, 5D).

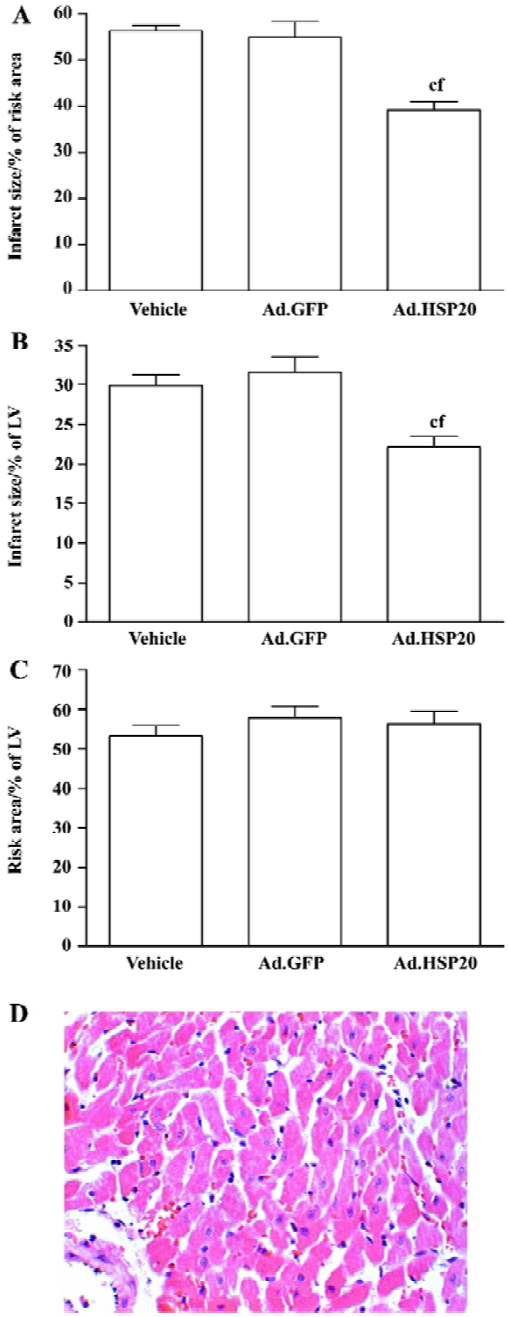

Figure 2. Effect of HSP20 gene delivery on infarct size. (A) Ratio of infarct sizes to risk areas. (B) Ratio of infarct sizes to left ventricle. (C) Ratio of risk areas to left ventricle. $n=6$. Mean±SD. $^{c}P<0.01$ *vs* Ad.GFP. (D) Typical reperfusion injury was observed in all groups by high-power microscopy (×40).

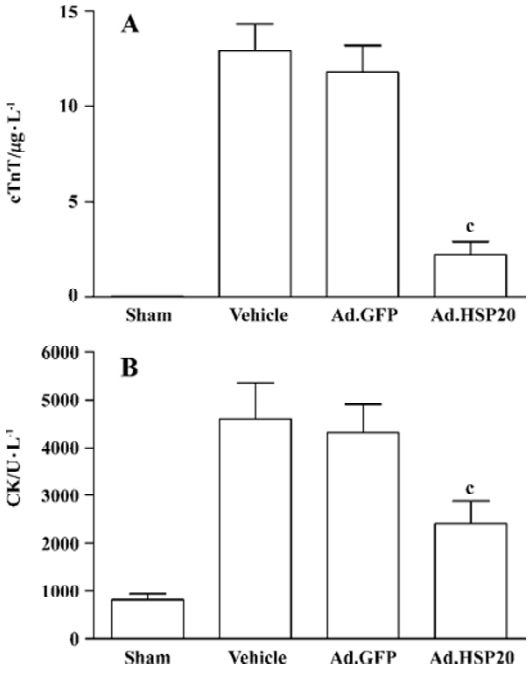

Figure 3. (A) Alterations in the serum levels of cardiac troponin T (cTnT). (B)Alterations in the serum levels of creatine phosphokinase (CK). n=6. Mean±SD. ^cP<0.01 *vs* Ad.GFP.

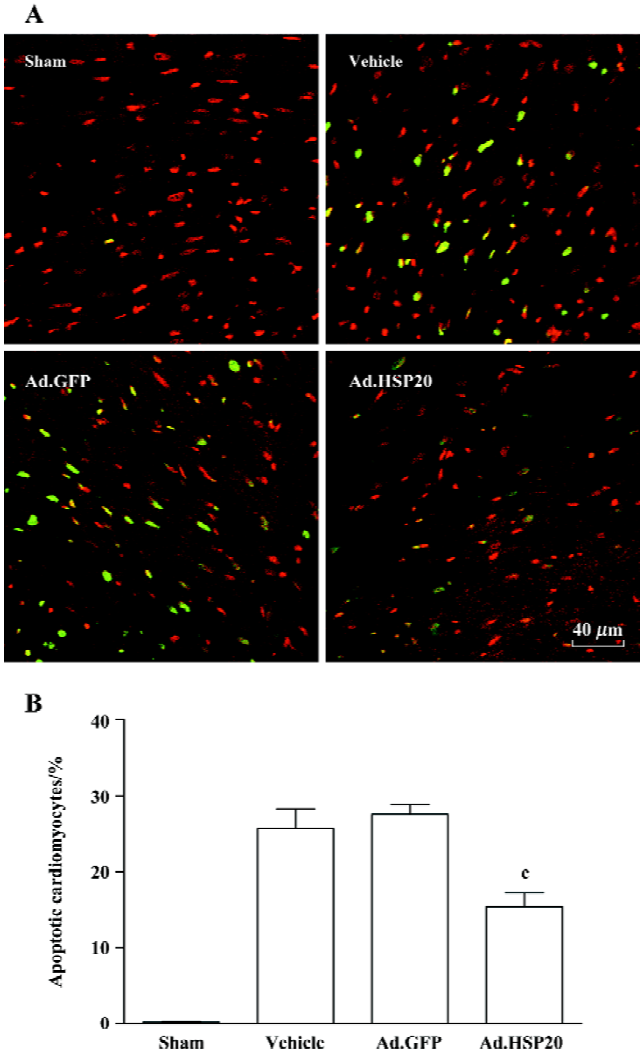

Figure 4. Terminal dUTP nick-end labelling staining showed *in vivo* cardiomyocyte apoptosis after 20-min ischemia and 120-min reperfusion. (A) Cardiomyocytes are depicted by red fluorescence from propidium iodide. Yellow fluorescence showed TUNEL-positive nuclei. (B) Proportion of positive TUNEL-stained nuclei in tissue sections from the no-vector control group or rat hearts exposed to ischemia/reperfusion under different treatments. n=3–4. Mean±SD. ^cP<0.01 *vs* Ad.GFP.

Discussion

Gene therapy has emerged as a genuine alternative therapy in coronary artery disease, including ischemic heart disease. One of the commonly used intramyocardial gene transfer methods is direct intramyocardial injection. There have been a few promising trials involving the use of direct intramyocardial injection in this area[18,19]. However, the technical problems with this method are that only a small volume of the myocardium is accessible for transfection and the distribution of transgenes in the myocardium is not homogeneous. To overcome these problems, we used an *in vivo* intracoronary gene delivery method that modified the approach of Hajjar *et al*[17] to transduce the HSP20 gene into the ventricular muscle with the use of recombinant adenoviral vectors. The adenoviral vectors are delivered into the myocardium via the coronary circulation. Using this delivery method, we sought to elucidate a direct cause and effect relationship between HSP20 and cardioprotective effects in the intact rat heart.

The present study demonstrates, for the first time, that gene transfer of Ad.HSP20 into the LV muscle causes robust expression of HSP20 as compared with vector or Ad.GFP transfer. Ad.GFP-treated LV showed no significant increase in HSP20 expression compared with vector-treated LV. These results suggest that the increased expression in the Ad.HSP20-treated hearts is not due to virus-related stress.

Infarct size was reduced significantly in the I/R hearts injected with Ad.HSP20. We also examined serum cTnT and CK levels independently. cTnT originating exclusively from the myocardium clearly differs from skeletal muscle troponin T. As a result of its high tissue specificity, cTnT is a cardiospecific, highly sensitive marker for myocardial damage[20]. Our results showed that the cTnT level in Ad.HSP20-treated

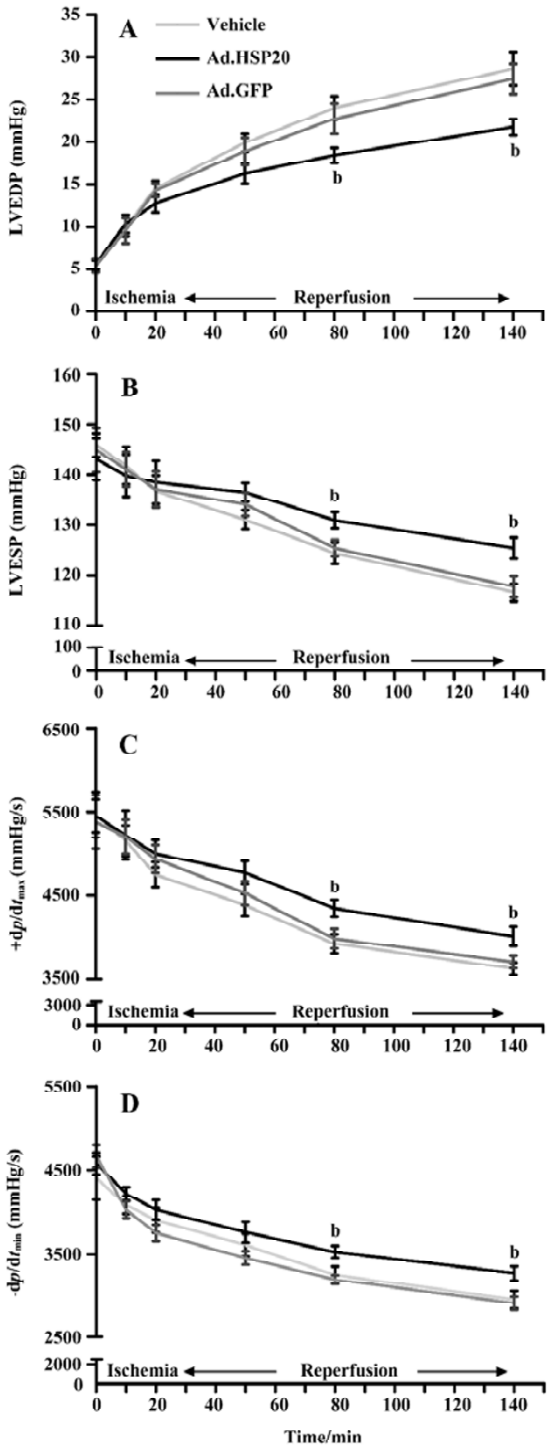

Figure 5. Cardiac function *in vivo*. Left ventricular end diastolic pressure (LVEDP) (A), left ventricular end systolic pressure (LVESP) (B), maximal rate of pressure increase ($+dp/dt_{max}$) (C), and decrease ($-dp/dt_{min}$) (D) were measured in the left ventricle at different time points in vector-, Ad.HSP20-, or Ad.GFP-treated hearts. n=6−8. Mean±SD. $^bP<0.05$ *vs* Ad.GFP.

hearts was reduced significantly as compared with that in vector- and Ad.GFP-treated hearts. Similar results were shown with CK. TUNEL staining showed that apoptosis of cardiomyocytes was reduced in Ad.HSP20-treated hearts.

The decrease of LVEDP and increase of LVESP, $+dp/dt_{max}$ and $-dp/dt_{max}$ in HSP20-treated hearts may be explained by HSP20 being an actin-associated protein. It is biochemically associated with αB-crystallin and localized to distinct transverse bands in a pattern similar to αB-crystallin and sarcomeric actin[21,22]. Phosphorylated HSP20 increases the contractility rate of cardiac myocytes, which indicates that HSP20 is involved in the regulation of myocardial contractility[6,7].

Heat shock proteins are a family of endogenous protective proteins. Various HSP have protective effects against stress injury. HSP70 prevents cell death by inhibiting apoptosis via associating with apoptosis protease activating factor-1 (Apaf-1) and blocking the assembly of a functional apoptosome[23]. Combined and individual mitochondrial HSP60 and HSP10 expression in cardiomyocytes protects mitochondrial function and decreases apoptotic cell death induced by simulated I/R accompanied by decreased mitochondrial cytochrome c release and caspase-3 activity[11]. HSP60 interacts with Bax and Bak to regulate apoptosis[24]. Overexpression of αB-crystallin in transgenic mice hearts provides resistance to I/R injury by negatively regulating myocyte and non-myocyte apoptosis[25]. HSP27 binds to cytochrome c released from the mitochondria into the cytosol and prevents cytochrome c-mediated interaction of Apaf-1 with procaspase-9[26]. These results highlight the notion that the protective effects of HSP are closely related to mitochondrial function. Thus, HSP are anti-apoptotic proteins in cardiomyocytes.

Myocardial ischemia is followed frequently by reperfusion. Reperfusion and the resultant re-oxygenation lead to the generation of oxygen radicals that can cause reperfusion injury. Our results *in vivo* are consistent with those of Fan *et al*[15], who showed that HSP20 and its phosphorylation at Ser16 might provide protective effects against β-agonist-induced apoptosis *in vitro*.

Death of cardiomyocytes due to I/R injury is caused by 2 distinct mechanisms, necrosis, and apoptosis, which contribute independently to myocardial infarction[25,27]. The infarct area represents cell death, including necrotic cell death and apoptotic cell death. cTnT and CK are indicators of myocardial necrosis, whereas TUNEL staining can reveal apoptosis. Thus, our results suggest that the protective effect of HSP20 is attributed to a reduction of necrosis and apoptosis in cardiomyocytes. In addition, our recent data shows that lactate dehydrogenase release and caspase-3

activity in H9c2 cells infected with Ad.HSP20 are also decreased. Therefore, the cardioprotective effect of HSP20 *in vivo* might be mediated mainly by inhibiting both cardiomyocyte necrosis and apoptosis.

In conclusion, our results show that overexpression of HSP20 protects against I/R injury *in vivo*, not only by inhibiting cardiomyocyte necrosis and apoptosis but also by increasing myocardial contractility. Our data suggest that HSP20 is a potential therapeutic protein for ischemic diseases and additional experiments should be carried out.

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