Heat Shock Transcription Factor 1 Protects Cardiomyocytes From Ischemia/Reperfusion Injury

Yunzeng Zou, MD, PhD*; Weidong Zhu, MD, PhD*; Masaya Sakamoto, MD; Yingjie Qin, MD; Hiroshi Akazawa, MD; Haruhiro Toko, MD, PhD; Miho Mizukami, MD; Norihiko Takeda, MD; Tohru Minamino, MD, PhD; Hiroyuki Takano, MD, PhD; Akira Nakai, MD, PhD; Issei Komuro, MD, PhD

Background—Because cardiomyocyte death causes heart failure, it is important to find the molecules that protect cardiomyocytes from death. The death trap is a useful method to identify cell-protective genes.

Methods and Results—In this study, we isolated the heat shock transcription factor 1 (HSF1) as a protective molecule by the death trap method. Cell death induced by hydrogen peroxide was prevented by overexpression of HSF1 in COS7 cells. Thermal preconditioning at 42°C for 60 minutes activated HSF1, which played a critical role in survival of cardiomyocytes from oxidative stress. In the heart of transgenic mice overexpressing a constitutively active form of HSF1, ischemia followed by reperfusion-induced ST-segment elevation in ECG was recovered faster, infarct size was smaller, and cardiomyocyte death was less than wild-type mice. Protein kinase B/Akt was more strongly activated, whereas Jun N-terminal kinase and caspase 3 were less activated in transgenic hearts than wild-type ones.

Conclusions—These results suggest that HSF1 protects cardiomyocytes from death at least in part through activation of Akt and inactivation of Jun N-terminal kinase and caspase 3. (Circulation. 2003;108:3024-3030.)

Key Words: ischemia ■ reperfusion ■ survival

Because the loss of functional cardiomyocytes causes heart failure, it is important to find the molecules that protect cardiomyocytes from death. The death trap is a useful method to identify cell-protective genes.1 After transfection of the cDNA library constructed using cardiac mRNA in the mammalian expression vector, COS7 cells were cultured with a lethal dose of H2O2. We isolated several cDNAs from the surviving cells, and one of them was the heat shock transcription factor 1 (HSF1). The HSF family (HSF1-4) regulates the transcription of heat shock protein (HSP) genes.2,3 In higher eukaryotes, expression of HSP genes is regulated primarily by HSF1 and HSF3 in response to various stresses and by HSF2 during development, whereas HSF4 seems to lack the activity as a positive transactivator. As a classical stress-responsive factor, HSF1 binds to heat shock element (HSE), which is present upstream of many HSP genes, and activates transcription of HSP genes under stress conditions. HSPs have been reported to be induced in various cardiovascular diseases and to have protective roles against various stresses.4–6 Although HSF1 has also been reported to be expressed in hearts,7 its role remains unknown. In the present study, using the transgenic mice expressing the active form of HSF1 (ΔHSF1),8 we examined the role of HSF1 in the heart subjected to ischemia/reperfusion injury.

Methods

Materials

[γ-32P]ATP was purchased from Du Pont-New England Nuclear Co. DMEM and FBS were from GIBCO BRL Co. pCMV SPORT heart cell expression cDNA library was from Life Technologies. The enhanced chemiluminescence reaction system was from Amersham. Other reagents were from Sigma.

Cloning of Cardioprotective Genes by Death Trap Method

COS7 cells cultured in DMEM supplemented with 10% FBS were resuspended in serum-free DMEM at 2×10^7 cells/mL immediately before transfection. Transfection of pCMV SPORT heart cell expression cDNA library was performed by electroporation (220 V, 960 μF) at 50 μg of plasmid cDNA per mL. Forty-eight hours later, the transfected cells were stimulated with 1 mmol/L H2O2 for 16 hours in serum-free DMEM to induce cell death and cDNA was recovered from the survived colonies. This procedure was repeated 4 times.1

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From the Department of Cardiovascular Science and Medicine (Y.Z., W.Z., M.S., Y.Q., H.A., H.T., M.M., T.M., H.T., T.N., I.K.), Chiba University Graduate School of Medicine, Chiba; Department of Cardiovascular Medicine (N.T.), University of Tokyo Graduate School of Medicine, Tokyo; and Department of Bio-Signal Analysis, Applied Medical Engineering Science (A.N.), Yamaguchi University Graduate School of Medicine, Yamaguchi, Japan.

*These authors contributed equally to this study.

Correspondence to Issei Komuro, MD, PhD, Department of Cardiovascular Science and Medicine, Chiba University, Graduate School of Medicine, 1-8-1 Inohana, Chuou-ku, Chiba 260-8670, Japan. E-mail komuro-tky@umin.ac.jp

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Cell Preparation and DNA Transfection
Primary cultures of cardiomyocytes were prepared from ventricles of 1-day-old Wistar rats, and 0.5 to 10 μg of HSF1 cDNA per dish was transfected into COS7 cells with the 1.5 to 20 μg of green fluorescence protein (GFP)-expressing vector by the standard calcium phosphate method.9,10

ΔHSF1 Transgenic Mice and Murine Ischemia/Reperfusion Model
Construction of the ΔHSF1 transgene and generation of the ΔHSF1 transgenic mice have been previously described.9 Ischemia/reperfusion injury was produced in 12-week-old male transgenic mice and their littermate wild-type mice by transiently ligating the left coronary artery.11 All protocols were approved by the guidelines of Chiba University.

Gel Mobility Shift Assay
DNA binding activities of HSF1 was examined as previously described using a self-complementary consensus HSE oligonucleotide (5′-CTAGAAGCTTCTAGAAGCTTCTAG-3′) (Sigma) as a probe.8

Antisense Experiment
Phosphothionate antisense oligonucleotides (5′-CTAGAAGCTTCTAGAAGCTTCTAG-3′) of HSF1 or scramble oligonucleotides (5′-AGTCAGATCTATAGATCTGAGTC-3′) (Sigma) were prepared and applied to the culture medium (10 μmol/L) before the thermal preconditioning treatment of cardiomyocytes.

Apoptosis Analysis
Apoptotic death of cardiomyocytes was determined by TUNEL and by DNA ladder analysis, as previously described.10,12

Western Blot Analysis
Total protein extracts from the heart or immunoprecipitates were electrophoresed on an SDS-polyacrylamide gel (SDS-PAGE) and transferred to Immobilon-p membrane (Millipore). The blotted membranes were incubated with antibodies to HSF1, HSP27, HSP90, HSP110, caspase3, Apaf1, Akt, phospho-Akt (Ser473), Jun N-terminal kinase (JNK), phospho-JNK (Thr 183/Tyr185), and α-actin (Santa Cruz Biotechnology), respectively. Immuno-reactivity was detected using an enhanced chemiluminescence reaction system according to the manufacturer’s instructions.

Statistics
Data are shown as mean±SE. Multiple group comparison was performed by one-way ANOVA followed by the Bonferroni procedure for comparison of means. A 2-tailed Student t test was used to compare transgenic with nontransgenic specimens under identical conditions. P<0.05 was considered statistically significant.

Results
Cloning of Cardioprotective Genes
COS7 cells were transfected with a heart cDNA library and then exposed to lethal dose of H2O2. Six cDNA clones were isolated from independent colonies of surviving cells, and 3 of them were identical to HSF1. To confirm the protective role of HSF1, we transfected the isolated HSF1 cDNA into COS7 cells. Overexpression of the HSF1 protected COS7 cells from H2O2-induced death (Figure 1A), whereas other cDNA, such as GFP, had no effect (data not shown).

To examine whether HSF1 protects cardiomyocytes against H2O2, cultured cardiomyocytes were exposed to H2O2 after thermal preconditioning at 42°C for 60 minutes followed by additional culture at 37°C for 24 hours. Electro-
preconditioning (Figure 1C), suggesting that the thermal preconditioning protects cardiomyocytes from H$_2$O$_2$-induced cell death through activation of HSF1.

HSF1 Transgenic Mice
To elucidate the protective role of HSF1 in the heart, we examined the transgenic mice that overexpress ΔHSF1. The transgenic mice were apparently healthy, and there were no significant differences in body weight, heart weight, blood pressure, and heart rate between the transgenic mice and littermate wild-type mice (data not shown).

Western blot analysis of heart extracts revealed that ΔHSF1 protein was expressed only in the adult transgenic mice, and there was no difference in expression levels of endogenous HSF1 between the transgenic and wild-type mice (data not shown). The expression of HSPs 27, 70, and 90 was markedly upregulated in the transgenic heart compared with wild-type heart (Figures 2A and 2B).

Ischemia/Reperfusion Injury
Mice were subjected to cardiac ischemia for 40 minutes followed by reperfusion for 120 minutes. There were no abnormalities in ECG before ischemia in both the transgenic mice and wild-type mice (Figure 3). When the left coronary artery was occluded, the ST-segment was rapidly elevated in both types of mice. There was no significant difference in ECG change during ischemia between the transgenic and wild-type mice. When the suture was released to allow reperfusion, the ST-segment returned to baseline within 15 minutes in the transgenic mice whereas the ST-segment elevation remained elevated over 30 minutes in wild-type mice.

Figure 2. Expression of HSPs in the heart. A, Expression of HSPs levels in hearts from wild-type (WT) and transgenic (TG) mice were examined by Western blot analysis using each antibody. A representative photograph from 3 independent experiments is shown. α-actin blot was also presented as a loading control. B, Quantitative analysis of HSP protein expression. Intensities of HSPs and α-actin bands were measured by densitometric scanning of the autoradiograms. Relative amounts of HSPs are expressed as percentage of α-actin. Data are presented as mean±SE from 3 independent experiments. *P<0.05.

Figure 3. ECG recordings. ECG was recorded before and during ischemia/reperfusion. Representative ECG recordings before ischemia (C), at 15 minutes after ischemia (I 15min), and at 1, 15, and 30 minutes after reperfusion (R 1min, R 15min, and R 30min, respectively). Arrows 1 through 4 indicate P wave, QRS wave, ST segment, and T wave, respectively.

Figure 4. AAR and AOI. A, Representative photographs of wild-type (WT) and transgenic (TG) hearts stained by Evans blue dye (EB) and triphenyltetrazolium chloride (TTC) after ischemia/reperfusion (I/R). Red areas in left photographs indicate AAR, and pale white areas in right photographs indicate AOI. B, AAR is presented as a percentage of whole LV mass. AOI is expressed as a percentage of AAR and of whole LV mass. Values are mean±SE of 3 independent experiments. *P<0.05.
These results suggest that ΔHSF1 exerts its protective effect on the electrical activity of myocardium against ischemia/reperfusion injury.

We also measured areas at risk (AAR) and areas of infarction (AOI) after ischemia/reperfusion. Because we ligated the left coronary artery at the most proximal portion, the occlusion consistently created a large ischemic area (AAR, red myocardium in Figure 4A, left), and there was no difference in the ischemic area between both groups. However, there was a significant difference in infarct size (AOI, pale white areas in Figure 4A, right). Both areas of infarction/left ventricle (LV) and areas of infarction/ischemic area were significantly smaller in the transgenic group than those in the nontransgenic mice (Figure 4B).

### Apoptosis in Mice

Ischemia/reperfusion injury has been reported to induce apoptosis in cardiomyocytes. Apoptotic death of cardiomyocytes was examined in the heart by TUNEL and DNA ladder analysis. Ischemia/reperfusion induced apoptosis in many cardiomyocytes (Figures 5A and 5B) and produced marked DNA ladder formation (Figure 5C) in the ischemic area of wild-type heart. The number of apoptotic cells and the DNA fragmentation were significantly less in the ΔHSF1 transgenic heart than the wild-type one (Figures 5A, 5B, and 5C). Caspase 3 was activated and Apaf1-cytochrome C complex was significantly increased in wild-type mice but less in the transgenic mice after ischemia/reperfusion (Figure 5D). Instead, more Apaf1-HSP70 association was observed in the transgenic heart than in the wild-type heart after ischemia/reperfusion (Figure 5D).

### Activation of Protein Kinases

It has been reported that many stresses, including ischemia/reperfusion, activate several protein kinases, including Akt/protein kinase B and JNK, and that activation of Akt induces survival of cells whereas activation of JNK usually triggers death-signaling pathways. In the basal state, there was no difference in the activity of Akt and JNK between the wild-type and the transgenic heart (Figures 6A through 6C). Ischemia/reperfusion induced a significant activation of Akt and JNK in the wild-type heart (Figures 6A through 6C), suggesting that both survival and death signaling were activated in response to ischemia/reperfusion. In the transgenic heart, activation of Akt in response to ischemia/reperfusion was more prominent whereas activation of JNK was weaker than in the wild-type heart (Figures 6A through 6C). Although there was no change in protein number is expressed as percentage of total cardiomyocytes. *P<0.05. C, DNA ladders. Genomic DNA from the heart was separated in 1.5% agarose gels and stained by ethidium bromide. D, Activation of caspase 3 and Apaf1. WT and the TG mice were subjected to I/R. Total cell extracts from the heart or immune complexes with cytochrome C or HSP70 were subjected to SDS-PAGE. Western blot analysis was performed using antibodies against caspase 3 or Apaf1. Pre-caspase 3 indicates precursor of caspase 3; a-caspase 3, activated caspase 3; and IP, immunoprecipitation. Representative autoradiograms from 3 independent experiments are shown.
levels of Akt and JNK after ischemia/reperfusion (Figure 6A), the amount of Akt and JNK that bound to HSP90 and HSP70, respectively, was more markedly increased in the transgenic heart compared with the wild-type heart after ischemia/reperfusion (Figures 7A through 7C).

Figure 6. Activation of Akt and JNK in the heart. A, Protein extracts from the total heart were subjected to SDS-PAGE, and Western blot analysis was performed. p-Akt indicates phosphorylated Akt; p-JNK, phosphorylated JNK. B and C, Quantitative analysis of Akt (B) and JNK (C) activities. The intensities of Akt or p-Akt and JNK or p-JNK bands were measured by densitometric scanning of the autoradiograms. Relative amounts of Akt or p-Akt and JNK or p-JNK are expressed as percentage of Akt and JNK, respectively, in sham-operated wild-type (WT) mice. TG indicates transgenic mice; I/R, ischemia/reperfusion. Data are mean±SE from 3 independent experiments. *,**P<0.05.

Figure 7. Association of Akt and JNK with HSPs. A, Protein extracts of the heart were immunoprecipitated (IP) using antibodies against HSP70 or HSP90. Immune complexes were subjected to SDS-PAGE, and the membranes were incubated with antibodies as indicated. B and C, Quantitative analysis of Akt (B) and JNK (C) bound to HSPs. Relative amounts of Akt, JNK, and HSP are expressed as fold of Akt, JNK, and HSPs, respectively, in sham-operated wild-type (WT) or transgenic (TG) mice. I/R indicates ischemia/reperfusion. Data are mean±SE from 3 independent experiments. *,**P<0.05.
Discussion

The functional cloning system, death trap, is designed to isolate molecules that protect cells from death. Using this method, HSF1 gene was isolated from heart cDNA library. HSF1, a major heat stress-responding factor, upregulates many HSP genes, including HSP110, HSP90, HSP70, and small HSPs. Expression levels of HSP90, HSP70, and HSP27 were significantly elevated in the heart of transgenic mice overexpressing HSF1 compared with wild-type mice. Although HSP70 has been reported to have a cardioprotective function, the role of other HSPs in the heart is largely unknown. The ΔHSF1 transgenic mouse provides a good model to examine the role of activated HSF1 and of multiple HSPs in the heart.

Hearts of the transgenic mice were more resistant to ischemia/reperfusion injury, as indicated by faster recovery of ST-segment elevation in ECG and smaller infarct size. There are several potential mechanisms underlying the cardioprotective effect of HSPs. HSPs are generally believed to help the correct folding of many proteins and restore their functional structures or target denatured proteins to the lysosome for degradation as molecular chaperones. These functions of HSPs as molecular chaperones play important roles in maintaining the normal cell functions and promoting cell survival. Functions of HSPs other than molecular chaperones have recently attracted much attention in many organs, including the heart. It has been reported that accumulation of HSPs in myocardium shows an ATP-sparing effect that prevents the heart from ischemia/reperfusion injury. Repeated coronary occlusions, called ischemic preconditioning, have been reported to induce rapid normalization of the elevated ST-segment during reperfusion by improving regional acidosis and hypokalemia. HSPs may be involved in the faster normalization of ST-segment after reperfusion through restoration of the metabolic and ionic balance.

We showed here that ischemia/reperfusion induced less apoptosis of cardiomyocytes in the ΔHSF1 transgenic mice than wild-type mice. Apoptosis is induced by defined biochemical mechanisms, including release of cytochrome C from mitochondria to cytoplasm and activation of Apaf1 through forming an apoptosome complex. The apoptosome complexes cleave and activate caspase 3, leading to the inevitable fate of cell death. It has been reported that overexpression of caspase 3 in the heart significantly increases infarct size and that the treatment with a caspase inhibitor conversely reduces infarct size. HSPs have been reported to inhibit the formation of the apoptosome complexes through forming cytosolic complexes with Apaf1 or cytochrome C and then inhibit activation of caspas. In this study, Apaf1 formed more complexes with HSP70 and less with cytochrome C in the transgenic heart than the wild-type one, and ischemia/reperfusion activated Apaf1 and caspase 3 more weakly in the transgenic heart than the wild-type one. These results and observations suggest that HSPs protect cardiomyocytes from ischemia/reperfusion-induced cell death at least in part through forming complexes with Apaf1 and inhibiting the activation of caspase 3.

Both Akt and JNK were activated in the heart after ischemia/reperfusion, as reported previously. Activation of Akt has been reported to protect cardiomyocytes against various stresses, such as oxidative stress and the ischemia/reperfusion injury, whereas JNK has been indicated to induce apoptosis during ischemia/reoxygenation in rat cardiomyocytes. In the present study, ischemia/reperfusion activated more Akt and less JNK in the transgenic heart than the wild-type one. HSP90 has been reported to bind to Akt and promote activation of Akt through inhibition of protein phosphatase 2A. Another recent report also showed that overexpression of HSP90 leads to an increased phosphorylation of Akt. On the other hand, Hsp70 family can suppress stress-activated signaling by directly binding to JNK. We here observed that association between both HSP90 and Akt and HSP70 and JNK was more enhanced in the HSF1 transgenic heart than in the wild-type heart. These results collectively suggest that HSPs exert cardioprotective effects through activation of Akt and suppression of JNK. Additional study is needed to elucidate which mechanism plays a major role in HSF1/HSP-induced protection of cardiomyocytes.

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