Reactive Oxygen Species Play an Important Role in the Activation of Heat Shock Factor 1 in Ischemic-Reperfused Heart

Junichiro Nishizawa, MD, PhD; Akira Nakai, MD, PhD; Katsuhiko Matsuda, MD, PhD; Masashi Komeda, MD, PhD; Toshihiko Ban, MD, PhD; Kazuhiro Nagata, PhD

Background—The myocardial protective role of heat shock protein (HSP) has been demonstrated. Recently, we reported that ischemia/reperfusion induced a significant activation of heat shock factor (HSF) 1 and an accumulation of mRNA for HSP70 and HSP90. We examined the role of reactive oxygen species (ROSs) in the induction of stress response in the ischemic-reperfused heart.

Methods and Results—Rat hearts were isolated and perfused with Krebs-Henseleit buffer by the Langendorff method. Whole-cell extracts were prepared for gel mobility shift assay using oligonucleotides containing the heat shock element. Induction of mRNA for HSP70 and HSP90 was examined by Northern blot analysis. Repetitive ischemia/reperfusion, which causes recurrent bursts of free radical generation, resulted in burst activation of HSF1, and this burst activation was significantly reduced with either allopurinol 1 mmol/L (an inhibitor of xanthine oxidase) or catalase 2.3 × 10^5 U/L (a scavenger of H_2O_2). Significant activation of HSF1 was observed on perfusion with buffer containing H_2O_2 150 μmol/L or xanthine 1 mmol/L plus xanthine oxidase 5 U/L. The accumulation of mRNA for HSP70 or HSP90 after repetitive ischemia/reperfusion was reduced with either allopurinol or catalase.

Conclusions—Our findings demonstrate that ROSs play an important role in the activation of HSF1 and the accumulation of mRNA for HSP70 and HSP90 in the ischemic-reperfused heart. (Circulation. 1999;99:934-941.)

Key Words: heat shock factor ■ reactive oxygen species ■ myocardium ■ ischemia ■ reperfusion

The induction of stress proteins in the heart has been observed under various physiological stresses, including ischemia and hyperthermia.1–5 These heat shock proteins (HSPs) are thought to have protective roles against various stresses. In fact, improvement of functional recovery and reduction of infarct size after ischemia in the heart were demonstrated in the transgenic mouse overexpressing HSP70, as well as after pretreatment with heat shock.9–11 Thus, there has been increasing interest in the heat shock/ stress response in the heart. However, the molecular mechanisms regulating this response in the heart remain unknown.

Heat shock gene regulation is mediated primarily at the transcriptional level by the activation of a preexisting transcription activator, heat shock factor (HSF). HSF binds to heat shock element (HSE), which is present upstream of all heat shock genes and induces heat shock gene transcription (for a review, see Reference 12).12 In higher eukaryotes, recent studies have identified a family of HSFs and have suggested that functional differences exist among the members of this family.13–17 Under stress conditions such as heat shock, ischemia/reperfusion, or exposure to heavy metals and amino acid analogues, HSF1 induces heat shock gene transcription through its trimerization and translocation into the nucleus and thereby acquisition of the DNA-binding activity.18,19 The activation of HSF2 is induced during erythroid differentiation of human K562 erythroleukemia cells with hemin treatment.20

Recently, we reported that ischemia/reperfusion induced significant activation of HSF1 and induced mRNA for HSP70 and HSP90.21 It is well known that a burst of oxygen free radical production is observed during the early moments of reperfusion of an ischemic heart.22 On the other hand, there is little agreement to the stress response caused by oxidative stress, despite a number of studies.23–31 Only few studies have been carried out so far on the role of reactive oxygen species (ROSs) in induction of stress response in the ischemic-reperfused heart.28 In the present study, we therefore examined the induction of the DNA-binding activity of HSF1...
during repetitive ischemia/reperfusion, which was reported to result in recurrent free radical generation, and during exposure to exogenous ROSs in isolated rat heart. We also determined the separate effects of 2 antioxidants, allopurinol and catalase, on the activation of HSF and on the accumulation of mRNA for HSP70 and HSP90 after repetitive ischemia/reperfusion. We found that the antioxidants reduced the activation of HSF1 and the accumulation of mRNA for HSP70 and HSP90.

Methods

Isolated Heart Perfusion

Male Sprague-Dawley rats (250 to 300 g) obtained from Shimizu Laboratory Supplies Co Ltd (Kyoto, Japan) were anesthetized with diethyl ether and given heparin 200 IU IV. The hearts were excised and then perfused, as described previously, by the Langendorff method with Krebs-Henseleit buffer (consisting of, in mmol/L: NaCl 118, NaHCO3 25, KCl 4.6, MgSO4 1.2, KH2PO4 1.2, CaCl2 2.5, and glucose 11) at 37°C at a constant pressure of 100 cm H2O. The perfusate was readjusted to 7.4 with HCl.34 Of which was added to the perfusate, and the pH of the gassed solution was indicated, throughout the whole perfusion period. In the study in which the hearts were subjected separately in the perfusion medium of some hearts, as indicated, during the stabilization period (37°C), temperature of perfusion buffer and heat shock period, and the hearts were contained in a water-jacketed chamber at the same temperature. The left ventricular (LV) pressure and LV dP/dt were monitored through the use of a fluid-filled latex balloon inserted into the LV via the left atrium and connected to a pressure transducer. The balloon volume was adjusted to obtain an LV end-diastolic pressure of 5 to 9 mm Hg. The LV developed pressure was calculated as the difference between the peak-systolic and end-diastolic pressures. Coronary flow was measured by timed collection of the overflow from the hearts. The creatine kinase (CK) concentration was determined according to the spectrophotometric method of Rosalki. All of the experiments were performed under conditions in compliance with the National Institutes of Health guidelines on the care and use of laboratory animals.

Experimental Protocols

The experimental protocols are summarized in Figure 1. In all hearts, after a 30-minute stabilization, baseline hemodynamic measurements were made. Then, in the heat shock experiments, warm (42°C) buffer was perfused for the indicated time periods. In the ischemia/reperfusion experiments, isolated hearts were subjected to 10-minute global ischemia by clamping of the aortic cannula followed by 10-minute reperfusion. Throughout the ischemic period only, the intraventricular balloon was kept deflated. Postischemic reperfusion was applied under the same conditions as during stabilization.

In the experiments on repetitive ischemia/reperfusion, 10-minute global ischemia and 10-minute reperfusion were repeated for the indicated times. Measurements of hemodynamic parameters and CK efflux were made at baseline and at the last minute of each of the first and second reperusions. In these experiments, allopurinol 1 mmol/L (an inhibitor of xanthine oxidase) or catalase 2×103 U/L (Sigma; specific activity, 41 000 IU/mg protein; a scavenger of H2O2) was included separately in the perfusion medium of some hearts, as indicated, throughout the whole perfusion period. In the study in which allopurinol was used, a solution was prepared by dissolving 1 mmol of the drug in 10 mL of 1 mol/L NaOH, the required volume of which was added to the perfusate, and the pH of the gassed solution was readjusted to 7.4 with HCl.34

In the experiments on exposure to exogenous ROSs, after stabilization, the perfusate was substituted with the buffer containing H2O2 150 μmol/L, xanthine 1 mmol/L plus xanthine oxidase 5 U/L, xanthine 1 mmol/L alone, or xanthine oxidase 5 U/L alone for 20 minutes.35 Thereafter, the hearts were perfused with Krebs-Henseleit buffer without ROSs for 30 minutes to allow recovery. Xanthine and xanthine oxidase were dissolved in the buffer solution and mixed for 90 minutes.

The hearts were perfused under the same conditions as during stabilization for 10 or 120 minutes after 30-minute stabilization. Hearts that developed ventricular fibrillation and did not return to normal sinus rhythm were excluded from the data analysis. At the end of each experiment, the ventricular tissue was quickly frozen in liquid nitrogen and then stored at –80°C.

Preparation of Cell Extracts

For preparation of whole-cell extracts from the hearts, the frozen samples were crushed and homogenized with a Polytron homogenizer (Kinematica) in high-salt buffer as described previously.21,36 The lysates were kept on ice for 5 minutes and then centrifuged at 100 000g for 5 minutes at 4°C. The supernatants were frozen in liquid nitrogen and stored at –80°C.

Gel Mobility Shift Assay

Whole-cell extracts from the hearts were assayed by gel mobility shift assay (GMSA) as described previously, with a double-stranded synthetic HSE. Binding reactions with protein extracts (40 μg) were performed for 20 minutes at 25°C in 25 μL of the binding buffer containing 0.2 ng of 32P-labeled probe and 0.5 μg of poly(dI-dC)·poly(dI-dC) (Pharmacia Biotech). The samples were then electrophoresed on nondenaturing 4% polyacrylamide gel, dried, and autoradiographed. For antibody supershift experiments, 2.0 μL of diluted (1:10 with PBS) specific antisera raised against recombinant chicken HSF1 (aHSF1β) or HSFP2 (aHSF2δ) were added to whole-cell extracts before the binding reaction.21,37 For the competition experiments, the binding reaction mixtures contained a 50-fold molar excess of unlabeled HSE oligonucleotides.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from the tissues by acid guanidinium thiocyanate–phenol-chloroform extraction. Total RNA (10 μg/lane) was separated on 1% agarose-formaldehyde gel and then transferred to a nylon membrane (Gene Screen Plus, Du Pont–New England Nuclear). The filter was hybridized with the 32P-labeled DNA probes. The membranes were exposed to X-ray film for 24 hours at −80°C.
Hemodynamic Changes During Repetition of Ischemia/Reperfusion

<table>
<thead>
<tr>
<th>Heart rate, bpm</th>
<th>Baseline</th>
<th>Rep 1</th>
<th>Rep 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>(–) (n=7)</td>
<td>324±8</td>
<td>310±12</td>
<td>313±12</td>
</tr>
<tr>
<td>Allopurinol (n=4)</td>
<td>330±11</td>
<td>323±14</td>
<td>323±14</td>
</tr>
<tr>
<td>Catalase (n=4)</td>
<td>320±7</td>
<td>310±12</td>
<td>305±10</td>
</tr>
<tr>
<td>Coronary flow, mL/min</td>
<td>(–)</td>
<td>12.9±0.4</td>
<td>12.6±0.5</td>
</tr>
<tr>
<td></td>
<td>Allopurinol</td>
<td>13.4±1.1</td>
<td>12.6±0.9</td>
</tr>
<tr>
<td></td>
<td>Catalase</td>
<td>13.4±0.9</td>
<td>13.2±1.0</td>
</tr>
<tr>
<td>LVDP, mm Hg</td>
<td>(–)</td>
<td>142±4</td>
<td>129±4</td>
</tr>
<tr>
<td></td>
<td>Allopurinol</td>
<td>138±5</td>
<td>124±7</td>
</tr>
<tr>
<td></td>
<td>Catalase</td>
<td>142±9</td>
<td>126±9</td>
</tr>
<tr>
<td>LV dP/dt, ×10² mm Hg/s</td>
<td>(–)</td>
<td>37.9±1.1</td>
<td>34.9±0.8</td>
</tr>
<tr>
<td></td>
<td>Allopurinol</td>
<td>36.3±1.4</td>
<td>33.3±1.7</td>
</tr>
<tr>
<td></td>
<td>Catalase</td>
<td>37.5±0.9</td>
<td>34.1±1.0</td>
</tr>
<tr>
<td>CK efflux, mU/min</td>
<td>(–)</td>
<td>49±14</td>
<td>394±120</td>
</tr>
<tr>
<td></td>
<td>Allopurinol</td>
<td>57±20</td>
<td>262±107</td>
</tr>
<tr>
<td></td>
<td>Catalase</td>
<td>64±22</td>
<td>355±143</td>
</tr>
</tbody>
</table>

Rep 1 indicates the first reperfusion; Rep 2, the second reperfusion; (–), absence of allopurinol or catalase; and LVDP, LV developed pressure. Results are expressed as mean±SEM of hearts.

*P<0.05 vs baseline value by ANOVA.

Statistical Analysis
All values are expressed as mean±SEM. Statistical comparisons between the time points in the hemodynamic study were assessed for significance with 1-way ANOVA followed by Bonferroni’s test. Comparisons were made between hearts perfused with allopurinol or catalase and hearts perfused with neither of them at individual time points by the unpaired t-test. Statistical significance was defined as P<0.05.

Results
The changes of hemodynamic parameters and CK release during repetitive ischemia/reperfusion are summarized in the Table. Hearts were subjected to repetition of 10-minute global ischemia and 10-minute reperfusion. Allopurinol 1 mmol/L (n=4), catalase 2×10⁷ U/L (n=4), or neither (n=7) was included in the perfusion medium as indicated throughout the whole perfusion period. All the hemodynamic parameters tended to be reduced with repetition of ischemia/reperfusion, but this did not reach a level of statistical significance except for the reduction in LV developed pressure and LV dP/dt at the second reperfusion in the untreated group. None of the hemodynamic data were affected by the addition of allopurinol or catalase. Although not significant, the CK efflux tended to be higher under baseline conditions but lower during reperfusion in the allopurinol- or catalase-treated group compared with that in the untreated group.

All GMSAs were repeated using at least 3 rats, and the representative data of reproducible results are shown. The Northern blot was repeated using 2 rats, and 1 set of the reproducible results is shown.

Effect of Repetitive Ischemia/Reperfusion on DNA-Binding Activity of HSF1
We investigated the effect of repetitive ischemia/reperfusion on HSF activation. The HSE-binding activities of HSF in hearts submitted to repetition of 10-minute global ischemia and 10-minute reperfusion were examined by GMSA using an end-labeled HSE oligonucleotide as a probe (Figure 2). As we reported previously, the HSE-binding activity of HSF after 10-minute ischemia (Figure 2, lane 3) was very weak, and it increased during reperfusion after 10-minute ischemia (lane 4) but was still significantly weaker than after heat shock at 42°C for 40...
minutes (lane 2). The DNA-binding activity during repetitive ischemia/reperfusion increased steadily, and after the third ischemia, it reached a level equal to or higher than that in the heat-shocked hearts (Figure 2, lanes 2 and 7). Competition by an excess of unlabeled HSE oligonucleotide eliminated protein binding to the labeled probe (see Figure 5, lane 11). We also demonstrated that HSF1 is the primary component of HSE-binding activity induced by repetition of ischemia/reperfusion, by antibody supershift experiments with antisera against HSF1 and HSF2 (Figure 5, lanes 2 to 4).

Effect of Allopurinol or Catalase on the DNA-Binding Activity of HSF1

Because the repetition of ischemia/reperfusion, which was reported to cause recurrent bursts of free radical generation, resulted in a burst of activation of HSF, we thought that oxygen free radicals might play an important role in the activation of HSF1 in the ischemic-reperfused heart. We examined the effect of allopurinol, an inhibitor of xanthine oxidase, or catalase, a scavenger of H2O2, on the binding activity. The hearts were submitted to the repetition of ischemia/reperfusion (Figure 3) in the absence or presence of allopurinol or catalase in the perfusion buffer throughout the experimental period. Each antioxidant reduced activity of HSF induced by repetition of ischemia/reperfusion significantly.

When both superoxide dismutase, a scavenger of O$_2^-$, and catalase are added to the perfusion buffer, the DNA-binding activity of HSF was almost the same level as with catalase alone (data not shown).

DNA-Binding Activity of HSF1 by Exposure to Exogenous ROSs

We next examined whether the treatment of the heart with exogenous ROSs would activate HSF1. Isolated hearts were perfused with the buffer containing H$_2$O$_2$ 150 μmol/L or xanthine 1 mmol/L plus xanthine oxidase 5 U/L for 20 minutes and thereafter with buffer containing no ROSs for 30 minutes as a recovery. Significant binding activity of HSF was observed with either oxidant. We also examined hearts perfused with buffer containing X 1 mmol/L or XO 5 U/L alone for 20 minutes followed by 30 minutes of recovery (B). HSF was not activated in hearts perfused with buffer containing X or XO alone.
loration of mRNA for HSP70 and HSP90 in ischemic-reperfused heart. This conclusion is based on the following 4 observations. First, repetitive ischemia/reperfusion, which was reported to cause recurrent bursts of free radical generation, resulted in a burst activation of HSF1. Second, this burst activation of HSF1 in the repetitive ischemic-reperfused heart was significantly reduced by treatment with either allopurinol, an inhibitor of xanthine oxidase, or catalase, a scavenger of H2O2. Third, significant binding activity of HSF1 was observed on perfusion with the buffer containing H2O2 or xanthine plus xanthine oxidase. And finally, the accumulation of mRNA for HSP70 or HSP90 after repetitive ischemia/reperfusion was reduced in the presence of either allopurinol or catalase.

It has been established that a burst of production of ROSs, including H2O2, superoxide radical (O2·−), and hydroxyl radical (·OH), occurs during the early moments of reperfusion of the ischemic heart. These ROSs are derived from a variety of sources, such as the xanthine oxidase system, activated neutrophils, the electron transport chain of mitochondria, and the arachidonic acid pathway. These ROSs have been suggested to be responsible for the postischemic myocardial dysfunction characterized as myocardial stunning and for postischemic arrhythmia. Xanthine plus xanthine oxidase, which is considered a potential source of ROSs during ischemia/reperfusion, releases O2·− and H2O2. H2O2 can cross the cell membrane and be converted to the more toxic ·OH by the Fenton reaction, but the intracellular penetration of exogenous ROSs may be significantly limited by plasma proteins and antioxidant systems. This might be the reason why the activation of HSF1 with exogenously added ROSs (H2O2 or xanthine plus xanthine oxidase) is weaker than that by repetition of ischemia/reperfusion (Figure 4A). Conversely, allopurinol is also a scavenger of ·OH as well as an inhibitor of xanthine oxidase. Catalase scavenges H2O2 and inhibits the formation of other radical species. It should therefore be concluded, from our results, that these ROSs play an important role in the induction of the DNA-binding activity of HSF1 in the ischemic-reperfused heart, although it remains to be determined which species is the more important. Our study corroborated the findings of Kukreja et al. They studied accumulation of HSP70 mRNA during exposure to exogenous ROSs and during postischemic reperfusion in isolated rat heart and concluded that one of the potential mechanisms of expression of HSP70 elicited by ischemia/reperfusion may involve oxygen radicals.

In the present study, the inhibitory effect of allopurinol on the binding activity of HSF to HSE was more remarkable than that of catalase. The effect of allopurinol on the repression of mRNA induction was also more significant than that of catalase, although there was a difference in the degree of the inhibition of HSP70 and HSP90 mRNAs. Catalase is a large molecule and most likely to exert its effect mainly on the extracellular space rather than in the intracellular environment. It seems reasonable to suppose that this is one of the reasons for the weakness of the inhibitory effect of

Discussion

Previously, we demonstrated that the induction of HSE-binding activity of HSF during ischemia was weak and the activity soon attenuated, that postischemic reperfusion significantly induced the binding activity of HSF and the accumulation of mRNA for HSP70 and HSP90, and that HSF1 mainly mediated the stress response in the ischemic-reperfused heart. Moreover, in the present study, our results suggested that ROSs play an important role in the induction of the binding activity of HSF1 and the accumulation of mRNA for HSP70 and HSP90.
catalase on the activation of HSF compared with that of allopurinol.

There was a difference concerning the inhibitory effect of allopurinol or catalase between HSP70 and HSP90 mRNAs. In our previous study,21 we observed the contrast between relative accumulation of HSP70 and HSP90 mRNAs induced by heat shock or ischemia/reperfusion. Differential regulations of HSP70 and HSP90 were also reported in lymphocytes and peripheral blood monocytes after the treatments with mitogens, phorbol esters, and heat shock.30,46 These findings suggested the involvement of additional regulatory mechanisms other than HSF.

Kukreja et al28 suggested 3 possibilities concerning the mechanisms of stress response in the ischemic-reperfused heart: changes in hemodynamics, decrease in intracellular ATP, and protein denaturation.28 In this study, none of the hemodynamic data were significantly affected by allopurinol or catalase during repetitive ischemia/reperfusion, although the binding activity of HSF was reduced markedly by both antioxidants. Differential regulations of HSP70 and HSP90 were also reported in lymphocytes and peripheral blood monocytes after the treatments with mitogens, phorbol esters, and heat shock.30,46 These findings suggested the involvement of additional regulatory mechanisms other than HSF.

Controversy continues as to the effects of oxidants on the stress response, despite a number of studies. Several studies have demonstrated the induction of HSPs by oxidative stress.23–25,27 Conversely, Bruce et al26 reported that although H2O2 or menadione induced DNA-binding activity of HSF, HSPs were not synthesized in NIH-3T3 cells. It was also reported that phorbol esters did not cause HSF induction but did induce HSP synthesis in human monocytes and that the increased mRNA stabilization was responsible for this induction of HSP.30 Recently, involvement of the redox mechanism in the heat shock signal transduction pathway has been suggested.29,31 Jacquier-Sarlin and Polla31 reported that H2O2 exerted a dual effect in the human premonocytic cells: it reversibly inhibited the binding activity of HSF as well as inducing the binding activity. Moreover, they proposed that the time required for thioredoxin induction provides an explanation for the lack of HSP synthesis on exposure to ROSs, despite the activation of HSF. Thus, a multistep and complex regulation of the stress response to oxidative stress is suggested, and the differences among the studies may be due to the cell specificity, the type of oxidative stress, and the subcellular location of the ROS generation.

In this article, we have established that HSF1 is efficiently activated with ROSs in ischemic-reperfused heart, which might provide a theoretical and experimental basis for the protection of the heart against ischemia/reperfusion. With further investigation of the mechanisms of HSP induction in the heart and less noxious stimuli that induce HSPs, we believe that it will be possible, by increasing the

Figure 6. Effect of allopurinol or catalase on HSP70 and HSP90 mRNA accumulation after repetitive ischemia/reperfusion. Total RNA was isolated from a 120-minute control heart (lane 1) and from hearts submitted to repetition of ischemia/reperfusion followed by 120-minute reperfusion after third ischemia (IRIRI, lanes 2 to 4). In perfusate of hearts submitted to repetitive ischemia/reperfusion, allopurinol or catalase addition was absent (−) or present throughout experimental period. RNA (10 μg) was electrophoresed, and Northern blot analysis was performed (A). After hybridization with β-actin cDNA probes as an internal control, filter was rehybridized with HSP70 and HSP90 cDNA probes successively. Relative expression of HSP70 (B) and HSP90 (C) mRNA is shown. Radioactivity of hybridization of each signal was determined with a bioimage analyzer, normalized with bands hybridized with β-actin probe. Radioactivity of band for control was detected by long exposure, which was adopted as a value of 1. Results are average of 2 independent experiments. Cont indicates control; Allo, allopurinol; and Cata, catalase.
endogenous protective faculty of the heart, to alter the progression of severe ischemic heart disease or heart transplantation.

In summary, our data indicate that ROSs play an important role in the induction of the binding activity of HSF1 and in the accumulation of mRNA for HSP70 and HSP90 in the ischemic-reperfused heart.

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