Activation of Signal Transducer and Activator of Transcription 3 Protects Cardiomyocytes from Hypoxia/Reoxygenation-Induced Oxidative Stress Through the Upregulation of Manganese Superoxide Dismutase

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Background—Mice with cardiac-specific overexpression of signal transducer and activator of transcription 3 (STAT3) are resistant to doxorubicin-induced damage. The STAT3 signal may be involved in the detoxification of reactive oxygen species (ROS).

Methods and Results—The effects of leukemia inhibitory factor (LIF) or adenovirus-mediated transfection of constitutively activated STAT3 (caSTAT3) on the intracellular ROS formation induced by hypoxia/reoxygenation (H/R) were examined using rat neonatal cardiomyocytes. Either LIF treatment or caSTAT3 significantly suppressed the increase of H/R-induced ROS evaluated by 2',7'-dichlorofluorescin diacetate fluorescence. To assess whether ROS are really involved in H/R-induced cardiomyocyte injury, the amount of creatine phosphokinase in cultured medium was examined. Both LIF treatment and caSTAT3 significantly decreased H/R-induced creatine phosphokinase release. These results indicate that the gp130/STAT3 signal protects H/R-induced cardiomyocyte injury by scavenging ROS generation. To investigate the mechanism of scavenging ROS, the effects of LIF on the induction of antioxidant enzymes were examined. LIF treatment significantly increased the expression of manganese superoxide dismutase (MnSOD) mRNA, whereas the expression of the catalase and glutathione peroxidase genes were unaffected. This induction of MnSOD mRNA expression was completely blocked by adenovirus-mediated transfection of dominant-negative STAT3. Moreover, caSTAT3 augmented MnSOD mRNA and its enzyme activity. In addition, the antisense oligodeoxyribonucleotide to MnSOD significantly inhibited both LIF and caSTAT3-mediated protective effects.

Conclusions—The activation of STAT3 induces a protective effect on H/R-induced cardiomyocyte damage, mainly by inducting MnSOD. The STAT3-mediated signal is proposed as a therapeutical target of ROS-induced cardiomyocyte injury. (Circulation. 2001;104:979-981.)

Key Words: antioxidants ■ hypoxia ■ signal transduction

In the heart, it has been reported that reactive oxygen species (ROS) contribute to cardiac dysfunction and myocardial damage under a variety of conditions, such as ischemia-reperfusion, congestive heart failure, and doxorubicin-induced cardiomyopathy. Recent studies have shown that gp130-mediated signals transduced both cytoprotective and hypertrophic responses in the heart. The signal transducer and activator of transcription-3 (STAT3) is a key molecule downstream of gp130, which is activated under various stressful conditions, such as pressure-overload and myocardial infarction. Transgenic mice with cardiac-specific overexpression of the STAT3 gene are protected against doxorubicin-induced cardiomyopathy. Therefore, the activation of STAT3 might induce a protective effect against oxidative stress–induced cardiomyocyte damage by scavenging ROS.

In the present study, we explored whether the gp130/STAT3 signal has a protective function against hypoxia/reoxygenation (H/R)-induced cardiomyocyte damage.

Methods

Cell Culture and H/R Experiments

Primary cultures of neonatal rat cardiomyocytes were prepared from the ventricles of 1-day-old Sprague-Dawley rats (Kiwa Dobutsu Wakayama, Japan), as previously described. Hypoxia was created by incubating cells in an airtight Plexiglas chamber with <1% O2 and 5% CO2, 95% N2 at 37°C for 2 hours using Gas Pak Plus (BBL). By replacing the medium saturated with 95% air and 5% CO2, the cells were exposed to normoxic atmosphere (reoxygenation). Anti-sense oligodeoxyribonucleotides (ODN) corresponding to the initiation sites of manganese superoxide dismutase (MnSOD) translation.
(22 mer: CAGCCGCGCCGACACAACTTG; 1.0 μmol/L) or sense ODN at the same site (22 mer: CAATGTTGTCGGGGCGGCGGTG; 1.0 μmol/L) were added to the cultured medium 18 hours before hypoxia until 24 hours after reoxygenation.7

**Generation of Recombinant Adenovirus and Protocol of Adenovirus Infection**

The recombinant replication-defective adenovirus expressing a dominant-negative form of STAT3 and constitutively activated STAT3 (caSTAT3), which were kindly provided by Drs J.F. Bromberg and J.E. Darnell, Jr (Laboratory of Molecular Cell Biology, The Rockefeller University, New York, NY),8 were prepared as described previously.6,9,10 Adenovirus vector expressing β-galactosidase (β-gal) was used as a control.

**Analysis of Intracellular ROS Generation**

The fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCF-DA), was used to assess intracellular ROS formation in cultured rat cardiomyocytes, as previously described.11

**Northern Blot Analysis and Western Blot Analysis**

Northern and Western blotting were performed as previously described.6 Specific cDNA probes for the detection of MnSOD, catalase, and glutathione peroxidase gene transcripts were reported previously.12

**Measurement of MnSOD Activity**

MnSOD activity was determined by the nitroblue tetrazolium method.7

**Measurement of Creatine Phosphokinase**

Creatine phosphokinase (CPK) activity in the culture medium was measured by the MERCK-1-TEST CK kit (Kantou Kagaku Co).13

**Statistical Analysis**

Statistical analysis was performed by Student’s t test and 1-way ANOVA followed by Bonferroni collection for multiple group comparisons. P<0.05 was considered significant.

**Results**

**Activation of Gp130/STAT3 Protects Cardiomyocytes from H/R Injury by Scavenging ROS**

To examine the effects of leukemia inhibitory factor (LIF) or caSTAT3 on the intracellular ROS formation induced by H/R, DCF-DA was used as a fluorescent probe. Both LIF treatment and caSTAT3 significantly suppressed the generation of ROS induced by H/R (Figure 1A). Furthermore, they both significantly decreased H/R-induced CPK release (Figure 1B). These results indicate that the gp130/STAT3 signal protects H/R-induced cardiomyocytes injury by scavenging ROS generation.

**The Protective Effects of STAT3 Are Involved in the Upregulation of MnSOD**

To explore the mechanism of protection against ROS, the effects of LIF on the induction of 3 important myocardial antioxidant enzymes (ie, MnSOD, catalase, and glutathione peroxidase) were examined. LIF treatment resulted in a significant increase of MnSOD mRNA level from 1 hour that continued for up to 24 hours, whereas the expressions of catalase and glutathione peroxidase mRNAs were unchanged throughout the time points examined (Figure 2A). Figure 2B shows the dose-dependent effect of LIF on the induction of MnSOD mRNA expression. These effects of LIF were inhibited by dominant-negative form of STAT3 (Figure 2C). Furthermore, significant enhancement of MnSOD mRNA expression was detected with caSTAT3, and this was accompanied by an increase in its enzyme activity. Thus, STAT3 is considered essential for LIF-induced MnSOD expression.

To evaluate whether the increase in MnSOD activity is indeed directly related to the protective effect of STAT3, antisense ODN against the MnSOD gene were used. Antisense ODN completely cancelled both LIF and caSTAT3-induced MnSOD activities, and sense ODN did not affect our experiments (data not shown). As shown in Figure 2D, these beneficial effects of LIF and caSTAT3 on CPK release were significantly decreased in the presence of antisense ODN. Thus, the protective effects of STAT3 against H/R are mainly related to the induction of MnSOD activity.

**Discussion**

The major new finding in the present study is that STAT3 activation protects cardiomyocytes from ROS caused by H/R through the upregulation of the MnSOD gene and its enzyme activity.

Endotoxin and cytokines such as tumor necrosis factor-α, interleukin-1β, and interleukin-6 are known to induce MnSOD, and several studies have confirmed the cardioprotective role of MnSOD.14–16 In view of the transcriptional regulation, 3 interferon-γ activation site motifs (TTCTCTTAA, TTCTCTCAA, and TTA-CATCAA) that are bound with activated STAT3 were identified in the region spanning from −2505 to −1104 in the MnSOD promoter region.17,18 This suggests that LIF induce MnSOD mRNA expression mainly via the STAT3 binding cis-element in cardiac myo-
cytes. It remains to be identified which motifs of the 3 discussed above are the most important for the induction of the MnSOD gene.

To clarify the role of MnSOD in H/R, we used an antisense strategy to suppress MnSOD induction. The responses to the pretreatment with antisense ODN in caSTAT3- or LIF-treated cells were different. The protective effects observed in caSTAT3 transduction were nearly completely abolished with antisense ODN, whereas the protective effects by LIF were not completely abolished with antisense ODN. These results suggest that induction of MnSOD by caSTAT3 is essential for caSTAT3-induced protection in H/R. The genes related to cardiac protection, such as the atrial natriuretic factor and vascular endothelial growth factor genes, increased in the hearts with cardiac-specific overexpression of STAT3; however, these genes have little effect on this condition.

However, LIF stimulation induces other anti-apoptotic molecules, such as Bcl-xL and Bcl-2, and also activates phosphatidylinositol 3-kinase-Akt and mitogen-activated protein kinase pathways, which would be involved in a protective effect against ROS. There-
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