Silent Information Regulator 1 Protects the Heart From Ischemia/Reperfusion

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Background—Silent information regulator 1 (Sir1), a class III histone deacetylase, retards aging and protects the heart from oxidative stress. We here examined whether Sir1 is protective against myocardial ischemia/reperfusion (I/R).

Methods and Results—Protein and mRNA expression of Sir1 is significantly reduced by I/R. Cardiac-specific Sir1−"" mice exhibited a significant increase (44±5% versus 15±5%; P=0.01) in the size of myocardial infarction/area at risk. In transgenic mice with cardiac-specific overexpression of Sir1, both myocardial infarction/area at risk (15±4% versus 36±8%; P=0.004) and terminal deoxynucleotidyl transferase dUTP nick end labeling–positive nuclei (4±3% versus 10±1%; P<0.003) were significantly reduced compared with nontransgenic mice. In Langendorff-perfused hearts, the functional recovery during reperfusion was significantly greater in transgenic mice with cardiac-specific overexpression of Sir1 than in nontransgenic mice. Sir1 positively regulates expression of prosurvival molecules, including manganese superoxide dismutase, thioredoxin-1, and Bcl-xL, whereas it negatively regulates the proapoptotic molecules Bax and cleaved caspase-3. The level of oxidative stress after I/R, as evaluated by anti-8-hydroxydeoxyguanosine staining, was negatively regulated by Sir1. Sir1 stimulates the transcriptional activity of FoxO1, which in turn plays an essential role in mediating Sir1-induced upregulation of manganese superoxide dismutase and suppression of oxidative stress in cardiac myocytes. Sir1 plays an important role in mediating I/R-induced increases in the nuclear localization of FoxO1 in vivo.

Conclusions—These results suggest that Sir1 protects the heart from I/R injury through upregulation of antioxidants and downregulation of proapoptotic molecules through activation of FoxO and decreases in oxidative stress. (Circulation. 2010;122:2170-2182.)

Key Words: cardioprotection ■ ischemia ■ oxidative stress ■ reperfusion injury

Silent information regulator 1 (Sir1) is a member of the sirtuin family of class III histone deacetylases.1 The class III histone deacetylases are distinguished from histone deacetylases in the other classes by their requirement of NAD+ for their enzyme activity.2 Sir1 is involved in gene silencing, differentiation, cell survival, metabolism, and longevity.1 Sir1 activity extends the lifespan of lower organisms, including yeast, Caenorhabditis elegans, and flies.3,4 In addition, resveratrol, which stimulates Sir1, extends the lifespan of mice fed a high-fat diet, suggesting that Sir1 may affect aging and/or lifespan in mammals.5 The beneficial effects of caloric restriction may be dependent on Sir1.5–8 Conversely, Sir1 knockout mice exhibit developmental abnormalities, including septal and valvular heart defects.9,10 Sir1 regulates the function of transcription factors and cofactors, including MyoD, Ku, p53, PGC1, and the FoxO family of transcription factors,11–19 through deacetylation.

Clinical Perspective on p 2182

Activation of molecular mechanisms extending lifespan generally increases the ability of the organism to survive against stress, a phenomenon that is termed hormesis.20 We have shown previously that upregulation of Sir1 inhibits apoptosis, protects against oxidative stress in cardiac myocytes, and retards the progression of aging in the mouse heart.21,22 By extending this observation, one can speculate that therapeutic activation of a longevity factor, such as Sir1, may protect the heart and the cardiac myocytes therein from pathologically relevant stress, such as ischemia and reperfusion.23 However, the protective effect of Sir1 against myocardial ischemia/ reperfusion (I/R), a major cause of myocardial injury in the clinical setting, has not been clearly demonstrated. Furthermore, the molecular mechanism by which Sir1 mediates its protective effects against I/R is unknown.
To address these issues, we used genetically altered mouse models in which expression of Sirt1 is either upregulated or downregulated in a cardiac myocyte–specific manner. In particular, we asked (1) how expression of Sirt1 is affected by I/R, (2) how upregulation or downregulation of Sirt1 in cardiac myocytes affects myocardial injury and cardiac function after I/R, and (3) whether stimulation of Sirt1 activates cell-protective mechanisms in the heart during I/R.

Methods

Genetically Altered Mouse Models

Cardiac-specific Sirt1 transgenic mice (Tg-Sirt1) were generated with the use of the α-myosin heavy chain promoter (courtesy of Dr J. Robbins, Children’s Hospital, Cincinnati, Ohio) on an FVB background. The baseline cardiac phenotype of Tg-Sirt1 mice (line 40) has been described.22 Cardiac-specific Sirt1 knockout (Sirt1−/−) mice were generated by crossing Sirt1lox/lox mice (Jackson Laboratory) with C57BL/6J background with α- myosin heavy chain promoter–driven Cre mice (αMHC-Cre, courtesy of Dr M. Schneider, Imperial College, London, UK). All Sirt1lox/lox,αMHC-Cre (control) and Sirt1lox/lox,αMHC-Cre (cardiac-specific Sirt1−/−) mice were back-crossed to C57BL/6J background. All animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey.

Antibodies

The antibodies used in this study include anti-Sirt1 and anti-manganese superoxide dismutase (MnSOD) antibodies (Millipore, Billerica, Mass), anti-acetylated-p53 (Lys-382) antibody (Abcam, Cambridge, Mass), anti-Bcl-XL antibody (BD Biosciences, San Jose, Calif), anti-Bax, anti-8-hydroxydeoxyguanosine (8-OHdG), and anti-FoxO1 antibodies (Santa Cruz Biotechnology Inc, Santa Cruz, Calif), anti-cleaved caspase-3 and anti-acetylated FoxO1 antibodies (Cell Signaling Technology, Danvers, Mass), anti-tri-tubulin antibody (Thermo Scientific, Waltham, Mass), and anti-actin and anti-tubulin antibodies (Sigma, St Louis, Mo). The anti-thioredoxin1 (Trx1) antibody has been described previously.24

I/R and Prolonged Ischemia In Vivo

Mice were housed in a temperature-controlled environment with 12-hour light/dark cycles when they received food and water ad libitum. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg). A rodent ventilator (model 683; Harvard Apparatus Inc) was used with 65% oxygen. The animals were kept warm with heat lamps. Rectal temperature was monitored and maintained between 36°C and 37°C. The chest was opened by a horizontal incision at the third intercostal space. I/R was achieved by ligating the anterior descending branch of the left coronary artery with an 8-0 Prolene suture, with silicon tubing (1 mm outer diameter) placed on top of the left anterior descending coronary artery, 2 mm below the border between the left atrium and left ventricle (LV). Ischemia was confirmed by ECG change (ST elevation). After occlusion for 45 minutes in mice of FVB background and 20 to 30 minutes in mice of C57BL/6J background, the silicon tubing was removed to achieve reperfusion, and the rib space and overlying muscles were closed. FVB mice were subjected to longer ischemia because they are more resistant to I/R injury. Some mice were subjected to alternating brief periods of ischemia (1.5 minutes, 4 times) and reperfusion (3.5 minutes, 4 times) before a longer period (20 minutes) of ischemia. When recovered from anesthesia, the mice were extubated and returned to their cages. They were housed in a climate-controlled environment. Twenty-four hours after reperfusion, the animals were reanesthetized and intubated, and the chest was opened. After the heart was arrested at the diastolic phase by KCl injection, the ascending aorta was canulated and perfused with saline to wash out blood. The left anterior descending coronary artery was occluded with the same suture, which had been left at the site of the ligation. To demarcate the ischemic area at risk (AAR), Alcian blue dye (1%) was perfused into the aorta and coronary arteries. Hearts were excised, and LVs were sliced into 1-mm-thick cross sections. The heart sections were then incubated with a 1% triphenyltetrazolium chloride solution at 37°C for 15 minutes. The infarct area (pale), the AAR (not blue), and the total LV area from both sides of each section were measured with the use of Adobe Photoshop (Adobe Systems Inc), and the values obtained were averaged. The percentages of area of infarction and AAR of each section were multiplied by the weight of the section and then totaled from all sections. AAR/LV and infarct area/AAR were expressed as percentages.25

Langendorff-Perfused Mouse Heart Model of Global I/R

Mice were anesthetized with pentobarbital (65 mg/kg IP) and treated intraperitoneally with 50 U of heparin. The heart was quickly removed and catheterized with a 22-gauge needle. The hearts were mounted on a Langendorff-type isolated heart perfusion system and subjected to retrograde coronary artery reperfusion with 37°C oxygenated Krebs-Henseleit bicarbonate buffer (mmol/L: NaCl 120, glucose 17, NaHCO3 25, KCl 5.9, MgCl2 1.2, CaCl2 2.5, EDTA 0.5), pH 7.4, at a constant pressure of 80 mm Hg. A balloon filled with water was introduced into the LV through the mitral valve orifice and connected to a pressure transducer via a plastic tube primed with water. LV pressures and LV dp/dt were recorded with a strip chart recorder (Astro-Med Inc). The LV end-diastolic pressure was set at 4 to 10 mm Hg at the beginning of perfusion by adjusting the volume of the balloon in the LV, and the volume was kept constant throughout an experiment. After a 30-minute equilibration period, the heart was subjected to 30 minutes of global ischemia (at 37°C) followed by 60 minutes of reperfusion.

Evaluation of Apoptosis in Tissue Sections

DNA fragmentation was detected in situ with the use of terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL), as described.25 Nuclear density was determined by manual counting of DAPI-stained nuclei in 6 fields for each animal with the ×40 objective, and the number of TUNEL-positive nuclei was counted by examining the entire section with the same power objective.

Immunoblot Analysis

For immunoblot analysis, heart samples were homogenized in lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 0.1% sodium dodecyl sulfate, 1% Igepal CA-630, 0.15 mol/L NaCl, 0.25% Na-deoxycholate, and 1 mmol/L EDTA supplemented with protease inhibitors).

Adenovirus Constructs

Adenovirus harboring Sirt1 has been described.23 Adenovirus harboring small hairpin RNA for FoxO1 (Ad-sh-FoxO1) was generated as described previously26 with the use of the following hairpin forming oligo 5′-CGCCAAACTCATAACCAATTCAAGAGAATGGGTATGTAGTGGTGGTGCTTTTTA-3′. The hairpin loop sequence is italicized.

Statistical Analysis

Data are expressed as mean±SEM, but nonparametric statistics were employed because of the small numbers of subjects. Differences in means between 2 groups and among >2 groups were evaluated with Mann-Whitney U test and Kruskal-Wallis test, respectively. The post hoc comparisons were performed by Mann-Whitney U test with Bonferroni correction when the multigroup comparisons were significant. Hemodynamic data between 2 groups at different times of reperfusion were compared with linear mixed models. All of the statistical analyses were performed with the use of SPSS 15.0 for Windows (SPSS Inc, Chicago, Ill). P values of <0.05 were considered statistically significant.
Results

Sirt1 Is Downregulated by I/R
C57BL/6J mice were subjected to 20 minutes of ischemia followed by 24 hours of reperfusion (I/R) (Figure 1A). Expression of Sirt1 was significantly reduced in hearts subjected to I/R compared with sham-operated hearts (66.6 ± 1.8%; \( P = 0.009 \) versus sham-operated hearts) (Figure 1B). Downregulation of Sirt1 was normalized (113.9 ± 12.0%) in hearts subjected to preconditioning before I/R (4 cycles of 1.5 minutes of ischemia followed by 3.5 minutes of reperfusion) (Figure 1A and 1B). Similar results were obtained in regard to the effect of I/R and preconditioning on mRNA expression of Sirt1 (Figure 1B).

Downregulation of Endogenous Sirt1 in the Heart Exacerbates Myocardial Injury Caused by I/R
To examine the role of endogenous Sirt1 in mediating survival and death of cardiac myocytes, we used cardiac-specific Sirt1\(^{-/-}\) mice. Expression of Sirt1 was selectively attenuated in the heart, and there was no compensatory increase in Sirt3 expression in cardiac-specific Sirt1\(^{-/-}\) mice (Figure I in the online-only Data Supplement). The cardiac phenotype in cardiac-specific Sirt1\(^{-/-}\) mice was normal at 3 months of age (Table I in the online-only Data Supplement). Cardiac-specific Sirt1\(^{-/-}\) mice or control wild-type mice were subjected to 30 minutes of ischemia followed by 24 hours of reperfusion. The AAR/LV in cardiac-specific Sirt1\(^{-/-}\) mice was not significantly different from that in control mice. The size of myocardial infarction/AAR was significantly greater in cardiac-specific Sirt1\(^{-/-}\) than in control mice (Figure 2A and 2B), suggesting that endogenous Sirt1 plays a protective role in the heart during I/R.

Overexpression of Sirt1 in the Heart Is Protective Against I/R Injury
Because downregulation of endogenous Sirt1 in the heart subjected to I/R appears to promote myocardial injury, we hypothesized that upregulation of Sirt1 in the heart prevents
myocardial injury in response to I/R. To evaluate the effect of increased expression of Sirt1 on myocardial injury caused by I/R, Tg-Sirt1 and nontransgenic mice generated on an FVB background were subjected to 45 minutes of ischemia followed by 24 hours of reperfusion (Figure 3A). We confirmed that Sirt1 is significantly upregulated in the myocardium of Tg-Sirt1 compared with nontransgenic mice (Figure IIA and IIB in the online-only Data Supplement). The level of acetylated p53 was significantly reduced in Tg-Sirt1, indicating that the activity of Sirt1 is elevated in Tg-Sirt1 (not shown). The size of AAR/LV was not significantly different between Tg-Sirt1 and nontransgenic mice (Figure 3B), but the size of myocardial infarction/AAR after I/R was significantly smaller in Tg-Sirt1 than in nontransgenic mice (Figure 3B). The number of TUNEL-positive cells in the ischemic border zone was also smaller in Tg-Sirt1 mice than in nontransgenic mice (Figure 3C).

The effect of Sirt1 on I/R injury was also evaluated in the Langendorff-perfused isolated heart model. Hearts isolated from Tg-Sirt1 or nontransgenic mice were subjected to 30 minutes of global ischemia followed by 60 minutes of reperfusion (Figure 4A). The baseline LV function of Tg-Sirt1 and nontransgenic mice was similar (Table II in the online-only Data Supplement). After reperfusion, recovery of LV systolic pressure and LV developed pressure was significantly greater in Tg-Sirt1 than in nontransgenic mice (Figure 4B and 4C). LV end-diastolic pressure did not differ significantly between Tg-Sirt1 and nontransgenic mice (Figure 4D). However, both systolic and diastolic dP/dt were significantly improved (Figure 4E and 4F). The size of myocardial infarction evaluated by triphenyltetrazolium chloride staining was significantly smaller in Tg-Sirt1 mice than in nontransgenic mice (Figure 4G). Taken together, these results suggest that Sirt1 plays a protective role against myocardial dysfunction and injury caused by I/R.

**Sirt1 Upregulates Antioxidant and Antiapoptotic Molecules and Downregulates Proapoptotic Molecules in the Heart**

We investigated the molecular mechanism by which Sirt1 protects the heart from I/R injury. Homogenates were prepared from the ischemic area in Tg-Sirt1 and nontransgenic hearts subjected to I/R, and expression of molecules involved in survival and death of cardiac myocytes was evaluated. Expression of MnSOD (6.6±1.8-fold versus nontransgenic) and Trx1 (2.4±0.3-fold) antioxidants was significantly higher in Tg-Sirt1 than in nontransgenic mice (Figure 5A through 5C). Expression of Bcl-xL (2.0±0.3-fold), an antiapoptotic molecule, was also increased (Figure 5A and 5D). On the other hand, expression of Bax (0.2±0.03-fold) and cleaved caspase-3 (0.4±0.14-fold), proapoptotic molecules, was significantly lower in Tg-Sirt1 than in nontransgenic mice (Figure 5A, 5E, and 5F).
Figure 3. I/R injury is attenuated in Tg-Sirt1 mice. A, The protocol used for I/R for Tg-Sirt1 and nontransgenic mice. Tg-Sirt1 or nontransgenic mice generated on an FVB background were subjected to 45 minutes of ischemia and 24 hours of reperfusion. B, top, Gross appearance of LV myocardial sections after Alcian blue and triphenyltetrazolium chloride (TTC) staining. B, bottom left, The AAR (% of LV) was comparable between nontransgenic (NTg) and Tg-Sirt1. B, bottom right, The infarction area/AAR was significantly smaller in Tg-Sirt1 than in nontransgenic. C, left, LV myocardial sections were subjected to TUNEL and DAPI staining. Representative images of the staining in the border zone are shown. C, right, The number of TUNEL-positive myocytes was expressed as a percentage of total nuclei detected by DAPI staining.
Figure 4. Myocardial I/R injury is attenuated in Tg-Sirt1 mouse hearts ex vivo. A, The protocol used for I/R for Tg-Sirt1 and nontransgenic (NTg) control mice in the Langendorff model. The hearts of Tg-Sirt1 or nontransgenic control mice were subjected to 30 minutes of global ischemia and 60 minutes of reperfusion. B to F, Hemodynamics of the Langendorff-perfused isolated mouse hearts of Tg-Sirt1 and nontransgenic control mice. $P<0.05$. B, LV systolic pressure; C, LV developed pressure (systolic pressure–diastolic pressure); D, LV end-diastolic pressure (LVEDP); E and F, systolic and diastolic dP/dt. In B to F, the level at baseline is expressed as 100%. G, top, Gross appearance of LV myocardial sections after triphenyltetrazolium chloride (TTC) staining. G, bottom, The infarction area/AAR (where AAR=total heart) was significantly smaller in Tg-Sirt1 than in nontransgenic mice.
Expression of these genes was reversed in cardiac-specific Sirt1−/− mouse hearts subjected to I/R (Figure III in the online-only Data Supplement).

To examine whether changes in the levels of the aforementioned molecules in Tg-Sirt1 occur in cardiac myocytes, immunostaining was conducted. We confirmed that both upregulation of Bcl-xL and downregulation of cleaved caspase-3 take place in cardiac myocytes in Tg-Sirt1 subjected to I/R (Figure 5G and 5H).

We have shown previously that Sirt1 reduces oxidative stress in the heart and cardiac myocytes therein under stress conditions. Because both MnSOD and Trx1 were upregulated in Tg-Sirt1 hearts subjected to I/R, we examined whether oxidative stress was attenuated in Tg-Sirt1 hearts. As expected, staining with anti-8-OHdG antibody, a marker of oxidative DNA damage, was significantly weaker in Tg-Sirt1 than in nontransgenic mice after I/R (Figure 5I). Conversely, the level of 8-OHdG staining was significantly enhanced in cardiac-specific Sirt1−/− mice compared with wild-type mice after I/R (Figure IV in the online-only Data Supplement).

### Sirt1-Induced Upregulation of MnSOD Is Partially Mediated by FoxO1

Sirt1 deacetylates FoxO transcription factors, thereby stimulating FoxO-mediated transcription of antioxidants. We therefore examined the role of FoxO in mediating Sirt1-mediated upregulation of MnSOD in cardiac myocytes. Overexpression of Sirt1 increases, whereas knockdown of Sirt1 decreases, total FoxO1 in cultured cardiac myocytes (Figure 6A and 6B). Sirt1-induced upregulation of FoxO1 was also observed at the mRNA level (Figure V in the online-only Data Supplement). On the other hand, overexpression of Sirt1 decreases, whereas knockdown of Sirt1 increases, an acetylated form of FoxO1 (Figure 6A and 6C). A sample prepared from heart cells treated with p300 acetyltransferase exhibited strong acetylation of FoxO1, thereby serving as a positive control for this experiment. Sirt1-mediated upregulation of MnSOD was significantly attenuated in the presence of FoxO1 knockdown in cultured cardiac myocytes (Figure 6D and 6E). These results suggest that FoxO1 plays an essential role in mediating Sirt1-induced upregulation of MnSOD. To test whether Sirt1 stimulates FoxO1-mediated transcription, we examined the effect of Sirt1 on the activity of
Figure 5. Sirt1 upregulates cardioprotective molecules in the heart. Heart homogenates were prepared from the ischemic area in Tg-Sirt1 and nontransgenic (NTg) mice subjected to I/R. Expression of Bax, MnSOD, Trx1, Bcl-xL, cleaved caspase-3, and tubulin was evaluated by immunoblots. A, Representative immunoblots are shown. B to F, The quantitative and statistical analyses of the immunoblots are shown. The relative expression of MnSOD (B), Trx1 (C), Bcl-xL (D), Bax (E), and cleaved (c.) caspase-3 (F) in Tg-Sirt1 vs nontransgenic mice is shown. The expression level of nontransgenic mice is expressed as 1. G to I, Representative immunostaining of Bcl-xL (G), cleaved caspase-3 (H), and 8-OHdG (I) in the ischemic area of LV myocardial sections in Tg-Sirt1 and nontransgenic mice subjected to I/R.
Sirt1 Plays an Important Role in Mediating Nuclear Localization of FoxO1 in the Mouse Heart

To clarify the role of Sirt1 in regulating FoxO1-mediated transcription in the heart in vivo, we examined localization of FoxO1 in the heart with immunostaining. In sham-operated nontransgenic or wild-type hearts, nuclear staining of FoxO1 was not prominent. However, I/R significantly increased nuclear staining of FoxO1 in control hearts. I/R-induced increases in the nuclear staining of FoxO1 were significantly enhanced in Tg-Sirt1 hearts, whereas they were significantly attenuated in cardiac-specific Sirt1−/− hearts (Figure 7). These results suggest that Sirt1 positively mediates nuclear localization of FoxO1 in the mouse heart in vivo.

Discussion

Our results suggest that Sirt1 plays a protective role against myocardial I/R in the heart in vivo. Together with the cardioprotective actions of Sirt1 against aging and oxidative stress, our results suggest that stimulation of Sirt1 could represent a novel modality to protect the heart from ischemic heart disease.

Our results suggest that the level of Sirt1 is downregulated by I/R. We have shown previously that expression of Sirt1 is upregulated by certain stresses in the heart, including aging, oxidative stress, and heart failure, thereby protecting the heart as a compensatory mechanism. Thus, the regulation of Sirt1 expression appears to be stimulus specific, and the

3xIRS-luc, a reporter gene containing 3 repeats of the FoxO1 binding site in the insulin-responsive sequence within the insulin-like growth factor-binding protein 1 promoter. Sirt1 significantly increased the activity of 3xIRS-luc, an effect that was abolished in the presence of sirtinol (Figure 6F). This result suggests that Sirt1 increases FoxO1-mediated transcription. To test whether Sirt1-induced upregulation of antioxidants reduces oxidative stress, we examined the effect of Sirt1 overexpression on increases in oxidative stress induced by chelerythrine treatment. We have shown previously that chelerythrine increases oxidative stress, thereby inducing apoptosis in cardiac myocytes. Sirt1 significantly attenuated chelerythrine-induced increases in oxidative stress, as evaluated with Dichlorofluorescin diacetate staining. Importantly, inhibition of oxidative stress by Sirt1 was reversed when FoxO1 was downregulated (Figure VI in the online-only Data Supplement). These results suggest that Sirt1 positively mediates nuclear localization of FoxO1 in the mouse heart in vivo.
compensatory mechanism of Sirt1 against stress does not operate in response to I/R. Because ischemic preconditioning induced by repetitive brief periods of ischemia prevented I/R-mediated downregulation of Sirt1, it is possible that expression of Sirt1 is also regulated by the extent of ischemia. At present, the molecular mechanism mediating downregulation of Sirt1 during I/R remains to be elucidated.

Both loss- and gain-of-function experiments suggest that Sirt1 plays a protective role against I/R injury in the heart. Because the reversal of Sirt1 downregulation through overexpression of Sirt1 in transgenic mice reduced I/R-induced myocardial injury, downregulation of endogenous Sirt1 during I/R may contribute to myocardial injury. We have shown previously that overexpression of Nampt, an enzyme critically regulating NAD\(^+\) synthesis in cardiac myocytes, in transgenic mice also reduced I/R-induced myocardial injury.\(^{28}\) Therefore, interventions to increase either protein expression or activity of Sirt1 during I/R appear to be protective.

Resveratrol protects the heart from I/R injury in experimental animals.\(^{29}\) Although resveratrol has the ability to stimulate Sirt1,\(^{3}\) whether stimulation of Sirt1 is mediated directly through interaction between the 2 molecules or secondarily through intermediates remains to be elucidated.\(^{30}\) Furthermore, the cardioprotective effect of resveratrol could be mediated by Sirt1-independent mechanisms, such as the antioxidant effect of resveratrol.\(^{31}\) Our results suggest that stimulation of Sirt1 can reduce I/R injury. It would be interesting to compare the molecular mechanisms through which specific stimulation of Sirt1 and resveratrol inhibits I/R injury, with the use of the animal models described here.

Our results suggest that Sirt1 upregulates cardioprotective molecules, including MnSOD, Trx1, and Bcl-xL, and downregulates proapoptotic molecules, including Bax. We have
shown previously that Sirt1 deacetylates p53, thereby inhibiting apoptosis of cardiac myocytes in response to serum starvation in vitro.21 Because Bax is positively regulated by p53,32 the protective effect of Sirt1 may be mediated in part through deacetylation and suppression of p53. In fact, the level of acetylated p53 is significantly lower in Tg-Sirt1 than in nontransgenic mice.22 In addition, Sirt1 deacetylates FoxO, thereby stimulating expression of genes mediating cell-protective effects.12 We show that Sirt1 stimulates transcription through FoxO1 and enhances I/R-induced nuclear localization of FoxO1 in the heart. Furthermore, Sirt1 stimulates expression of MnSOD in cultured cardiac myocytes, and FoxO1 plays an essential role in mediating Sirt1-induced upregulation of MnSOD. Importantly, Sirt1 inhibits oxidative stress in cardiac myocytes through FoxO1-dependent mechanisms (Figure VI in the online-only Data Supplement). We propose that coordinated regulation of these cell-protective and cell death–promoting molecules mediates the protective effect of Sirt1 under I/R. Consequently, upregulation of Sirt1 significantly attenuates oxidative stress and activation of caspase-3 in the heart during I/R. Many of the molecular changes by either upregulation or downregulation of Sirt1 were observed only in the context of I/R but not at basal conditions. Thus, additional I/R-dependent mechanisms regulating expression of antiapoptotic/proapoptotic molecules and/or nuclear localization of FoxO1 appear to exist. The manner in which Sirt1 protects the heart during I/R in vivo and the role of FoxO1 in mediating the protective effect of Sirt1 require further investigation.

We have shown recently that Nampt, an enzyme stimulating NAD synthesis, protects the heart from prolonged ischemia.28 Because Nampt increases the activity of Sirt1,28 Sirt1 may protect the heart from I/R not only by reducing reperfusion injury but also by protecting the heart from prolonged ischemia. We have found recently that Sirt1 and FoxO coordinately activate autophagy in response to glucose starvation in cardiac myocytes in vitro (N. Hariharan, BS, et al, unpublished data, 2010). Although the molecular mechanism through which Sirt1 protects against myocardial injury caused by prolonged ischemia remains to be elucidated in vivo, Sirt1 might stimulate autophagy, thereby inhibiting myocardial cell death during prolonged ischemia. In any case,
stimulation of Sirt1 is an attractive modality to reduce myocardial injury that can be caused by both ischemia and reperfusion in patients suffering from acute myocardial infarction.

Sirt1 is involved in lifespan extension in lower organisms under conditions of calorie restriction. Stimulation of Sirt1 by a small-molecule compound reduces the mortality of obese mice on a high-calorie diet. Although the molecular mechanism mediating the lifespan extension remains to be elucidated in mammals, currently well-accepted hypotheses include the hormesis hypothesis in which accumulation of stress resistance conferred by nonlethal stress leads to lifespan extension. Stimulation of Sirt1 may confer resistance to I/R injury to the heart, which could be a novel mechanism of cardioprotection against cardiac stress. Endogenous mechanisms of lifespan extension are stimulated by low-grade stress, such as calorie restriction. The fact that downregulation of Sirt1 under I/R is attenuated in the presence of preconditioning suggests that stimulation of Sirt1 by a low grade of repetitive stress may partly mediate the beneficial effect of preconditioning.

We have shown previously that the antiaging effect of Sirt1 is dose dependent and that a very high dose of Sirt1 actually induces heart failure. It has also been shown that the cardioprotective effect of resveratrol is dose dependent. Thus, the extent of Sirt1 stimulation should be addressed carefully when stimulation of Sirt1 is considered for treatment of I/R injury in the future.

In summary, Sirt1 has a cardioprotective effect against I/R. Sirt1 upregulates cardioprotective molecules and downregulates proapoptotic molecules during I/R, thereby attenuating oxidative stress and inhibiting apoptosis. Thus, activation of Sirt1 could be a novel method of cardioprotection against cardiac I/R.

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Disclosures

None.

References


**CLINICAL PERSPECTIVE**

Reperfusion injury is a significant health problem in Western countries, but there is no medical treatment to effectively reduce it. We have shown previously that silent information regulator 1 (Sirt1), a class III histone deacetylase and a member of the sirtuin family, inhibits cell death in cardiomyocytes in response to stress and retards aging in the heart. The sirtuin family proteins play an important role in mediating lifespan extension induced by caloric restriction in lower organisms, and stimulation of sirtuins appears to increase lifespan in vertebrates in certain conditions. Because many mechanisms inducing lifespan extension make organisms resistant to stress, we hypothesized that stimulation of sirtuin may also make the heart more resistant to ischemic injury. In this study, we investigated the role of Sirt1 in mediating cardioprotection in a mouse model of ischemia/reperfusion. Although downregulation of endogenous Sirt1 exacerbated myocardial injury, upregulation of Sirt1 attenuated it, in response to ischemia/reperfusion. Sirt1 enhanced nuclear accumulation of FoxO1, a transcription factor regulating antioxidants and cell death/survival mechanisms, which in turn attenuated oxidative stress in the heart. Our results suggest that enhancing the function of Sirt1 is a promising modality to reduce myocardial injury in patients with acute myocardial infarction.
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SUPPLEMENTAL MATERIALS

Supplemental methods:

Immunohistochemistry

The heart specimens were fixed with formalin, embedded in paraffin, and sectioned at 6-μm thickness. Formalin-fixed paraffin-embedded sections were deparaffinized and rehydrated in PBS. Pretreatments included microwave antigen retrieval in a 10 mmol/L citrate buffer for 20 min. Immunohistochemical staining was performed with the ImmunoCruz staining system (streptavidin-biotin peroxidase method, Santa Cruz Biotechnology), and sections were counterstained with hematoxylin. Bcl-xL rabbit polyclonal antibody (BD Biosciences), cleaved caspase-3 rabbit polyclonal antibody (Cell Signaling Technology), 8-OHdG goat polyclonal antibody (Chemicon), FKHR (FoxO1) rabbit monoclonal antibody (Epitomics), and Troponin T mouse monoclonal antibody (Thermo scientific) were used.

Quantitative Reverse Transcription-PCR (qRT-PCR)

Total RNA was extracted from cultured cardiac myocytes with TRIsol (Invitrogen). cDNA was synthesized with the RETROscript kit (Ambion) following the manufacturer's instructions. Real time-PCR was carried out as described previously. The following primer pairs were used –

FoxO1: Sense – CAGATCTACGAGTGGATGGT

Antisense – ACTTGCTGTGTAGGGACAGA

Sirt1: Sense – CTC CTG TTG ACC GAT GGA CT

Antisense – GCG TCA TAT CAT CCA GCT CA

GAPDH: Sense – GAGCTGAACGGGAAGCTCACT

Antisense – TTGTCATACCAGGAAATGAGC

Reporter Gene Assays
Reporter gene assays were conducted with a 3x insulin responsive sequence (IRS) promoter-luciferase reporter (3 x IRS-Luc)\(^2\) as described previously\(^3\). To generate 3 x IRS-Luc, the two oligonucleotides

\[
\text{CTTCAAAATAAGTTTGTTTTGCTTCAAAATAAGTTTGTTTTGCTTCAAAATAAGTTTGT} \\
\text{TTTGCA} \\
\text{AGCTTGCAAAACAAACTTATTTTGAAGCAAAACAAACTTATTTTGAAGCAAAACAAAC} \\
\text{TTATTTTGAAGAGCT}
\]

were annealed and ligated into the SacI, HindIII sites of the pGL3-basic vector (Promega, Madison).

**Fluorescence Microscopic Measurement of ROS Production**

We used 5-(and-6)-chloromethyl-2', 7'-dichloro-dihydrofluorescein diacetate (CM-H2DCFDA, Molecular Probes) to detect intracellular generation of reactive oxygen species (ROS). Cardiac myocytes were loaded with 5 \(\mu\text{mol/L}\) CM-H2DCFDA for 15 min in the dark. After loading, cells were washed with warm HBSS without phenol red. During loading, the acetate groups on CM-H2DCFDA are removed by intracellular esterase, trapping the probe inside the myocyte. Cells loaded with CM-H2DCF were treated with chelerythrine (10 \(\mu\text{mol/L}\)) and analyzed by fluorescence microscopy.

Production of ROS can be measured by changes in fluorescence due to intracellular production of CM-DCF (5-(and-6)-chloromethyl-2', 7'-dichlorofluorescein) caused by oxidation of CM-H2DCF. CM-DCF fluorescence was measured at an excitation wavelength of 480 nm and an emission wavelength of 520 nm.
Supplemental Table S1. Organ weight of cardiac specific Sirt1 knockout mouse

<table>
<thead>
<tr>
<th></th>
<th>Cardiac specific Sirt1 -/- (N=3)</th>
<th>Wild type (N=5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart weight/ BW, mg/ g</td>
<td>5.2±0.3</td>
<td>5.1±0.2</td>
</tr>
<tr>
<td>LV weight/ BW, mg/ g</td>
<td>3.9±0.1</td>
<td>3.8±0.1</td>
</tr>
<tr>
<td>Lung weight/ BW, mg/ g</td>
<td>5.9±0.1</td>
<td>5.9±0.2</td>
</tr>
<tr>
<td>Liver weight/ BW, mg/ g</td>
<td>52.3±3.9</td>
<td>48.5±0.9</td>
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</tbody>
</table>

Values are mean ± SEM; BW, body weight; LV, left ventricle.
### Supplemental Table S2 Baseline characteristics of Tg-Sirt1 and NTg control mice

<table>
<thead>
<tr>
<th></th>
<th>LVSP</th>
<th>LVEDP</th>
<th>LVDP</th>
<th>+ dP/dt</th>
<th>- dP/dt</th>
<th>HR</th>
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</thead>
<tbody>
<tr>
<td>NTg</td>
<td>90.8±15.2</td>
<td>4.8±0.8</td>
<td>86±14.7</td>
<td>2620±465</td>
<td>1360±240</td>
<td>273±46</td>
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<tr>
<td>Tg</td>
<td>106±7.4</td>
<td>5.6±0.7</td>
<td>100.4±7.9</td>
<td>3160±444</td>
<td>1660±60</td>
<td>274±29</td>
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</tbody>
</table>

NTg, non-transgenic mice; Tg, transgenic mice with cardiac specific overexpression of Sirt1
LVSP, left ventricular systolic pressure (mmHg);
LVEDP, left ventricular end-diastolic pressure (mmHg);
LVDP, left ventricular developed pressure (mmHg); HR, heart rate (beats/min)
Supplemental Fig. S1

A

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>CKO</th>
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</thead>
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<td>Heart</td>
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<tr>
<td>Lung</td>
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<tr>
<td>Liver</td>
<td></td>
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<tr>
<td>Brain</td>
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</table>

B

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>CKO</th>
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</thead>
<tbody>
<tr>
<td>Sirt1</td>
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<tr>
<td>Sirt3</td>
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<td></td>
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<tr>
<td>Tubulin</td>
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</tbody>
</table>
Supplemental Fig. S2

A  Sirt1
   Tubulin
   NTg  Tg

B
   NTg  Tg  50 μm
Supplemental Fig. S3

A

B

C

D

E

F

<table>
<thead>
<tr>
<th>Protein</th>
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<tr>
<td>Bax</td>
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<tr>
<td>MnSOD</td>
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<td>Trx1</td>
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<tr>
<td>Bcl-xL</td>
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<td>Cleaved caspase 3</td>
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<tr>
<td>Sirt1</td>
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<td>Tubulin</td>
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<th>Relative MnSOD expression</th>
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<th>Relative Trx1 expression</th>
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<table>
<thead>
<tr>
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<th>Relative Bcl-xL expression</th>
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<td>Sirt1 -/-</td>
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<table>
<thead>
<tr>
<th>Protein</th>
<th>Relative Bax expression</th>
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<tbody>
<tr>
<td>WT</td>
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<tr>
<td>Sirt1 -/-</td>
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</table>
Supplemental Fig. S5

The figure shows a comparison of the relative FoxO1 mRNA level between Ad-LacZ and Ad-Sirt1. The data indicates a significant difference with a p-value of 0.0155.
Supplemental Fig. S6

A

B

Chele  -  +  +  +  +
Ad-Lacz +  +  -  -  -
Ad-Sirt1 -  -  +  +  +
Ad-sh-FoxO1 -  -  -  +  +

P=0.002  P=0.024

arbitrary units
**Figure legends for supplemental Figures**

Supplemental Figure S1 Homogenates were prepared from indicated organs (A) and the heart (B) in wild type (WT) or cardiac specific Sirt1 -/- (CKO) mice and immunoblot analyses were conducted with anti-Sirt1, anti-Sirt3, and anti-α-tubulin antibodies.

Supplemental Figure S2 Expression of Sirt1 in Tg-Sirt1. (A) Homogenates were prepared from Tg-Sirt1 (Tg) and non-transgenic (NTg) hearts. The level of Sirt1 in the heart was evaluated by immunoblot analysis. (B) The level of Sirt1 expression in the myocardium was evaluated with immunostaining with anti-Sirt1 antibody.

Supplemental Figure S3 Heart homogenates were prepared from the ischemic area in wild type (WT) or cardiac specific Sirt1 -/- mice subjected to I/R. Expression of Bax, MnSOD, Trx1, Bcl-xL, cleaved caspase-3, Sirt1 and tubulin was evaluated by immunoblots. (A) Representative immunoblots are shown. (B-F) The quantitative and statistical analyses of the immunoblots are shown. The relative expression of MnSOD (B), Trx1 (C), Bcl-xL (D), Bax (E) and cleaved caspase-3 (c. caspase 3) (F) in Sirt1 -/- versus WT is shown. The expression level of WT mice is expressed as 1.

Supplemental Figure S4 Representative immunostaining of 8-OHdG in left ventricular myocardial sections obtained from wild type (WT) and cardiac specific Sirt1 -/- mice subjected to either sham operation or I/R.

Supplemental Figure S5 Cultured cardiac myocytes were transduced with either adenovirus harboring LacZ (Ad-LacZ) or adenovirus harboring Sirt1 (Ad-Sirt1). After forty-eight hours, the level of FoxO1 mRNA was evaluated with quantitative PCR analyses. GAPDH was used as an internal control. n=4 each.

Supplemental Figure S6 Cultured cardiac myocytes were transduced with Ad-LacZ, Ad-Sirt1 and/or Ad-sh-FoxO1 as indicated. The total amount of adenovirus was 10MOI in each sample. After forty-eight hours, myocytes were subjected to DFCDA staining as described in the Method section in the presence or absence of chelerythrine (10 μmol/L).
References

