Cardiac Sirt1 mediates the cardioprotective effect of caloric restriction by suppressing local complement system activation after ischemia-reperfusion

Tsunehisa Yamamoto,1 Kayoko Tamaki,1,3 Kohsuke Shirakawa,1 Kentaro Ito,1 Xiaoxiang Yan,1 Yoshinori Katsumata,1 Atsushi Anzai,1 Tomohiro Matsuhashi,1 Jin Endo,1 Takaaki Inaba,2 Kazuo Tsubota,2 Motoaki Sano,1 Keiichi Fukuda,1 and Ken Shinmura1,3

1Department of Cardiology, Keio University School of Medicine, Tokyo, Japan; 2Department of Ophthalmology, Keio University School of Medicine, Tokyo, Japan; and 3Department of General Medicine, Hyogo College of Medicine, Nishinomiya, Japan

Submitted 25 August 2015; accepted in final form 4 February 2016

NEW & NOTEWORTHY

Sirt1 in cardiomyocytes is essential for the development of caloric restriction-induced cardioprotection and regulates expression levels of component 3 in cardiomyocytes. Thus we conclude that caloric restriction attenuates myocardial ischemia-reperfusion injury by suppressing local complement activation during ischemia-reperfusion in a Sirt1-dependent manner.

CALORIC RESTRICTION (CR) is the established intervention scientifically proven to have anti-aging effects (17, 22, 36, 40). CR profoundly affects age-related physiological and pathophysiological alterations and markedly increases both mean and maximal lifespan in several species, including mammals. Recent experimental and clinical investigations demonstrated that CR exerts pleiotropic effects on the cardiovascular system (26, 28, 29, 37, 40, 42). CR prevents the progression of atherosclerosis and vascular aging via direct and indirect mechanisms (28, 40). CR prevents cardiac senescence by attenuating oxidative damage and enhancing cardiac autophagy, leading to improved cardiac function in aged animals (33). CR improves myocardial ischemic tolerance in rodents of all ages (30–32, 42). CR may mitigate metabolic cardiomyopathy associated with obesity and type 2 diabetes mellitus (37).

Thus the clinical application of CR and the development of CR mimetics that can replicate the effects of CR on the cardiovascular system have considerable potential as novel therapeutic approaches to manage patients with cardiovascular diseases. The mechanisms underlying the beneficial cardiovascular effects of CR are multifaceted, but remarkable progress has been made in the past decade toward their understanding. Recent investigations, including our studies, have revealed that CR triggers an active defense response against stressful conditions and exerts preconditioning-like cardioprotection (26, 28, 29, 40). At the center of this response are cardiovascular protective signals, which include adenosine monophosphate-activated kinase, sirtuins, and endothelial nitric oxide (NO) synthase (eNOS).

Increasing evidence demonstrates that Sirt1 mediates the preferable effects of CR in various organs (4, 7, 9, 14). Our laboratory found that prolonged CR significantly increases the expression levels of Sirt1 in the nuclear fraction and decreases the expression levels of acetyl-histone H3 (30), suggesting that the activated form of Sirt1 increases in the hearts of CR-fed rats. Hsu and colleagues demonstrated that cardiac Sirt1 protects the myocardium from ischemia-reperfusion (I/R) injury...
by upregulating the antioxidants, manganese superoxide dismutase (MnSOD) and thioredoxin 1 (Trx1), and an anti-apoptotic protein, Bel-xy, and by downregulating the proapoptotic molecules, Bax and cleaved caspase-3 (19). Cardiac Sirt1 deacetylates the transcription factor Forkhead box O1 (FoxO1), and FoxO1 translocates into the nucleus and mediates Sirt1-dependent cardioprotection. They recently reported that the protective effect of CR against myocardial infarction was not significant in the cardiomyocyte-specific Sirt1 knockout (CM-Sirt1−/−) mice (42). However, direct evidence that demonstrated that cardiac Sirt1 is necessary for the cardioprotective effect of CR against I/R was insufficient.

We observed the interaction between eNOS and Sirt1 because this interaction plays an obligatory role in the development of vascular protection (31). By using eNOS-deficient (eNOS−/−) mice, we demonstrated that the increased NO production, which is mainly derived from eNOS, is essential for CR-induced cardioprotection. Furthermore, we found that eNOS is located upstream of cardiac Sirt1 activation during CR. Although it is likely that Sirt1 is a downstream mediator of eNOS, it is located upstream of cardiac Sirt1 activation during CR. The second aim was to investigate the mechanisms of Sirt1-mediated cardioprotection during CR. Hsu et al. (19) reported that overexpression of cardiac Sirt1 attenuates myocardial I/R injury in vivo and ex vivo by enhancing oxidative defense and suppressing cardiomyocyte apoptosis. However, whether the same mechanisms are involved in transgenic mice and CR-treated mice remains to be known. In addition to the enhancement of oxidative defense and suppression of cardiomyocyte apoptosis, we expected the third mechanism of Sirt1-mediated cardioprotection during CR and found that cardiac Sirt1 mediates cardioprotection against I/R injury by attenuating local complement system activation after I/R.

MATERIALS AND METHODS

All procedures in the present study conformed to the principles outlined in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee of Keio University School of Medicine.

Mice

CM-Sirt1−/− mice were generated by crossing Sirt1fl/fl mice on a C57BL/6J background with α-myosin heavy chain promoter-driven Cre mice (αMHC-Cre). Floxed Sirt1 mice to delete exon 4 of the Sirt1 gene were provided by Dr. D. Sinclair. All Cont mice (Sirt1fl/fl) and Sirt1fl/fl,αMHC-Cre (CM-Sirt1−/−) mice were backcrossed to the C57BL/6J background. Eight-week-old mice deficient in complement component 3 (C3−/−) mice; originally purchased from the Jackson Laboratory and bred in our animal laboratory) were compared with age-matched C57BL/6J mice as wild-type (Wt; Jackson Laboratory, Bar Harbor, ME). Animals were maintained on a standard chow diet and kept on a normal 12:12-h light-dark cycle with a minimum of 14 days allowed for local vivarium acclimation before experimental use.

CR Protocols

CR was performed as described previously (32, 33). Briefly, 8-wk-old male CM-Sirt1−/− mice and the corresponding Cont mice, as well as C3−/− and Wt mice were housed in individual cages according to institutional protocols at the Keio University Experimental Animal Centre and fed AL for 2 wk with a modified semipurified diet A (Oriental Yeast, Tokyo, Japan). The average caloric intake was calculated from the daily food intake over this 2-wk period. After weaning, mice were randomly divided into two groups. AL mice continued to be fed AL using the Cont diet Afor the subsequent 16 wk. CR mice were fed 90% of the average caloric value of caloric intake during the AL period for 2 wk (10% restriction), followed by 60% of that for 12 wk (40% restriction) using modified semipurified diets B and C, respectively. Modified semipurified diets B and C comprise the same amount of calories per weight, but are enriched in vitamins and minerals by 11 and 67%, respectively, compared with diet A. We reduced the daily caloric intake by decreasing food weight to 90 and 60% in the modified semipurified diets B and C. Thus daily intake of vitamins and minerals was constant during the CR period.

Echocardiography

At the age of 26 wk, the mice were anesthetized with 1.5% isoflurane inhalation and then were anchored to a positioning platform in the supine position. Short-axis echocardiographic measurements were made using the Vevo 660 system (Visual Sonics) with a 6000 series real-time microvisualization scanhead probe (33). The left ventricular (LV) internal end-systolic (LVEDS), LV end-diastolic pressure (LVEDP), and LV peak positive and negative change in pressure over time (dP/dt) were calculated from the following formula: FS (%) = [(LVEDD − LVEDD/LVEDD) × 100. Heart rate did not differ significantly among the groups during the echocardiographic assessments.

Langendorff Perfusion of the Heart

Mice were anesthetized by concomitantly administering pentobarbital sodium (300 mg/kg) with heparin (50 units) via an intraperitoneal injection. Then the hearts were excised quickly and perfused with modified Krebs-Henseleit buffer (120 mmol/l NaCl, 25 mmol/l NaHCO3, 5.9 mmol/l KCl, 1.2 mmol/l MgSO4, 1.75 mmol/l CaCl2, and 10 mmol/l glucose) gassed with 95% O2/5% CO2 at 37°C, according to the Langendorff procedure, as described previously. Coronary perfusion pressure was maintained at 80 cmH2O. A plastic catheter with a balloon was inserted into the LV through the left atrium, as described previously. Before the induction of ischemia, the LV end-diastolic pressure (LVEDP) was adjusted to 10 mmHg by filling the balloon with water. Indexes of LV function [heart rate (HR)], LV systolic pressure (LVSP), LV developed pressure (LVEDP), and LV peak positive and negative change in pressure over time (dP/dt) were recorded every 5 min before the induction of ischemia and after reperfusion. After finishing the entire experiment, we excluded mouse hearts that showed poor LV function during the initial perfusion (LVSP < 90 mmHg) or massive leakage from the aorta from further analysis.

I/R Protocol and Measurement of the Lactate Dehydrogenase Activity

After 40 min of perfusion (stabilization) in a non-recirculating mode, all hearts were subjected to 25 min of global no-flow ischemia.
followed by 60 min of reperfusion. The coronary effluent perfusate was collected throughout reperfusion by inserting a cannula into the right atrium. The collected perfusate was stored on ice until the end of reperfusion, and the total volume was measured. Then the perfusate was stored in the deep freezer in 1-ml aliquots, and later lactate dehydrogenase (LDH) activity was measured using commercially available spectrophotometric assays (Sigma-Aldrich, St. Louis, MI). Total LDH release was calculated as the product of effluent concentration \( \times \) effluent volume during 60 min of reperfusion and was corrected for ventricular weight (VW).

**Gene Expression Analysis by Quantitative RT-PCR**

For quantitative real-time polymerase chain reaction (qRT-PCR), total RNA samples from cultured cardiomyocytes and hearts were prepared using TRIzol reagent (Invitrogen). Samples of total RNA (2 \( \mu \)g) were reverse-transcribed using an RNA PCR kit (Takara Biotechnology, Otsu, Shiga, Japan), and the resulting cDNA was used as a PCR template. RNA levels were then determined by RT-PCR using the ABI PRISM7700 Sequence Detector (Applied Biosystems). Pre-designed gene-specific primer and probe sets (TaqMan Gene Expression Assays) were used, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was amplified as an internal control. Relative gene expression levels (i.e., the amount of target gene normalized against housekeeping genes) were calculated using the comparative cycle threshold (Ct) method as \( 2^{-\Delta\Delta Ct} \).

**Western Blotting**

Standard SDS-PAGE Western blotting techniques were used to assess the expression levels of eNOS, Sirt1, MnSOD, Trx1, catalase, C3, acetylated (Ac)-FoxO1, GAPDH, heat shock protein (HSP) 70, and lamin A/C, as described previously (31, 34). Total protein was extracted from frozen hearts. Equal amounts of total proteins (20–40 \( \mu \)g) were subjected to SDS-PAGE. The primary antibodies used in the present study were anti-eNOS, anti-phosphorylated eNOS (P-eNOS) at the serine 1177 residue (Ser1177), anti-P-eNOS at the threonine 495 residue (Thr495), anti-Sirt1 (for subcellular fractions), anti-Trx1, anti-lamin A/C (Cell Signaling Technology, Danvers, MA), anti-C3 (Cälbiochem, La Jolla, CA), anti-Sirt1 (for total homogenate), anti-catalase, anti-Ac-FoxO1, anti-HSP70 (Santa Cruz, Dallas, TX), anti-MnSOD (Enzo Lifesciences, Farmingdale, NY), and anti-GAPDH (Millipore, Billerica, MA). For assessment of the subcellular distribution of Sirt1 protein, cytosolic and nuclear fractions were prepared according to the manufacturer’s instructions (28, 30). Densitometric analyses of the proteins were normalized using reference proteins such as GAPDH, HSP70, or lamin A/C. The densitometric measurement of P-eNOS was normalized using the corresponding value of total eNOS protein and expressed as the ratio of P-eNOS to total eNOS (P-eNOS/total eNOS).

**Histological Analysis**

Mice were anesthetized and killed. The hearts were then immediately perfused with phosphate-buffered saline, fixed with 10% formalin neural buffer solution, and embedded in paraffin. After deparaffinization, the sections were incubated in 0.3% \( \mathrm{H}_2\mathrm{O}_2 \) for 15 min and blocked in 5% BSA for 30 min at room temperature. Next, the sections were incubated with anti-C3 (Hyctul Biotech no. HM1045 1:50) overnight at 4°C. Secondary antibodies conjugated with horse-radish peroxidase were applied for 30 min at room temperature. Antibody binding was detected by incubating the sections in 3,3-diaminobenzidine tetrahydrochloride substrate for 10 min at room temperature.

**Cell Culture and Resveratrol Treatment**

Primary cultures of neonatal rat ventricular cardiomyocytes were prepared as described previously (34). In brief, neonatal ventricular myocytes from 1- to 2-day-old Sprague-Dawley rats were killed by decapitation, and the hearts were immediately removed and subjected to Percoll gradient centrifugation and differential plating to enrich the cardiac myocyte population and to deplete nonmyocytes. Cardiomyocytes were cultured in a mixture of Dulbecco’s modified Eagle’s medium and M-199 with 10% fetal bovine serum (Biowest, Nuaille, France). Cardiomyocytes were stimulated with 50 \( \mu \)M resveratrol (Sigma-Aldrich, St. Louis, MO) for 24 h. This dose of resveratrol was chosen to activate Sirt1 in cardiomyocytes, as described in a previous report (43). Resveratrol was dissolved in DMSO, and the control cardiomyocytes were treated with the same volume of DMSO as the vehicle.

**Measurement of Serum C3 Concentration**

Serum C3 levels were measured by immunoturbidimetric method using commercially available kits (Nittobo Medical, Tokyo, Japan and Mannheim, Germany).

**Statistical Analysis**

Data are presented as means \( \pm \) SE. For intergroup comparisons, data were analyzed by using Mann-Whitney’s U-test, and one-way or two-way ANOVA, followed by Scheffé’s post hoc test. A \( P < 0.05 \) was considered significant.

**RESULTS**

**Phase I Experiment: Role of Cardiac Sirt1 in CR-induced Cardioprotection**

**Body and ventricular weights in the Cont and CM-Sirt1 \(^{-/-}\) mice.** There was no difference in body weight (BW) at 14 wk of age between the Cont and CM-Sirt1 \(^{-/-}\) mice (data not shown). During the protocol, BW increased in the AL group and decreased in the CR group in both mice, but the difference in the change in BW was not significant between the Cont and CM-Sirt1 \(^{-/-}\) mice (Table 1). The VW was greater in the AL group, and the VW-to-BW ratio was higher in the CR group in both mice (Table 1). However, there was no difference in them between the Cont and CM-Sirt1 \(^{-/-}\) mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Body Weight, g</th>
<th>Ventricular Weight, g</th>
<th>Ventricular Weight/Body Weight, %</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phase I experiment: role of cardiac Sirt1 in CR-induced cardioprotection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cont mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AL group</td>
<td>13</td>
<td>36.2 ± 0.9</td>
<td>0.117 ± 0.003</td>
<td>0.323 ± 0.011</td>
</tr>
<tr>
<td>CR group</td>
<td>13</td>
<td>24.8 ± 0.5*</td>
<td>0.091 ± 0.001*</td>
<td>0.369 ± 0.005*</td>
</tr>
<tr>
<td>CM-Sirt1 (^{-/-}) mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AL group</td>
<td>13</td>
<td>35.2 ± 1.4</td>
<td>0.146 ± 0.005</td>
<td>0.422 ± 0.016</td>
</tr>
<tr>
<td>CR group</td>
<td>13</td>
<td>24.4 ± 0.4*</td>
<td>0.120 ± 0.005*</td>
<td>0.492 ± 0.025*</td>
</tr>
<tr>
<td><strong>Phase II experiment: role of C3 in CR-induced cardioprotection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AL group</td>
<td>7</td>
<td>38.0 ± 1.7</td>
<td>0.132 ± 0.007</td>
<td>0.353 ± 0.025</td>
</tr>
<tr>
<td>CR group</td>
<td>7</td>
<td>25.7 ± 0.5*</td>
<td>0.091 ± 0.016*</td>
<td>0.433 ± 0.031*</td>
</tr>
<tr>
<td>C3 (^{-/-}) mice</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AL group</td>
<td>13</td>
<td>32.1 ± 1.0</td>
<td>0.128 ± 0.006</td>
<td>0.398 ± 0.012</td>
</tr>
<tr>
<td>CR group</td>
<td>13</td>
<td>25.1 ± 0.4*</td>
<td>0.110 ± 0.004*</td>
<td>0.440 ± 0.016*</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SE; n, no. of mice. Values are at the age of 26 wk. Cont, control; CM-Sirt1 \(^{-/-}\), cardiomyocyte-specific Sirt1 knockout; Wt, wild type; C3 \(^{-/-}\), conventional component 3 knockout; AL, ad libitum; CR, caloric restriction. \( *P < 0.05 \) compared with the corresponding AL group.
In vivo evaluation of cardiac function by echocardiography demonstrated no significant difference in cardiac parameter at baseline between the Cont and CM-Sirt1\(^{-/-}\) mice (Fig. 1A). Expression levels of cardiac Sirt1 in the Cont and CM-Sirt1\(^{-/-}\) mice. The expression levels of myocardial Sirt1 in the CM-Sirt1\(^{-/-}\) mice were ~25% at the mRNA level and ~20% at the protein level of those in the Cont mice (Fig. 1, B and C). In the Cont mouse hearts, CR increased the expression levels of Sirt1 mRNA, but there was no difference in the expression levels of Sirt1 protein in the total homogenate between the AL and CR groups (Fig. 1, B and C). However, CR increased the expression levels of Sirt1 protein in the nuclear fractions, but not in the cytosolic fractions (Fig. 1, D and E), indicating that CR enhances cardiac Sirt1 activity in the Cont mice. There were no differences in the expression levels of myocardial Sirt1 with CR in the CM-Sirt1\(^{-/-}\) mice (Fig. 1, B and C).

Myocardial I/R injury in the Cont and CM-Sirt1\(^{-/-}\) mice. There was no difference in LV function at baseline between the Cont and CM-Sirt1\(^{-/-}\) mice. There were no differences in the expression levels of myocardial Sirt1 with cardiac Sirt1 activity in the Cont mice. There were no differences in LV function at baseline between the AL and CR groups (Fig. 1, B and C). In the Cont mice, CR increased the expression levels of Sirt1 protein in the total homogenate between the AL and CR groups (Fig. 1, B and C). There was no difference in LV function at baseline between the Cont and CM-Sirt1\(^{-/-}\) mice. There were no differences in LV function at baseline between the AL and CR groups (Fig. 1, B and C). In the Cont mice, CR increased the expression levels of Sirt1 protein in the total homogenate between the AL and CR groups (Fig. 1, B and C). There was no difference in LV function at baseline between the Cont and CM-Sirt1\(^{-/-}\) mice. There were no differences in LV function at baseline between the AL and CR groups (Fig. 1, B and C). In the Cont mice, CR increased the expression levels of Sirt1 protein in the total homogenate between the AL and CR groups (Fig. 1, B and C). There was no difference in LV function at baseline between the Cont and CM-Sirt1\(^{-/-}\) mice. There were no differences in LV function at baseline between the AL and CR groups (Fig. 1, B and C).
by Sirt1 (18, 25). Cardiac Sirt1 is known to upregulate several genes involved in antioxidant defense (e.g., catalase, MnSOD, and Trx), that might be upregulated through activation of FoxO1 (15, 19, 35, 38). In fact, we found that expression levels of PGC-1α, FoxO1, MnSOD, Trx1, and catalase mRNAs increased with CR in the Cont mouse heart (Fig. 3A). Furthermore, we confirmed that CR increased the expression levels of MnSOD, Trx1, and catalase proteins in the Cont mouse hearts (Fig. 3B).

Expression levels of total and P-eNOS in the Cont and CM-Sirt1−/− mice. We demonstrated that CR upregulates eNOS protein expression and activates eNOS by promoting Thr495 dephosphorylation (31). However, we could not clarify the mechanism by which CR promotes Thr495 dephosphorylation in eNOS. Hence, we evaluated the ratio of P-eNOS to total eNOS (P-eNOS/total eNOS) in the Cont and CM-Sirt1−/− mouse hearts. In the present study, CR did not affect P-eNOS (Ser1177)/total eNOS, but decreased P-eNOS (Thr495)/total eNOS in the Cont mouse heart (Fig. 3E). In contrast, CR failed to increase P-eNOS (Ser1177)/total eNOS or decrease P-eNOS (Thr495)/total eNOS in the CM-Sirt1−/− mouse heart (Fig. 3F). These results suggest that Sirt1 is responsible for the dephosphorylation of eNOS at Thr495.

Cardiac C3 expression in the Cont and CM-Sirt1−/− mice. To gain some insight into the possible mechanisms by which cardiac Sirt1 protects myocardium from I/R injury during CR, we performed microarray analysis of the heart obtained from the Cont and CM-Sirt1−/− mice. Pathway analysis revealed that genes related to the complement and coagulation cascades were downregulated with CR in a Sirt1-dependent manner (Gene Expression Omnibus accession no. GSE75558). Thus we noticed the expression levels of complement C3 with CR because C3 plays a central role in the activation of the complement system (2, 3, 13, 20). The
expression levels of C3 protein significantly decreased with CR in the Cont mouse heart (Fig. 4A), although the expression levels of C3 mRNA did not change with CR (Fig. 4B). In contrast, the expression levels of C3 mRNA and protein in the heart were not changed in the CM-Sirt1−/− mice treated with CR (Fig. 4, C and D). There was no difference in serum C3 levels between the AL and CR groups in both strains (Fig. 4E), suggesting that the decrease in myocardial C3 protein is independent of systemic C3 production during CR. Immunohistological staining revealed that accumulation of C3 and its fragments in the ischemia-reperfused myocardium was decreased with CR in the Cont mice, but not in the CM-Sirt1−/− mice (Fig. 4F).

Phase II Experiment: Role of C3 in CR-induced Cardioprotection

Expression levels of C3 in cardiomyocytes after Sirt1 activation. The expression levels of C3 in cultured neonatal rat ventricular cardiomyocytes were evaluated in the absence and presence of resveratrol (Fig. 5). Treatment with resveratrol increased the expression levels of Sirt1 in the nuclear fractions but not in the cytosolic fractions (Fig. 5, A–C) and decreased the expression levels of Ac-FoxO1 (Fig. 5, A and D). Treatment with resveratrol did not change the expression levels of C3 mRNA (data not shown), but significantly decreased the expression levels of C3 protein in cardiomyocytes 24 h later.
Cont CM-Sirt1-/– the Cont mouse heart treated with CR.

BW increased in the AL group of difference in them between the Wt and CR group of change in BW between the Wt and CR. (Fig. 5, A)

BW and VW in the Wt and C3−/− mice. During the protocol, BW increased in the AL group of C3−/− mice and decreased in the CR group of C3−/− mice, and there was no difference in the change in BW between the Wt and C3−/− mice (Table 1). VW was greater in the AL group, and the VW-to-BW ratio was higher in the CR group of C5−/− mice (Table 1). However, there was no difference in them between the Wt and C3−/− mice (Table 1).

Myocardial I/R injury in the Wt and C3−/− mice. Although recoveries of LVDP, +dP/dt, −dP/dt, and HR after I/R in the C3−/− mice fed AL were similar to those in the Wt mice fed AL, the increase in LVEDP was significantly attenuated in the C3−/− mice fed AL, compared with that in the Wt mice (Fig. 6, A–E). Furthermore, the LDH release into the perfusate during reperfusion was significantly attenuated in the C3−/− mice fed AL, and it was at the same level as that in the Wt mice treated with CR (Fig. 6F). CR failed to improve the recovery of LV function in the C3−/− mice and did not further attenuate the LDH release in the C3−/− mice (Fig. 6).

Expression levels of cardiac Sirt1, Sirt1-target genes, and antioxidant enzymes in the Wt and C3−/− mice. The mRNA levels of myocardial Sirt1 and Sirt1-target genes increased with CR in the C3−/− mice (Fig. 7A). CR significantly
increased the expression levels of MnSOD, Trx1, and catalase proteins in the C3−/− mice (Fig. 7B). CR increased the expression levels of SirT1 protein in the nuclear fractions but not in the cytosolic fractions in the C3−/− mice (Fig. 7, C and D). These results indicate that SirT1 is activated in the heart of C3−/− mice treated with CR.

**DISCUSSION**

The major findings of the present study were as follows: 1) CR-induced cardioprotection against I/R injury was not observed in the CM-SirT1−/− mice; 2) activation of SirT1 suppresses the expression levels of cardiac C3 protein in vivo and in vitro; and 3) CR-induced cardioprotection against I/R injury was absent in the C3−/− mice, despite SirT1 activation with CR.

SirTunis, notably SirT1, have been studied for their role in CR, the prevention of age-related diseases, and the maintenance of metabolic homeostasis (4, 7, 9, 14). Although it is still controversial whether SirT1 mediates lifespan extension afforded by CR, it is a fact that SirT1 regulates various aspects of the CR response, namely, glucose homeostasis, insulin secretion, fat metabolism, stress resistance, and physical activity. Activation of SirT1 confers anti-oxidative and anti-inflammatory effects in the vasculature, resulting in attenuated vascular senescence (23, 27). In addition, SirT1 plays an important role in the cardiac adaptive response to various stresses, such as I/R, oxidative stress, and starvation (1, 15, 19). However, direct evidence that SirT1 in cardiomyocytes plays a key role in the development of favorable effects of CR on the cardiovascular system is still lacking. Yamamoto et al. (42) performed in vivo myocardial infarction experiment in Cont and CM-SirT1−/− mice fed AL or treated with CR. They found that the expression levels of nicotinamide phosphoribosyltransferase and myocardial content of nicotinamide adenine dinucleotide increased with CR. Infarct size was significantly attenuated in the Cont mice treated with CR, but not in the CM-SirT1−/− mice treated with CR. Their results are quite consistent with our findings. Taken together, it is conclusive that CR activates SirT1 in the heart, and that cardiac SirT1 plays an essential role in CR-induced cardioprotection against I/R. From the aspect of myocardial I/R injury, SirT1 existing in cardiomyocytes, but not in non-cardiomyocytes, including endothelial cells and vascular smooth muscle cells, is necessary for the development of CR-induced cardioprotection. However, these results cannot deduce the cardiovascular protection afforded by lifelong CR, because the effect of CR on vasculature might play a bigger role in aged animals than in the adult animals used in these studies (6 mo old).

CR increased the expression levels of the total eNOS protein and decreased P-eNOS (Thr495), although the expression levels of P-eNOS (Ser1177) were comparable between the AL and CR hearts (31). Thus we speculate that increased total eNOS protein level and enhanced eNOS activity via the promotion of Thr495 dephosphorylation are responsible for the increase in...
NO bioavailability in the CR hearts. Enhancement in eNOS activity via the promotion of Thr495 dephosphorylation is reported as the mechanism by which evodiamine, a transient receptor potential vanilloid 1 ligand, enhances eNOS activity (8). Our results suggest the involvement of cardiac Sirt1 in dephosphorylating eNOS at Thr495 during CR (Fig. 3, E and F). As Sirt1 cannot dephosphorylate eNOS directly, Sirt1 is likely to modify some phosphatase activity directly or indirectly. In fact, Sirt1 is reported to modulate phosphatase and tensin homolog activity, and localization by deacetylation in mouse embryonic stem cells (6). Another possibility is that deacetylation of eNOS by Sirt1 causes a conformational change in eNOS, resulting in the decrease in phosphorylation at Thr495.

Hsu and colleagues (19) demonstrated that cardiac Sirt1 upregulates the antioxidants, MnSOD and Trx1, and the anti-apoptotic protein, Bcl-xL, and downregulates the proapoptotic molecules, Bax and cleaved caspase-3, via the activation of FoxO1. Reduced oxidative stress and decrease in cardiomyocyte apoptosis in the heart with Sirt1 activation might contribute to Sirt1-mediated cardioprotection (35, 38). However, whether these molecules are essential for Sirt1-mediated cardioprotection remains to be clarified. In the present study, we confirmed that the expression levels of FoxO1, MnSOD, and catalase increase with CR in a Sirt1-dependent manner. Although CR enhanced the expression levels of antioxidant genes in the C3-/− mice, CR-induced cardioprotection was not observed in these mice. These results strongly suggest that the increase in reactive oxygen species production during I/R contributes to activation of the complement system.

Evidence indicates that the complement system is activated after I/R in various organs (2, 3, 13, 20). Three different pathways have been identified to activate the complement system, and C3 plays a central role in the complement system. Increasing evidence demonstrates that I/R activates the complement system via the classic and lectin pathways. Deposition of bioactive complements and the membrane attack complex progresses tissue damage and enhances inflammation after I/R.
Thus blockade of the complement system may become a therapeutic target for attenuating I/R injury. In fact, complement depletion and administration of C5-specific antibodies, soluble complement receptor, or C1-esterase inhibitor were reported to protect the myocardium from I/R injury in experimental models (2, 3, 13, 20). In conjunction with these facts, previous reports demonstrated that the C3−/− mice are relatively resistant to cerebral, hepatic, renal, and myocardial I/R injuries (10, 16, 21, 24). Accordingly, we speculated that CR attenuates myocardial I/R injury, in part, by suppressing C3 expression during I/R in a Sirt1-dependent manner.

Compared with the Wt mice fed AL, the increase in LVEDP after I/R was suppressed, and the relative value of LDH activity released during reperfusion was lower in the C3−/− mice fed AL (Fig. 6). These results indicate that the C3−/− mice are resistant to myocardial ischemia, as reported by Lu et al. (21). Loss of additional protection by CR in the C3−/− mice strongly supports that cardiac Sirt1 mediates CR-induced cardioprotection by regulating complement activation through C3 expression. Based on the experimental evidence, a reduction in infarct size by administration of specific antibodies to C5 could be expected. However, in all of the clinical studies, the effectiveness of treatment was not confirmed (3, 13). As one of the reasons why clinical studies ended in failure, the essential role of C3 in postinfarct LV remodeling was proposed by Wysoczynski et al. (41). They found that activation of the complement cascade through C3 contributes to myocardial preservation and regeneration during the chronic phase after myocardial infarction. Another possibility is the distinct role of C3 between systemic and local complement activation (20). Complement components, including C3, are mainly produced in the liver, but local production of C3 is confirmed in the kidney, heart, and brain. Farrar et al. (11) demonstrated using a mouse isograft model that local synthesis of C3, which is identified mainly in the tubular epithelium, is necessary for complement-mediated I/R injury in the kidney, whereas circulating C3 had a negligible effect. Regarding this point, Thurman et al. (39) recently reported a positive correlation between the expression levels of local C3 mRNA and the degree of renal I/R injury. We evaluated the effect of CR on myocardial I/R injury.
independent of circulating factors. Thus it is possible that cardiac Sirt1 attenuates myocardial I/R injury by suppressing local complement activation during I/R via C3 expression. Figure 7E represents our hypothesis regarding Sirt1-mediated cardioprotection during CR.

The mechanism by which cardiac Sirt1 suppresses cardiac C3 expression remains unknown. Sirt1 was reported to regulate adipose inflammation via deacetylation of nuclear factor-kB (12). In addition to proinflammatory cytokines, C3 transcript levels were increased by Sirt1 knockout in white adipose tissue, suggesting that Sirt1 suppresses C3 expression by suppressing nuclear factor-kB activation. Chromatin immunoprecipitation followed by a sequencing methodology using murine hearts revealed that the C3 promoter is bound by Sirt1 (5). However, we found that both CR and Sirt1 activation decreased the expression levels of C3 protein, but not C3 mRNA, suggesting that Sirt1 suppresses C3 expression at the posttranscriptional levels or the process of degradation. Further investigations are needed to clarify this issue.

In conclusion, the present study demonstrated the essential role of cardiac Sirt1 in CR-induced cardioprotection and, for the first time, the regulation of C3 expression in cardiomyocytes by Sirt1. Because CR decreases cardiac C3 expression without affecting systemic C3 production, suppression of local complement activation during I/R has the potential as an alternative therapeutic strategy for attenuating myocardial I/R injury. Thus elucidation of the mechanisms underlying cardiac Sirt1-regulated C3 expression is important for the development of effective sirtuin-activating compounds. The results of the present study support the close interaction between eNOS and Sirt1 as key molecules to mimic the cardiovascular protection afforded by CR.

REFERENCES


