Indispensable role of endothelial nitric oxide synthase in caloric restriction-induced cardioprotection against ischemia-reperfusion injury

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1Department of Cardiology, Keio University School of Medicine, Tokyo, Japan; 2Department of Biochemistry, Keio University School of Medicine, Tokyo, Japan; 3Japan Science and Technology Agency, Exploratory Research for Advanced Technology, Suematsu Gas Biology Project, Tokyo, Japan; and 4Department of Biochemistry, Keio University Graduate School of Pharmaceutical Sciences, Tokyo, Japan

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Shinmura K, Tamaki K, Ito K, Yan X, Yamamoto T, Katsumata Y, Matsushashi T, Sano M, Fukuda K, Suematsu M, Ishii I. Indispensable role of endothelial nitric oxide synthase in caloric restriction-induced cardioprotection against ischemia-reperfusion injury. Am J Physiol Heart Circ Physiol 308: H894–H903, 2015. First published February 13, 2015; doi:10.1152/ajpheart.00333.2014.—Caloric restriction (CR) confers cardioprotection against ischemia-reperfusion injury (IRI). We previously found that treatment with N\textsubscript{G}-nitro-L-arginine methyl ester completely abrogates CR-induced cardioprotection and increases nuclear sirtuin 1 (Sirt1) expression. However, it remains unclear whether endothelial nitric oxide (NO) synthase (eNOS) plays a role in CR-induced cardioprotection and Sirt1 activation. We subjected eNOS-deficient (eNOS\textsuperscript{-/-}) mice to either 3-mo ad libitum (AL) feeding or CR (40%). Isolated perfused hearts were subjected to 25-min global ischemia followed by 60-min reperfusion. The degree of myocardial IRI in AL-fed eNOS\textsuperscript{-/-} mice was more severe than that in AL-fed wild-type mice. Furthermore, CR did not exert cardioprotection in eNOS\textsuperscript{-/-} mice. eNOS\textsuperscript{-/-} mice exhibited elevated blood pressure and left ventricular hypertrophy compared with wild-type mice, although they underwent CR. Although nuclear Sir1 content was increased, the increases in cardiac Sirt1 activity with CR was absent in eNOS\textsuperscript{-/-} mice. However, it remains unclear whether endothelial nitric oxide (NO) synthase (eNOS) plays a role in CR-induced cardioprotection and Sirt1 activation. We subjected eNOS-deficient (eNOS\textsuperscript{-/-}) mice to either 3-mo ad libitum (AL) feeding or CR (40%). Isolated perfused hearts were subjected to 25-min global ischemia followed by 60-min reperfusion. The degree of myocardial IRI in AL-fed eNOS\textsuperscript{-/-} mice was more severe than that in AL-fed wild-type mice. Furthermore, CR did not exert cardioprotection in eNOS\textsuperscript{-/-} mice. eNOS\textsuperscript{-/-} mice exhibited elevated blood pressure and left ventricular hypertrophy compared with wild-type mice, although they underwent CR. Although nuclear Sirt1 content was increased, the increases in cardiac Sirt1 activity with CR was absent in eNOS\textsuperscript{-/-} mice. However, CR-induced cardioprotection was not observed. Resveratrol enhanced cardiac Sirt1 activity but failed to mimic CR-induced cardioprotection in eNOS\textsuperscript{-/-} mice. Finally, combination therapy with resveratrol and hydralazine attenuated myocardial IRI and reduced infarct size in eNOS\textsuperscript{-/-} mice, and their effects were comparable with those observed in CR-treated wild-type mice. These results demonstrate the essential roles of eNOS in the development of CR-induced cardioprotection and Sirt1 activation during CR. The combination of a relatively low dose of resveratrol with an adequate vasodilator therapy might be useful for managing patients with endothelial dysfunction associated with impaired NO bioavailability.

ischemia-reperfusion; myocardial infarction; nitric oxide synthase; resveratrol; sirtuin 1

Caloric restriction (CR) is the established intervention scientifically proven to have antiaging effects (11, 20, 31, 37, 40). CR profoundly affects age-related physiological and pathophysiological alterations and markedly increases both mean and maximal lifespan in several species, including mammals. The effect of CR is clearly distinct from that of malnutrition and starvation. In general, there is no evidence of malnutrition or cachexia in animals treated with standard CR regimens if caloric intake is restricted to 50–70% of ad libitum (AL) quantities. On the basis of the findings of experimental studies, CR is expected to attenuate the incidence of age-associated diseases such as dementia and cardiovascular diseases (CVDs) (11, 31, 40, 41). Furthermore, CR exerts pleiotropic effects on the cardiovascular system (8, 17, 31–35, 40, 44). We have previously demonstrated that CR confers cardioprotection against ischemia-reperfusion injury (31–33). Thus, the clinical application of CR and 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 CVDs.

The exact mechanism by which CR confers cardiovascular protection has not been completely clarified. In the short-term CR setting, we have established an obligatory role of the activation of adiponectin-AMP-activated protein kinase (AMPK) signaling in CR-induced cardioprotection against ischemia-reperfusion (33). However, the role of adiponectin-AMPK signaling in cardioprotection afforded by prolonged CR is undefined. We noticed the specific role of nitric oxide (NO) as a mediator of CR-induced cardiovascular protection. Increasing evidence demonstrates that NO, either endogenous or exogenous, represents one of the most important defenses against myocardial ischemia-reperfusion injury (15, 16). Furthermore, NO appears to be a common mediator of the protection afforded by a wide array of seemingly unrelated pharmacological and nonpharmacological interventions in the cardiovascular system (2, 15). In contrast, aging (as well as risk factors for atherosclerosis) impairs the bioavailability of NO in the cardiovascular system (1, 12, 22, 36, 38, 40, 42). Age-related impairment of NO bioavailability is likely to promote vascular inflammation and atherogenesis and may result in severe myocardial damage after ischemia-reperfusion. Therefore, it is likely that the increased NO bioavailability by CR plays an obligatory role in the development of CR-induced cardiovascular protection.

The interaction between endothelial NO synthase (eNOS) and Sirt1 (Sirt1) seems to be essential for the development of the vascular protection afforded by CR. We previously found that prolonged CR significantly increases Sirt1 and decreases acetyl-histone H3 in the nuclear fraction (32), suggesting that Sirt1 is activated in CR-treated hearts. Chronic inhibition of NO by giving CR-treated rats N\textsuperscript{G}-nitro-\textsuperscript{l}-arginine methyl ester (\textsuperscript{l}-NAME) in their drinking water for 4 wk...
completely abrogated not only CR-induced cardioprotection against ischemia-reperfusion injury but also the increase in nuclear Sirt1 content. These results strongly suggest that NOS is located upstream, not downstream, of Sirt1 activation. However, it is still unknown which NOS isoform is the most important for this phenomenon. L-NNAME is a nonselective NOS inhibitor and inhibits the activities of all three NOS isoforms: eNOS, inducible NOS (iNOS), and neuronal NOS (nNOS). Therefore, it is also unclear whether the interaction between eNOS and Sirt1 is essential for CR-induced cardioprotection against ischemia-reperfusion injury.

To confirm whether the eNOS isoform is essential for CR-induced cardioprotection, we subjected eNOS-deficient (eNOS<sup>−/−</sup>) mice and control wild-type (WT) mice to either 3 mo of CR or AL feeding. The first aim of the present study was to investigate whether eNOS deficiency abrogates CR-induced cardioprotection and the increase in nuclear Sirt1 content. eNOS<sup>−/−</sup> mice exhibited elevated blood pressure (BP) and left ventricular (LV) hypertrophy (LVH); it was possible that elevated BP and the subsequent LVH prevented the development of CR-induced cardioprotection in eNOS<sup>−/−</sup> mice. Thus, the second aim of the present study was to investigate whether CR could induce cardioprotection in eNOS<sup>−/−</sup> mice under normotensive conditions. We considered it clinically useful if we could protect eNOS<sup>−/−</sup> mouse hearts from ischemia-reperfusion injury by activating Sirt1. Sirt1 is expected to be located downstream of eNOS activation during CR and to mediate cardiovascular protection afforded by CR. The third aim of the present study was to investigate whether chronic administration with resveratrol (RSV), a sirtuin-activating compound, without or with hypertensive treatment could mimic CR-induced cardioprotection in eNOS<sup>−/−</sup> mice.

**MATERIALS AND METHODS**

All procedures in the present study conformed with principles outlined in the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, Revised 1996) and were approved by the Institutional Animal Care and Use Committee of Keio University School of Medicine.

Eight-week-old eNOS<sup>−/−</sup> (NOS III deficient) mice (originally purchased from Jackson Laboratory and bred in our animal laboratory) were compared with age-matched C57BL/6J mice (WT mice; 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 (Phase I Experiment)**

CR was performed as previously described (33, 34). Briefly, 8-wk-old male eNOS<sup>−/−</sup> 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 modified semipurified diet A (Oriental Yeast, Tokyo, Japan). The diet was given once a day at 7 PM. We measured the rest of the diet the next day and calculated the daily food intake for 2 wk. The average caloric intake was calculated from 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 control diet A for the subsequent 16 wk. CR mice were fed 90% of the average 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, which were enriched in vitamins and minerals. Thus, daily intake of vitamins and minerals was constant during the CR period.

**Langendorff Perfusion of the Heart**

Mice were anesthetized with pentobarbital sodium (300 mg/kg) with heparin (50 units) given concomitantly by an intraperitoneal injection. Hearts were then quickly excised and perfused with modified Krebs-Henseleit buffer (120 mmol/l NaCl, 25 mmol/l NaHCO<sub>3</sub>, 5.9 mmol/l KCl, 1.2 mmol/l MgSO<sub>4</sub>, 1.75 mmol/l CaCl<sub>2</sub>, and 10 mmol/l glucose) gassed with 95% O<sub>2</sub>-5% CO<sub>2</sub> at 37°C according to the Langendorff procedure as previously described (33, 39). Coronary perfusion pressure was maintained at 80 cmH<sub>2</sub>O. A plastic catheter with a balloon was inserted into the LV through the left atrium as previously described. Before the induction of ischemia, 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 (LVDP), LV peak positive and negative 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.

**Ischemia-Reperfusion Protocol**

After 40 min of perfusion (stabilization) in a nonrecirculating mode, all hearts were subjected to 25 min of global no-flow ischemia followed by 60 min of reperfusion. After reperfusion, the LV was sliced into four thin cross-sections. Slices were incubated with 1% 2,3,5-triphenyltetrazolium chloride in phosphate buffer and fixed in 10% formaldehyde. Infarct size was calculated by computerized videomicroscopy as previously described (33, 39).

**Measurement of BP**

Systolic BP was measured noninvasively and periodically by the tail-cuff method with a BP-98A-L apparatus (BP-98A-L, Softron, Tokyo, Japan).

**Western Blot Analysis**

Standard SDS-PAGE Western immunoblot techniques were used to assess the expression of eNOS, iNOS, nNOS, GAPDH, Sirt1, and p300, as previously described (32, 35). Total protein was extracted from frozen hearts. Equal amounts of total proteins (20–40 μg) were subjected to SDS-PAGE. The primary antibodies used in the present study were anti-eNOS, anti-phospho-eNOS (Ser1177), anti-phospho-iNOS, anti-phospho-nNOS (Thr<sup>495</sup>) (Cell Signaling Technology, Danvers, MA), anti-nNOS (BD Bioscience, San Jose, CA), anti-iNOS, anti-Sirt1, anti-p300 (Upstate, Lake Placid, NY), and anti-GAPDH (Millipore, Billerica, MA). For the assessment of the subcellular distribution of Sirt1 protein, cytosolic and nuclear fractions were prepared according to the manufacturer’s instructions (32).

**Measurement of Myocardial Nitrite/Nitrate Content**

Frozen hearts were homogenized and centrifuged (30). Supernatants were collected to measure the concentration of nitrite/nitrate (NO<sub>x</sub>) using a Nitrite/Nitrate Fluorometric Assay kit (Cayman Chemical, Ann Arbor, MI).

**Measurement of Sirtuin Activity**

NAD<sup>+</sup>-dependent deacetylase activity in total heart homogenates was measured using a commercially available kit (SIRT1/Sir2 Deacetylase Fluorometric Assay Kit, CycLex, Ina, Japan) (35).
**Hypertensive Treatment with Hydralazine (Phase II Experiment)**

Ten-week-old WT and eNOS−/− mice were given hydralazine (HY; Sigma-Aldrich, St. Louis, MO) in the drinking water (200 mg/l) for 16 wk before they were euthanized (HY group). At the age of 12 wk, the CR protocol was started in eNOS−/− mice treated with HY as described above (CR + HY group). This dose of HY was chosen to achieve a BP value comparable with that in WT mice fed AL (21). At the age of 26 wk, isolated hearts were subjected to the same ischemia-reperfusion protocol (n = 6 each). Body weight and BP were measured every week.

**RSV Treatment With or Without HY (Phase III Experiment)**

Ten-week-old WT and eNOS−/− mice were randomly divided into the following two groups: the RSV group [fed AL with control diet containing RSV (Wako Pure Chemicals, Osaka, Japan) for 16 wk (200 mg/kg in control diet, ~25 mg·kg−1·day−1)] and the RSV + HY group [fed AL with control diet containing 200 mg/kg RSV (~25 mg·kg−1·day−1) and given HY in the drinking water (200 mg/l) for 16 wk]. In a pilot study, we administered three different doses (~5, 25, and 125 mg·kg−1·day−1) of RSV to eNOS−/− mice for 4 wk. Middle- and higher-dose RSV enhanced cardiac Sirt1 activity (data not shown). We then chose a middle dose of RSV for the phase III experiment. At the age of 26 wk, isolated hearts were subjected to the same ischemia-reperfusion protocol (n = 7 each). Body weight and BP were measured every week.

**Statistical Analysis**

Data are presented as means ± SE. For intergroup comparisons, data were analyzed using one-way or two-way ANOVA followed by a Scheffe’s post hoc test. P values of <0.05 were considered significant.

**RESULTS**

**Phase I Experiment**

Body weight, ventricular weight, and BP. There was no difference in body weight at 10 wk of age between WT and eNOS−/− mice (data not shown). During the protocol, body weight increased in the AL group and decreased in the CR group in both strains, and there was no difference in the change in body weight between WT and eNOS−/− mice (Table 1). Ventricular weight was greater in eNOS−/− mice, and the ratio of ventricular weight to body weight was higher in eNOS−/− mice compared with those in WT mice (Table 1).

In WT mice, CR significantly lowered BP (Table 1). eNOS−/− mice exhibited higher BP compared with that in WT mice. CR significantly decreased BP in eNOS−/− mice, but BP in eNOS−/− mice treated with CR was still higher than that in WT mice treated with CR.

**Myocardial ischemia-reperfusion injury.** At baseline, there was no difference in LV function between WT and eNOS−/− mice (Fig. 1, A–E). CR improved the recovery of LV function after ischemia-reperfusion and attenuated LDH release into the perfusate after ischemia-reperfusion as well as infarct size detected by 2,3,5-triphenyltetrazolium chloride staining in WT mice (Fig. 1, A–G), indicating CR-induced cardioprotection against ischemia-reperfusion. The degree of myocardial ischemia-reperfusion injury was more severe in eNOS−/− mice fed AL compared with that in WT mice fed AL (Fig. 1, A–E). In contrast, total LDH release into the perfusate during reperfusion was less in eNOS−/− mice (Fig. 1F). Coronary flow during reperfusion was significantly lower in eNOS−/− mice fed AL (data not shown), which was responsible for relatively low LDH release despite severe myocardial ischemia-reperfusion injury. The difference in infarct sizes between WT and eNOS−/− fed AL did not reach statistical significance (Fig. 1G). In eNOS−/− mice, CR significantly improved the recovery of HR during reperfusion (Fig. 1C) but failed to improve the recovery of LV function or attenuate infarct size after ischemia-reperfusion (Fig. 1, A–G).

**Myocardial NOx content and sirtuin activity.** In WT mice, CR significantly increased myocardial NOx content and sirtuin

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Table 1. Body weight, ventricular weight, ventricular weight/body weight, systolic blood pressure, and heart rate in each group at the age of 26 wk

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<th>Body Weight, g</th>
<th>Ventricular Weight, g</th>
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<th>Systolic Blood Pressure, mmHg</th>
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<td>AL group</td>
<td>7</td>
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<td>HY group</td>
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<td>RSV group</td>
<td>6</td>
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<td>0.440 ± 0.008</td>
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<td>RSV group</td>
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<td>132 ± 4</td>
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Data are means ± SE; n, number of mice/group. WT, wild-type; eNOS−/−, endothelial nitric oxide synthase deficient; AL, ad libitum; CR, caloric restriction; HY, hydralazine; RSV, resveratrol. *P < 0.05 compared with the corresponding AL group; †P < 0.05 compared with the corresponding WT group; ‡P < 0.05 vs. eNOS−/− mice fed AL; ‡P < 0.05 vs. the HY group; †P < 0.05 vs. the RSV group.
activity (Fig. 1, H and J). In eNOS−/− mice, myocardial NOx content was lower and CR failed to increase myocardial NOx content and sirtuin activity.

Expression levels of NOS isoforms and Sirt1. In WT mice, CR increased expression levels of eNOS and nNOS but not those of iNOS (Fig. 2, A–D). In addition, CR increased expression levels of Sirt1 in nuclear fractions in WT mice (Fig. 2, A and E). Expression levels of phospho-eNOS at Ser1177 were comparative between AL and CR hearts when they were normalized by expression levels of total eNOS protein (Fig. 2, F and G). In contrast, expression levels of phospho-eNOS at Thr495 were decreased in CR hearts (Fig. 2, A, F, and H), indicating that increased total eNOS protein and enhanced eNOS activity by promoting Thr495 dephosphorylation are responsible for the increase in myocardial NOx content in CR hearts. eNOS−/− mice fed AL showed an increase in expression levels of nNOS and a decrease in expression levels of iNOS (Fig. 2, A, C, and D). eNOS−/− mice fed AL also showed an increase in expression levels of nuclear Sirt1, but CR did not further increase expression levels of nNOS and Sirt1 in eNOS−/− mice (Fig. 2, A, D, and E).

Phase II Experiment

From the results of the phase I experiment, it was difficult to exclude the influence of hemodynamic changes and the subsequent LVH on the loss of CR-induced cardioprotection in eNOS−/− mice treated with CR. To exclude the influence of elevated BP on the development of CR-induced cardioprotection, we performed hypertensive treatment with HY in eNOS−/− mice.

As a result, ventricular weight, the ventricular weight-to-body weight ratio, and BP in eNOS−/− mice treated with HY became comparable with those in WT mice treated with CR (Table 1).

Although HY lowered BP in WT mice (Table 1), treatment with HY neither improved the recovery of LV function (data not shown) nor attenuated infarct size in WT mice (Fig. 3G). Treatment with HY neither improved the recovery of LV function nor attenuated infarct size in eNOS−/− mice fed AL (Fig. 3, A–G). Treatment with HY rather exacerbated the increase in LVEDP during reperfusion (Fig. 3B), but the infarct size in eNOS−/− mice treated with HY was similar to that in
eNOS−/− mice fed AL (Fig. 3G). In addition, treatment with HY failed to restore the cardioprotection afforded by CR in eNOS−/− mice (Fig. 3).

**Phase III Experiment**

To investigate whether chronic treatment with RSV, a sirtuin-activating compound, could rescue eNOS−/− mouse hearts from severe damage after ischemia-reperfusion, both WT and eNOS−/− mice were treated with RSV in the presence or the absence of HY treatment for 16 wk.

Treatment with RSV did not affect body weight, ventricular weight, the ventricular weight-to-body weight ratio, and BP in WT mice (Table 1).

Although body weight and ventricular weight in eNOS−/− mice treated with RSV were lighter than those in eNOS−/− mice fed AL, there was no difference in BP between eNOS−/− mice treated with and without RSV (Table 1). In the HY + RSV group, ventricular weight, the ventricular weight-to-body weight ratio, and BP became comparable with those in WT mice.

Treatment with RSV tended to improve the recovery of LV function (data not shown) and attenuated infarct size in WT mice (Fig. 4G). However, treatment with RSV neither improved the recovery of LV function nor attenuated infarct size in eNOS−/− mice fed AL (Fig. 4, A–G). Treatment with RSV rather exacerbated the increase in LVEDP during reperfusion (Fig. 4B), but the infarct size in eNOS−/− mice treated with RSV was similar to that in eNOS−/− mice fed AL (Fig. 4G).

The recovery of LV function after ischemia-reperfusion in the HY + RSV group was the best when eNOS−/− mice were treated with both HY and RSV; their recoveries were comparable with those observed in WT mice fed AL (Figs. 1, A–E, and 4, A–E). In addition, the infarct size in the HY + RSV group was the smallest and was comparable with that observed in WT mice treated with CR (Figs. 1G and 4G).

Myocardial
Sirt1 activity was enhanced by chronic treatment with RSV in both WT and eNOS\(^{-/-}\) mice, but the combination of HY with RSV did not further enhance Sirt1 activity in either strain (Fig. 4H).

**DISCUSSION**

The major findings of the present study were as follows: 1) CR-induced cardioprotection against ischemia-reperfusion injury was not observed in eNOS\(^{-/-}\) mice, 2) the essential role of eNOS in CR-induced cardioprotection was independent of its effect on BP, 3) eNOS was located upstream of Sirt1 activation during CR, 4) activation of Sirt1 with a relatively low dose of RSV was not powerful enough to mimic CR-induced cardioprotection in eNOS\(^{-/-}\) mice, and 5) the combination of RSV with antihypertensive therapy could attenuate myocardial ischemia-reperfusion damage in eNOS\(^{-/-}\) mice to an extent similar to that observed in WT mice fed AL.

Mounting evidence demonstrates that the frequency of CVDs increases with aging, suggesting that the aging process is closely associated with the development of CVDs, including LVH, heart failure, and atrial fibrillation (18). We have previously demonstrated that CR exerts several beneficial effects on the cardiovascular system (31–35). However, it is still controversial whether CR initiated at middle age confers the same effects as those of CR initiated at a younger age. Starting at middle age, one suffers from vascular aging that is characterized by increased thickening and stiffness of arteries as well as endothelial dysfunction (4, 10, 18). In addition, one often has several comorbidities such as hypertension, diabetes mellitus, and hypercholesterolemia. All of these risk factors for atherosclerosis could exacerbate endothelial function by impairing NO bioavailability (4, 10, 40). The impairment of NO bioavailability is aggravated by an age-related decline in eNOS expression (5, 12, 22, 38, 42), reduced availability of tetrahydrobiopterin (36) and intracellular L-arginine (1), and imbalance between eNOS and iNOS (44). Therefore, we investigated whether the development of CR-induced cardioprotection is eNOS independent or dependent.

In the present study, CR-induced cardioprotection was not observed in eNOS\(^{-/-}\) mice (Fig. 1). In accordance with pre-
vious reports, eNOS−/− mice exhibited elevated BP, LVH, and impaired myocardial ischemic tolerance (Table 1 and Fig. 1). eNOS−/− mice fed AL showed increased nNOS and nuclear Sirt1 expression levels (Fig. 1). These changes resembled those observed in CR-treated WT mice and may be a compensatory mechanism for the loss of eNOS. Experimental studies have demonstrated that animals subjected to CR exhibited a decrease in BP and HR compared with controls fed AL (14, 43). A clinical observation in which individuals who had been on a CR diet for an average of 6 yr were compared with age-matched healthy individuals on a typical American diet indicated a change in BP similar to that observed in animal experiments (9). Activation of eNOS and improved NO bioavailability with CR are likely to be responsible for the decrease in BP (3, 40). In fact, BP in CR-treated eNOS−/− mice was still higher than that in CR-treated WT mice (Table 1). In addition, it is well known that LVH exacerbates myocardial damage after ischemia-reperfusion (6). We thought that it was difficult to exclude the influence of hemodynamic changes and the subsequent LVH on loss of CR-induced cardioprotection in eNOS−/− mice. Although BP and the LV weight-to-body weight ratio in eNOS−/− mice became comparable by HY treatment with those in WT mice, HY treatment failed to restore CR-induced cardioprotection in eNOS−/− mice (Table 1 and Fig. 3). Finckenberg et al. (8) demonstrated that CR provides powerful, BP-independent, protection against mitochondrial remodeling and LVH in double transgenic rats harboring human renin and angiotensinogen genes. Therefore, CR exerts a preferable effect on the myocardium independent of its hemodynamic effect, and it is possible that eNOS also plays a role in this direct effect.

We have previously demonstrated that 1-NAME treatment completely abrogates both CR-induced cardioprotection against ischemia-reperfusion and the increase in nuclear Sirt1 content during CR (32). In the present study, CR increased neither expression levels of nuclear Sirt1 content nor Sirt1 activity in eNOS−/− mice (Figs. 1 and 2). Taken together, we concluded that NO derived from eNOS is essential for Sirt1 activation. Our results were in agreement with those of a previous report by Nisoli et al. (24), which demonstrated that CR enhances mitochondrial biogenesis by inducing eNOS and that enhanced mitochondrial biogenesis and an enhanced ex-
pression of Sirt1 are strongly attenuated in eNOS−/− mice. They further indicated that NO donors upregulated Sirt1 expression in white adipocytes and that the induction of Sirt1 by CR was reduced in white adipocytes obtained from eNOS−/− mice. Regarding this finding, we also confirmed that the administration of an NO donor, S-nitroso-N-acetylpenicillamine, could increase nuclear Sirt1 expression in hearts from eNOS−/− mice (data not shown). Although the exact mechanism by which NO regulates Sirt1 expression has not been clarified yet, several reports (19, 26) have demonstrated that NO can regulate Sirt1 expression. Ota et al. (26) reported that diethylentriamine/NO adduct, another NO donor, upregulated Sirt1 expression in endothelial cells and suppressed oxidative stress-induced endothelial cell senescence. Lemarie et al. (19) demonstrated that uncoupling of eNOS leads to decreased expression of Sirt1 and subsequently accelerates endothelial progenitor cell senescence. Because CR activates eNOS (3, 17, 23, 24, 40), it is plausible that CR prevents endothelial senescence by enhancing Sirt1 expression in an eNOS-dependent manner. These findings strongly suggest that Sirt1 is a downstream mediator of cardiovascular protection afforded by CR.

Although eNOS−/− mice showed an increase in nuclear Sirt1 expression (Fig. 2, A and E), Sirt1 activity was kept at the similar level to that in WT mice fed AL (Fig. 1F). Therefore, we speculated that it might be possible to activate Sirt1 further in eNOS−/− mice. RSV is not a specific Sirt1 activator, but it has been reported that a relatively low dose of RSV is effective for Sirt1 activation (28). In contrast, a higher dose of RSV was reported to prevent hypertension and LVH in spontaneously hypertensive rats and angiotensin II-infused mice, probably by preserving the liver kinase B1-AMPK-eNOS signaling axis (7). Therefore, we investigated whether activation of Sirt1 by a relatively low dose of RSV (25 mg·kg−1·day−1), compared with previous reports (7, 27, 29) in which RSV was administered in diseased mice, could mimic the cardioprotection afforded by CR. As expected, chronic treatment with RSV enhanced cardiac Sirt1 activity in both WT and eNOS−/− mice (Fig. 4H). Although treatment with RSV conferred cardioprotection in WT mice, it failed to do so in eNOS−/− mice (Fig. 4, A–G), suggesting that RSV is not powerful enough at low doses to rescue diseased hearts when eNOS is deficient and/or depleted. On the contrary, CR may regulate eNOS activity and its expression via Sirt1 activation. Experimental studies (23, 40) have previously demonstrated that Sirt1 and eNOS are colocalized in endothelial cells and that Sirt1 deacetylates eNOS, thereby stimulating eNOS activity and increasing NO production in endothelial cells. Sirt1 overexpression or Sirt1 activators have been shown to induce eNOS expression in endothelial cells (25, 40). Thus, eNOS and Sirt1 may form a network of cardiovascular protection during CR, and both are necessary for the development for CR-induced cardioprotection. Figure 5 shows our hypothesis regarding the relationship between eNOS and Sirt1 in the development of CR-induced cardioprotection. We speculate that eNOS might play a dual role of both a trigger and a mediator in the development of CR-induced cardioprotection as it does in the development of late preconditioning (2). This hypothesis can explain the failure to restore CR-induced cardioprotection by activation of Sirt1 with RSV in eNOS−/− mice.

Finally, we found that the combination therapy of RSV with HY could attenuate myocardial ischemia-reperfusion injury in eNOS−/− mice to the same degree as that with CR in WT mice (Fig. 4). The existence of endothelial dysfunction precedes clinical disease and predicts a higher risk for developing clinical atherosclerosis, coronary artery disease, and stroke (4, 10). The impairment of NO bioavailability is mainly attributed to endothelial dysfunction associated with aging and comorbidities. Thus, the combination of RSV with an adequate vasodilator therapy might be useful for managing patients with endothelial dysfunction, as the dosage of RSV used in the present study was relatively low and safe.

Evidence, including the results of the present study, strongly suggests that NO derived from eNOS regulates Sirt1 activity, but the exact mechanism(s) by which NO activates Sirt1 remains to be elucidated. Although our results indicate the importance of a close interaction between eNOS and Sirt1 in the development of CR-induced cardioprotection, we have not confirmed that Sirt1 is essential for CR-induced cardioprotection yet. Thus, we are currently investigating whether targeted disruption of the Sirt1 gene abrogates CR-induced cardioprotection and whether Sirt1 deficiency impairs NO bioavailability. RSV is a cardioprotective compound but is not specific for Sirt1 activation. In addition, it has been previously reported that RSV protects the myocardium from ischemia-reperfusion injury in an eNOS-dependent manner (13). Therefore, we would like to evaluate the effect of more specific Sirt1 activators on myocardial ischemia-reperfusion injury in eNOS−/− mice in the near future.

In conclusion, the present study demonstrated the essential role of eNOS in CR-induced cardioprotection and the close interaction between eNOS and Sirt1 during the development of CR-induced cardioprotection. Because population aging and obesity have become social health problems in developed countries, CR and CR mimetics are promising interventions for both preventing and managing CVDs associated with these...
pathologies. Thus, the elucidation of mechanisms underlying cardiovascular protection afforded by CR is extremely important for the clinical application of CR and development of CR mimetics. The results of the present study do suggest an effective interaction between eNOS and Sirt1 as key molecules to mimic the cardiovascular protection afforded by CR.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


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