Caloric Restriction Primes Mitochondria for Ischemic Stress by Deacetylating Specific Mitochondrial Proteins of the Electron Transport Chain


Rationale: Caloric restriction (CR) confers cardioprotection against ischemia/reperfusion injury. However, the exact mechanism(s) underlying CR-induced cardioprotection remain(s) unknown. Recent evidence indicates that SirTunis, NAD⁺-dependent deacetylases, regulate various favorable aspects of the CR response. Thus, we hypothesized that deacetylation of specific mitochondrial proteins during CR preserves mitochondrial function and attenuates production of reactive oxygen species during ischemia/reperfusion.

Objective: The objectives of the present study were (1) to investigate the effect of CR on mitochondrial function and mitochondrial proteome and (2) to investigate what molecular mechanisms mediate CR-induced cardioprotection.

Methods and Results: Male 26-week-old Fischer344 rats were randomly divided into ad libitum–fed and CR (40% reduction) groups for 6 months. No change was observed in basal mitochondrial function, but CR preserved postischemic mitochondrial respiration and attenuated postischemic mitochondrial H₂O₂ production. CR decreased the level of acetylated mitochondrial proteins that were associated with enhanced SirTunin activity in the mitochondrial fraction. We confirmed a significant decrease in the acetylated forms of NDUFS1 and cytochrome bc₁ complex Rieske subunit in the CR heart. Low-dose Resveratrol treatment mimicked the effect of CR on deacetylating them and attenuated reactive oxygen species production during anoxia/reoxygenation in cultured cardiomyocytes without changing the expression levels of manganese superoxide dismutase. Treatment with nicotinamide completely abrogated the effect of low-dose Resveratrol.

Conclusions: These results strongly suggest that CR primes mitochondria for stress resistance by deacetylating specific mitochondrial proteins of the electron transport chain. Targeted deacetylation of NDUFS1 and/or Rieske subunit might have potential as a novel therapeutic approach for cardioprotection against ischemia/reperfusion. (Circ Res. 2011;109:396-406.)

Key Words: ischemia ▶ mitochondria ▶ nutrition ▶ reactive oxygen species ▶ reperfusion

The prevalence of cardiovascular diseases increases with age.¹ Mortality of coronary artery disease in the elderly is higher than that in the middle-aged.²³ Consistent with clinical investigations, experimental studies demonstrate that hearts from senescent animals are more susceptible to ischemia than those from young animals.⁴⁻⁶ Both age-related increased prevalence of systemic diseases, referred to as cardiovascular risk factors, and development of cardiovascular aging contribute to the association between aging and cardiovascular diseases.¹ Therefore, development of novel therapeutics for the purpose of controlling cardiovascular aging is urgently required to provide for population aging in developed countries.

Caloric restriction (CR) has been widely investigated in experimental animals as a powerful intervention that can prevent and reverse age-related changes.⁷⁻¹⁰ The daily caloric intake in CR-treated animals was 50% to 70% of the average food intake of animals fed ad libitum (AL). Although there is no evidence stating that CR prolongs lifespan in humans, it markedly increases life span in several species, including rhesus monkey.⁷¹⁰ In addition, there is mounting evidence...
that CR profoundly affects the physiological and pathophysiological alterations associated with aging.7–11

We have demonstrated that both short-term (4 weeks) and prolonged (6 months) CR improves myocardial ischemic tolerance in rats of different ages.5,12 In the short-term CR setting, we have established an obligatory role for the activation of adiponectin-AMP activated protein kinase signaling in CR-induced cardioprotection.13 However, the mechanism(s) by which prolonged CR confers cardioprotection remains unknown.12 In the past decade, silent information regulator (Sir) 2, a longevity gene, has been reported to mediate lifespan extension by CR in lower organisms such as Caenorhabditis elegans.14–16 In addition, recent evidence indicates that mammalian Sirtuin, which is an ortholog of Sir2 and consists of 7 members of sirt proteins, regulates various aspects of CR response.14–16 We have found that cardioprotection afforded by prolonged CR is associated with increased level of Sir1 protein and decreased level of acetyl-histone H3 in the nuclear fraction.12 These findings suggest the involvement of Sirtuin in CR-induced cardioprotection; however, how Sirtuin works in the CR heart remains to be elucidated.

Because mitochondria produce the energy required for maintaining cellular homeostasis and reactive oxygen species (ROS) as a byproduct, the mitochondrion is believed to be the key organelle for cardioprotection against ischemia/reperfusion injury.17–20 Several investigations have demonstrated that CR restores aged-related mitochondrial dysfunction and attenuates oxidative damage in mitochondria.8,9,11,21–24 These results strongly suggest that CR directly attenuates ROS production in mitochondria. However, how CR attenuates ROS production and what molecular mechanisms regulate ROS production in mitochondria remain unknown, despite fragmentary information from previous studies.25 Recently, a comprehensive analysis of the mitochondrial proteome was undertaken to clarify the effect of aging and CR on mitochondrial proteins; the findings showed that CR has a minor effect on age-related changes in proteins.26 The failure to identify CR-associated changes in mitochondrial protein expression using conventional proteomics techniques strongly suggests that posttranslational modification of mitochondrial proteins might be more important than transcriptional changes for the development of CR effects.

Thus, we hypothesized that deacetylation of specific mitochondrial proteins preserves mitochondrial function and attenuates ROS production during ischemia/reperfusion. The aims of the present study were to investigate (1) the effects of prolonged CR on cardiac mitochondrial function, including ROS production, (2) whether the expression levels of mitochondrial proteins and the degree of acetylation in cardiac mitochondrial proteins are affected by prolonged CR, and (3) whether any CR mimetics, such as Sirtuin-activating compounds, can reproduce the effects of CR on mitochondrial protein deacetylation and ROS production during hypoxia/reoxygenation in cultured rat cardiomyocytes.

**Methods**

An expanded Methods section is available in the Online Supplement at http://circres.ahajournals.org.

**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AL</td>
<td>ad libitum</td>
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<tr>
<td>CR</td>
<td>caloric restriction</td>
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<tr>
<td>DIGE</td>
<td>dimensional fluorescence difference gel electrophoresis</td>
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<tr>
<td>ETC</td>
<td>electron transport chain</td>
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<tr>
<td>MnSOD</td>
<td>manganese superoxide dismutase</td>
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<tr>
<td>NAD+</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>STAT3</td>
<td>signal transducer and activator of transcription 3</td>
</tr>
<tr>
<td>Sir2</td>
<td>silent information regulator 2</td>
</tr>
<tr>
<td>YC</td>
<td>young control</td>
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**CR Protocols**

Twenty-six–week-old Fischer344 male rats were randomly divided into 2 groups. For the next 26 weeks, AL rats continued to be fed ad libitum with control diet A. CR rats were fed with 90% of the average caloric intake during the AL period for 2 weeks (10% restriction), followed by 60% of that for 24 weeks (40% restriction) with modified, semipurified diets B and C, as described previously.27 Finally, 26 and 20 rats were assigned to the AL and CR groups, respectively.

**Phase I Experiments**

**Langendorff Perfusion of Hearts**

Isolated rat hearts were perfused according to Langendorff procedure.6,12 Four hearts from each group were immediately used after initial perfusion to assess basal mitochondrial function. Eight hearts from each group were subjected to 25 minutes of global ischemia, followed by either 3 minutes or 5 minutes of reperfusion.

**Isolation of Mitochondria and Assessment of Mitochondrial Function**

Mitochondria were freshly isolated from perfused hearts before the induction of ischemia (baseline) or at 3 minutes of reperfusion after 25 minutes of global ischemia. We evaluated (1) individual enzymatic activity of complexes I, II, III, and IV; (2) mitochondrial respiration; (3) mitochondrial H2O2 production rate; and (4) Ca2+ -induced opening of mitochondrial permeability transition pore.

**Phase II Experiments**

**Ettan 2–Dimension Fluorescence Difference Gel Electrophoresis**

Mitochondrial proteins from AL hearts and CR hearts were labeled with Cy2 dye and Cy5 dye, respectively. An identical amount of each sample (300 μg) was then mixed and separated by 2D electrophoresis. The fluorescence of each spot was analyzed using the Ettan 2-dimension fluorescence difference gel electrophoresis (DIGE) Imager. In another set of experiments, with mitochondrial proteins from AL or CR hearts, 700 μg of unlabeled proteins was added to 150 μg of Cy5-labeled proteins and separated by 2D electrophoresis. The Western immunoblotting technique with Cy2-labeled 2nd antibodies was used to assess acetylated proteins at lysine residues. The same experiments were performed using mitochondrial proteins from young control hearts (YC: 8-week-old Fischer344 male) and AL hearts to clarify the effect of aging per se on the differences with or without CR. After spots were cut out, protein identification was carried out using matrix-assisted laser desorption/ionization-time-of-flight/mass spectrometry and the Mascot and ProFound search engines. We repeated the same experiments 3 times using different samples.
**Confirmation and Quantification of Modified Mitochondrial Proteins**

The results of matrix-assisted laser desorption/ionization-time-of-flight/mass spectrometry were confirmed and quantified by immunoprecipitation and Western immunoblotting.

**Phase III Experiments**

**Myocyte Isolation and Western Immunoblotting**

Adult ventricular myocytes were isolated from 26-week-old Fischer 344 male rats and treated with either of vehicle (dimethyl sulfoxide), Resveratrol, Resveratrol+nicotinamide, or Kaempferol for 12 hours.

**Cell Culturing and Western Immunoblotting**

Neonatal cardiomyocytes from 1- to 2-day-old Sprague-Dawley rats were treated with either of vehicle (dimethyl sulfoxide), Resveratrol, Resveratrol+nicotinamide, or Kaempferol for 24 hours.

**Glucose-Free Hypoxia and Reoxygenation**

Cultured neonatal cardiomyocytes were exposed to 5 hours of hypoxia, followed by 1 hour of reoxygenation. Cell viability and ROS production were compared among groups.

**Statistical Analysis**

Data are reported as mean+SEM. Data were compared by unpaired Student t test. In vitro experimental data were analyzed by a 1-way ANOVA, followed by a Scheffe post hoc test. A value of P<0.05 was considered statistically significant. Statistical analyses were performed using Stat-View 5.0 software (SAS Institute, Cary, NC) for Windows.

**Results**

**Phase I Experiments**

**Mitochondrial Protein and DNA Content**

CR decreased body weight [AL: 416±6 (n=20) versus CR: 318±3 (n=20) g; P<0.05] and heart weight [AL: 1.42±0.03 (n=8) versus CR: 1.08±0.02 (n=8) g; P<0.05], but the ratio of heart weight to body weight was similar between AL and CR groups [AL: 0.426±0.009 (n=8) versus CR: 0.431±0.006 (n=8) %; not significant]. There was no difference in the mitochondrial protein and DNA content between the 2 groups (Online Figure IA).

**Mitochondria Function**

1. **Enzymatic Activity of Complexes I, II, III, and IV**

   There was no difference in the enzymatic activity of each electron transport chain (ETC) complex between the 2 groups (Online Figure IB).

2. **Mitochondrial Respiration**

   At baseline, mitochondrial state 3 respiration was higher in the CR heart in the presence of succinate as a substrate but not in the presence of pyruvate/malate (Figure 1A). In contrast, CR preserved state 3 respiration and increased respiratory control index in the presence of pyruvate/malate in the ischemic-reperfused heart. These findings suggest that the mitochondria in the CR heart are well coupled during the ischemia/reperfusion sequence.

3. **Mitochondrial H2O2 Production Rate**

   At baseline, there was no difference in mitochondrial H2O2 production between the 2 groups (Figure 1B). However, CR attenuated maximal H2O2 production; this was assessed by adding rotenone (complex I inhibitor) in the presence of pyruvate/malate. Mitochondria obtained from ischemic-reperfused CR hearts produced less H2O2 in the presence of pyruvate/malate, suggesting that the mitochondria in the CR heart produce less ROS during early reperfusion.

4. **Ca2+-Induced Opening of Mitochondrial Permeability Transition Pore**

   At baseline, Ca2+-induced mitochondrial swelling was less in the CR heart, but the difference did not reach statistical significance (Figure 1C). Mitochondria from ischemic-reperfused CR hearts were definitely resistant to swelling induced by Ca2+ loading.

**Western Immunoblotting**

Oxyblot analysis demonstrated that protein carbonyls were decreased in the ischemic-reperfused CR hearts (Figure 2A). Western immunoblotting indicated that the level of proteins acetylated at lysine residues increased with age in the mitochondrial fraction and that these changes were partially attenuated with CR (Figure 2B). However, no difference was observed in the expression levels of Sirt1 and Sirt3 in mitochondrial and cytosolic fractions between the 2 groups (Figure 2C and 2D). With regard to Sirt3, we analyzed only a 28-kDa band because it is an active form of Sirt3.28 The short form of Sirt3 was detected only in the mitochondria fraction. In addition, CR did not increase the expression levels of manganese superoxide dismutase (MnSOD) in the mitochondrial fraction.

**Nicotinamide Adenine Dinucleotide+‐Dependent Deacetylase Activity**

CR significantly increased NAD+‐dependent deacetylase activity in total heart homogenate (Figure 2E). Furthermore, CR increased NAD+‐dependent deacetylase activity in the mitochondrial fraction by 30%.

**Phase II Experiments**

**Proteomics Analysis of Mitochondrial Proteins**

The Ettan 2-DIGE system was used to identify changes in the expression levels and acetylated state of mitochondrial proteins. Figure 3 represents the Cy2, Cy5, and overlay images of mitochondrial proteins. The green spot indicates reduced protein content. Finally, we identified 2 proteins that increased with CR and 4 proteins that decreased with CR (Table, A). Figure 4 illustrates ECL detection of acetylated proteins and the overlay image of the PVDF membrane in the AL and CR groups. By comparing these images, we identified 3 candidate proteins whose acetylation was increased by CR and 7 candidate proteins whose acetylation was decreased by CR (Table, B). These altered proteins are involved in the tricarboxylic acid cycle, ETC, creatine shuttle, and mitochondrial permeability transition pore, as well as include chaperone proteins.

As shown in Online Table II and Online Figure II, we found that aging increased the expression levels of 2 mitochondrial proteins and decreased those of 4 mitochondrial proteins. Furthermore, we identified 7 mitochondrial proteins that were increasingly acetylated with aging. CR restored
age-associated alterations in 5 of 6 mitochondrial proteins and deacetylated 3 of 7 mitochondrial proteins whose acetylation increased with aging.

**Confirmation and Quantification of Modified Mitochondrial Proteins**

Among candidate proteins, we focused on the following 2 mitochondrial proteins involved in the ETC: NADH-ubiquinone oxidoreductase 75-kDa subunit (NDUFS1) and cytochrome bc1 complex Rieske subunit. Western immunoblotting revealed that CR decreased NDUFS1 by 13% and did not change the expression levels of the Rieske subunit (Figure 5A and 5B). Immunoprecipitation analysis demonstrated that the acetylated form of NDUFS1 and Rieske subunit significantly decreased in the CR heart (Figure 5A).
Deprivation of fetal bovine serum caused apoptosis in freshly isolated adult cardiomyocytes and treatment with high-dose Resveratrol improved cell viability 12 hours later (data not shown). In contrast, treatment with either low-dose Resveratrol, low-dose Resveratrol/nicotinamide, or Kaempferol (20 \(\mu\)mol/L) failed to decrease the amount of acetylated forms of NDUF51 and Rieske subunit and to upregulate the expression levels of MnSOD (Figure 5C through 5E).

In the cultured neonatal cardiomyocytes, treatment with low-dose of Resveratrol (5 \(\mu\)mol/L) for 24 hours decreased the amount of acetylated forms of both NDUF51 and Rieske subunit without affecting the expression levels of MnSOD as seen in isolated adult cardiomyocytes (Figure 6A through 6D). Treatment with nicotinamide (20 mmol/L) completely abrogated the effects of low-dose Resveratrol on deacetylating mitochondrial proteins as seen in isolated adult cardiomyocytes (data not shown).

**Hypoxia/Reoxygenation Experiment**

In cultured neonatal cardiomyocytes, treatment with either low-dose or high-dose Resveratrol attenuated the increase in the fluorescent intensity of 2', 7'-dichlorodihydrofluorescein after hypoxia/reoxygenation (Figure 6E and 6F), suggesting that treatment with Resveratrol decreases ROS production during early reoxygenation. As a result, pretreatment with either low-dose or high-dose Resveratrol protected the cardiomyocytes from cell death caused by glucose-free hypoxia=reoxygenation (Figure 6G and 6H). In contrast, cytoprotective effect against hypoxia/reoxygenation was not observed when cardiomyocytes were treated with Kaempferol. Treatment with nicotinamide completely abrogated the effects of low-dose Resveratrol on
ROS production and cell survival, indicating that the effects of low-dose Resveratrol were dependent of Sirtuin activity.

**Discussion**

This study provides 4 major findings: (1) prolonged CR attenuated myocardial oxidative damage after ischemia/reperfusion by suppressing mitochondrial ROS production during early reperfusion, (2) prolonged CR had little effect on the expression levels of mitochondrial proteins, but it decreased the amount of acetylated forms of several mitochondrial proteins belonging to the ETC that were associated with enhanced Sirtuin activity, (3) low-dose Resveratrol mimicked the effect of CR on deacetylating NDUFS1 and Rieske subunit of cytochrome bc1 complex in freshly isolated adult cardiomyocytes and cultured neonatal cardiomyocytes, and (4) in cultured neonatal cardiomyocytes, low-dose Resveratrol resulted in decreased ROS production and improved cell survival after hypoxia/reoxygenation without increasing the expression levels of MnSOD in a Sirtuin-dependent manner.

Increasing evidence demonstrates that CR can prevent and reverse the physiological and pathophysiological alterations associated with aging. In fact, our results indicate that CR partially antagonizes age-related alterations in mitochondrial proteins. In addition, our results strongly suggest that CR influences the mitochondrial proteome by age-related and -unrelated mechanisms. These findings may explain how CR protects myocardium from ischemia/reperfusion injury at different ages (young, middle-aged, and elderly).

A robust increase in ROS production after ischemia/reperfusion is one of the main causes of myocardial ischemia/reperfusion injury. Oxyblot analysis in the present study demonstrated that CR attenuated myocardial oxidative damage after ischemia/reperfusion. The mitochondrion is the major source of ROS during the ischemia/reperfusion sequence, therefore, it is likely that CR directly attenuates ROS production in mitochondria during ischemia/reperfusion. In fact, several investigations proved that CR attenuated the levels of 8-hydroxy-2'-deoxyguanosine in mitochondrial DNA, a marker of mitochondrial oxidative damage, in the heart. However, the mechanisms—molecular or otherwise—underlying CR-induced attenuation of ROS production in the heart and modulation of mitochondrial function remain unclear.

**Table. Mitochondrial Proteins Altered by CR (A) and Those Acetylated or Deacetylated by CR (B)**

<table>
<thead>
<tr>
<th>Name</th>
<th>Calculated MW</th>
<th>Calculated pl</th>
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<tbody>
<tr>
<td><strong>A-1. Increased proteins with CR (red No.)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Cytochrome bc1 complex subunit 1</td>
<td>53 815</td>
<td>5.57</td>
</tr>
<tr>
<td>2. Aconitase hydratase</td>
<td>85 380</td>
<td>7.87</td>
</tr>
<tr>
<td><strong>A-2. Decreased proteins with CR (white No.)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Prohibitin</td>
<td>29 802</td>
<td>5.57</td>
</tr>
<tr>
<td>2. Cytochrome bc1 complex subunit 2</td>
<td>48 366</td>
<td>9.16</td>
</tr>
<tr>
<td>3. Succinate dehydrogenase flavoprotein subunit</td>
<td>71 570</td>
<td>6.75</td>
</tr>
<tr>
<td>4. NADH-ubiquinone oxidoreductase 75-kDa subunit (NDUFS1)</td>
<td>79 362</td>
<td>5.65</td>
</tr>
<tr>
<td><strong>B-1. Acetylated proteins with CR (red No.)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. ATP synthase subunit δ</td>
<td>18 752</td>
<td>6.17</td>
</tr>
<tr>
<td>2. ATP synthase subunit β</td>
<td>56 318</td>
<td>5.19</td>
</tr>
<tr>
<td>3. ATP synthase subunit α</td>
<td>59 717</td>
<td>9.22</td>
</tr>
<tr>
<td><strong>B-2. Deacetylated proteins with CR (white No.)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Cytochrome bc1 complex subunit Rieske</td>
<td>29 427</td>
<td>9.04</td>
</tr>
<tr>
<td>2. Voltage-dependent anion-selective channel protein 1</td>
<td>30 737</td>
<td>8.62</td>
</tr>
<tr>
<td>3. Creatine kinase</td>
<td>47 335</td>
<td>8.76</td>
</tr>
<tr>
<td>4. Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex</td>
<td>67 123</td>
<td>8.76</td>
</tr>
<tr>
<td>5. Succinate dehydrogenase flavoprotein subunit</td>
<td>71 570</td>
<td>6.75</td>
</tr>
<tr>
<td>6. NADH-ubiquinone oxidoreductase 75 kDa subunit (NDUFS1)</td>
<td>79 362</td>
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<tr>
<td>7. Aconitase hydratase</td>
<td>85 380</td>
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**Figure 4. Western immunoblots showing acetylated proteins and overlay images showing total and acetylated proteins in the mitochondrial function.**

A. Representative Western immunoblots showing proteins acetylated at lysine residues in the mitochondrial fraction from an AL heart. B. Representative overlay image showing both total proteins and acetylated proteins in the mitochondrial fraction from an AL heart. The green spot indicates the acetylated form of each protein at lysine residues and the red spot, total expression levels of each protein. C. Representative Western immunoblots showing proteins acetylated at lysine residues in the mitochondrial fraction from a CR heart. D. Representative overlay image showing total mitochondrial proteins and acetylated proteins in the mitochondrial fraction from a CR heart. The number in D corresponds to that in the Table, B.
One possible mechanism by which CR modifies mitochondrial ROS production is enhanced mitochondrial biogenesis associated with amplified antioxidant mechanisms. Activation of Sirt1 increased mitochondrial biogenesis through activation of peroxisome proliferator-activated receptor γ coactivator 1 in the skeletal muscle. Nisoli et al demonstrated that 3 months of CR enhanced mitochondrial biogenesis in the murine heart and that this phenomenon was dependent on endothelial nitric oxide synthase. Activation of Sirt1 also contributes to decreased oxidative damage in CR-treated hearts by upregulating antioxidant enzymes including MnSOD, as seen in the failing hearts of TO2 hamsters. However, we did not find any increase in the mitochondrial DNA and protein content, and in the expression levels of MnSOD, indicating that CR did not enhance mitochondrial biogenesis associated with amplified antioxidant mechanisms in middle-aged rat hearts.

Another possible mechanism is CR-induced modification of specific mitochondrial proteins involved in ROS production. A comprehensive analysis of the mitochondrial proteome had been undertaken to clarify the effect of aging and CR on mitochondrial proteins. The authors concluded that CR has a minor effect on age-related protein changes, even in the heart. These results are consistent with our results and demonstrate that changes in the expression levels of mitochondrial proteins were relatively lesser during the CR period. The failure to identify CR-related changes in mitochondrial protein expression by using conventional proteomics techniques strongly suggests that posttranslational modifications of mitochondrial proteins might be more important than transcriptional changes for CR to be effective. Among many forms of protein modifications, we focused on deacetylation of mitochondrial proteins in the CR heart, because we found that the activated form of Sirt1 was increased in the CR heart. Among the 7 Sirtuin members, Sir3, Sirt4, and Sirt5 target mitochondria. Thus, we speculated that activated Sirtuin during CR attenuates mitochondrial ROS production by deacetylating specific mitochondrial proteins.

Regarding the enzymatic activity of the ETC, baseline mitochondrial respiration and mitochondrial H2O2 production in the CR heart were comparable to those in the AL heart. It emerged that ischemia/reperfusion increases electron leakage.
from the ETC and results in a robust increase in mitochondrial ROS production during early reperfusion. As shown in Figure 1C and 1D, preserved mitochondrial respiration with attenuated H₂O₂ production in the CR heart subjected to ischemia/reperfusion strongly suggested that mitochondria in the CR heart were more resistant to ischemia/reperfusion. Our findings correspond well with those of Broderick et al: they found that the beneficial effect of CR on the recovery of left ventricular function after ischemia/reperfusion were associated with improvement in mitochondrial respiration. Analysis of mitochondrial H₂O₂ production rate (Figure 1B) suggested that electron leakage from complex I and/or III might be attenuated in the CR heart. In addition, we found that mitochondria from the ischemic-reperfused CR heart were resistant to Ca²⁺ overload. Although it is still unknown whether this phenomenon is dependent on or independent of reduced mitochondrial ROS production during ischemia/reperfusion, this finding supported our hypothesis that CR primes mitochondria for ischemic stress because Ca²⁺ overload is another cause of myocardial ischemia/reperfusion injury.

The effect of CR on basal mitochondrial function in the heart remains controversial. Gredilla et al demonstrated that 12 months of CR significantly decreased mitochondrial H₂O₂ production in the presence of pyruvate/malate but did not modify mitochondrial oxygen consumption in 24-month-old Wistar rat hearts. They speculated that CR decreased mitochondrial free radical generation at complex I because CR failed to decrease mitochondrial H₂O₂ production in the presence of succinate. Colom et al found that 3 months of CR attenuated mitochondrial H₂O₂ production in the presence of pyruvate/malate or succinate in 18-month-old Wistar rat hearts. They also confirmed that CR decreased the enzymatic activities of both complexes I and III. In contrast, other studies failed to find any changes in mitochondrial respiration and H₂O₂ production in the CR heart. Recently, Niemann et al demonstrated that 6 months of CR improved mitochondrial respiration and enhanced the enzymatic activity of complex I in 30-month-old rat hearts and that those changes were not observed in 12-month-old rat hearts. Our results were consistent with their findings with middle-aged rat
hearts, suggesting that CR has little effect on basal mitochondrial function in the middle-aged heart, probably because mitochondrial function is maintained at a high level.

As expected, enhanced Sir2uin activity was associated with a decrease in the amount of acetylated forms of mitochondrial proteins in the CR heart. Interestingly, most of the deacetylated proteins were related to cellular metabolism (Table). Among them, we focused on 2 proteins, NDUFS1 and Rieske subunit of cytochrome bc1 complex, which belong to complexes I and III, respectively. NDUFS1 and Rieske subunit were deacetylated in the CR heart, and low-dose Resveratrol could mimic the effect of CR on deacetylation of these proteins and ROS production during hypoxia/reoxygenation. These findings strongly suggested that deacetylation of NDUFS1 and Rieske subunit is closely associated with the decrease in mitochondrial ROS production after ischemia/reperfusion in the CR heart. Although it is not yet clear how the modification of these proteins reduces electron leakage from the ETC during the ischemia/reperfusion sequence, it is certain that each of these proteins plays an important role in electron transfer through the ETC. NDUFS1 is the largest subunit of complex I and is a component of the iron-sulfur fragment of the enzyme. It faces the mitochondrial matrix, receives electrons from NADH and passes them to the downstream iron-sulfur protein clusters. Mutations in the NDUFS1 gene are a recognized cause of Leigh syndrome. Impaired cellular oxidative metabolism is observed in patients with mutations on the NDUFS1 gene.43 The Rieske subunit is also a component of the iron-sulfur fragment of cytochrome bc1 complex and transfers electrons to cytochrome c. Liu et al44 showed that 4 subunits from the ETC including the Rieske subunit were remarkably nitrated at the tyrosine residues in the ischemic-reperfused heart. Thus, deacetylation of these proteins might protect them from detrimental protein modifications during ischemia/reperfusion. Further investigations are needed to clarify this issue.

Although we demonstrated that Sir2uin activity was enhanced in the CR heart, we could not identify which Sir2uin protein was responsible for deacetylation of NDUFS1 and Rieske subunit. We found no significant increase in the expression levels of Sirt3 in the mitochondrial fraction between the AL and CR hearts (Figure 2C and 2D). However, Sirt3 is still one of the leading candidates responsible for CR-induced modification of mitochondrial function. Mounting evidence indicates that Sirt3 plays a major role in fatty acid oxidation and maintenance of cellular ATP levels by regulating the acetylation levels of key metabolic enzymes.36,38,45 Thus, it is possible that NDUFS1 and Rieske subunit are novel targets of Sirt3. However, Kaempferol, which increases the expression and mitochondrial localization of Sirt3,46 failed to mimic the effect of CR on the deacetylation of mitochondrial proteins in the present study. In addition to Sirt3, Sirt4 and Sirt5 are located in mitochondria and regulate mitochondrial function and mitochondrial gene expression.38 Sirt4 and/or Sirt5 might regulate the acetylation levels of NDUFS1 and Rieske subunit in the mitochondria. Further evaluations are needed to determine which Sir2uin protein is responsible for the effect of CR on mitochondrial protein deacetylation and function.

The present study has several limitations. We performed proteomics analysis of mitochondrial proteins with the Ettan 2-DIGE system. This method is not quantitative, and it is possible that we overlooked important target proteins of mitochondrial Sir2uins. Mounting evidence demonstrates that decreased expression levels of connexin 43 and the signal transducer and activator of transcription 3 (STAT3) localized in mitochondria is involved in age-associated loss of cardioprotection afforded by ischemic preconditioning and postconditioning.47–49 Immunoprecipitation and Western immunoblotting revealed no changes in their acetylated levels with CR, although we verified that the expression levels of mitochondrial connexin 43 significantly reduced with aging and CR attenuated this decrease (data not shown). We speculate that connexin 43 and STAT3 are not targets for mitochondrial Sir2uins. However, it is possible that CR restores cardioprotection in aged animals by modulating connexin 43–mediated and STAT3–mediated signaling. We should evaluate whether STAT3-mediated and connexin 43–mediated signaling toward mitochondria is regulated by their acetylation/deacetylation states. Next, we evaluated the effect of deacetylation of mitochondrial proteins by CR mimetics on ROS production and cell viability using cultured neonatal cardiomyocytes. We recognize that mitochondrial function and dynamics in ROS production in neonatal cardiomyocytes were entirely different from those in middle-aged cardiomyocytes. However, we speculate that the effect of deacetylation of specific mitochondrial proteins by CR is universal, independent of the aging process. Finally, 2 different populations of mitochondria exist in cardiomyocytes: subsarcolemmal and interfibrillar mitochondria.50 Increasing evidence demonstrates that these mitochondrial populations differ in morphology, function, and the signal transduction in cardioprotection.50,51 Thus, we should pay attention to the distinct effect of aging and CR on subsarcolemmal and interfibrillar mitochondria in the future.

In summary, this study demonstrates that CR primes cardiac mitochondria into a stress-resistant state, resulting in improvements in myocardial ischemic tolerance. On the basis of the finding that enhanced Sir2uin activity was associated with a decrease in the amount of acetylated mitochondrial proteins in the CR heart, we proposed that the beneficial effect of CR on mitochondrial function is mediated by deacetylating specific mitochondrial proteins. Among numerous CR-induced deacetylated proteins, we speculated that the deacetylation of NDUFS1 (complex I) and/or Rieske subunit of cytochrome bc1 complex (complex III) plays a key role in the reduced ROS production in the mitochondria during early reperfusion. Figure 7 illustrates possible mechanism(s) by which CR and low-dose Resveratrol attenuate ROS production from the ETC during ischemia/reperfusion sequence. Thus, targeted modification of NDUFS1 and/or Rieske subunit might have potential as a novel therapeutic approach for cardioprotection against ischemia/reperfusion. Pharmacological inhibition of the ETC, especially complexes I and III, is expected to protect cardiac mitochondria and decrease myocardial ischemia/reperfusion injury.17 In contrast, deacetylation of NDUFS1 and/or
Rieske subunit has great advantage of attenuating ROS production after ischemia/reperfusion without lowering baseline mitochondrial function. However, further investigations are needed to clarify which Sirtuin is involved in this phenomenon before the clinical application of CR mimetics targeting the mitochondria.

Acknowledgments
We thank Yoshiko Miyake and Kiyomi Nishimaki for technical assistance.

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Disclosures
None.

References


**Novelty and Significance**

**What Is Known?**

- Caloric restriction (CR) confers cardioprotection against ischemia/reperfusion injury.
- Mammalian Sirtuin family, an ortholog of \textit{Sir2}, regulates various aspects of CR response.
- Among the 7 Sirtuin members, Sirt3, Sirt4, and Sirt5 target mitochondria and modify mitochondrial function.

**What New Information Does This Articles Contribute?**

- CR attenuates myocardial oxidative damage by suppressing mitochondrial reactive oxygen species (ROS) production during early reperfusion.
- NDUFS1 and Rieske subunit of cytochrome bc1 complex that belong to the electron transport chain are significantly deacetylated in the CR heart.
- Deacetylation of these mitochondrial proteins plays a key role in reduced ROS production in the mitochondria and is essential for CR-induced cardioprotection.

Several reports suggest that CR directly attenuates ROS production in the mitochondria and thus suppresses oxidative damage in various tissues. However, how ROS production is attenuated during CR and what molecular mechanisms regulate its production in the mitochondria remains partially unknown. Data in the present study suggest that mitochondrial Sirtuin deacetylates specific mitochondrial proteins that belong to the electron transport chain during CR and, consequently, cardiac mitochondria are primed into a stress-resistant state, resulting in improved myocardial ischemic tolerance. NDUFS1 and Rieske subunit of cytochrome bc1 complex, which belong to complexes I and III, respectively, are novel target proteins for mitochondrial Sirtuin. Targeted modification of these mitochondrial proteins might have potential as a novel therapeutic approach for cardioprotection against ischemia/reperfusion. Deacetylating NDUFS1 and/or Rieske subunit by a specific Sirtuin-activating compound has the advantage of attenuating mitochondrial ROS production during early reperfusion because this modification is expected not to affect mitochondrial function at baseline.
Caloric Restriction Primes Mitochondria for Ischemic Stress by Deacetylating Specific Mitochondrial Proteins of the Electron Transport Chain


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Experimental procedures

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 USA National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the Institutional Animal Care and Use Committee, Keio University School of Medicine.

CR protocols

Twenty-two week-old male Fischer 344 rats were obtained from Charles-River Japan. Rats were housed in individual cages and fed ad libitum for 4 weeks with a modified, semi-purified diet A (Oriental Yeast Co.). The average caloric intake was calculated from the daily food intake during 2 weeks (AL period). After weaning, 26 week-old rats were randomly divided into 2 groups. For the next 26 weeks, AL rats continued to be fed ad libitum with control diet A. CR rats were fed with 90% of the average caloric intake during the AL period for 2 weeks (10% restriction), followed by 60% of that for 24 weeks (40% restriction) with modified, semi-purified diets B and C, which were enriched in vitamins and minerals, as described previously. Thus, the daily intake of vitamins and minerals was constant during the CR period. Finally, 26 and 20 rats were assigned to the AL and CR groups, respectively.

PHASE I EXPERIMENTS

Langendorff-perfusion of hearts

Under anesthesia, rat hearts were excised quickly and perfused with modified Krebs-Henseleit buffer gassed with 95% O₂/5% CO₂ at 37°C according to Langendorff procedure. All hearts underwent 5 min of washout perfusion in a non-recirculating mode, followed by 10 min of initial perfusion in a recirculating mode. Then, 4 hearts from each group were immediately used to assess basal mitochondrial function. Eight hearts from each group were subjected to 25 min of global ischemia, followed by either 3 min or 5 min of reperfusion. Four hearts from each group subjected to 25 min of ischemia/3 min of reperfusion were immediately used to assess mitochondrial function after...
ischemia/reperfusion; the other hearts were stored at –140°C until use.

**Mitochondrial protein and DNA content**

Protein concentration in the mitochondrial fractions was measured by Bradford method. Mitochondrial DNA isolated from heart was analyzed by real-time polymerase chain reaction (PCR) using Thermal Cycler Dice Real Time System TP800 (TAKARA BIO INC.). All samples were normalized to genomic DNA content. Primer and probe sequences for each PCR are shown in Supplemental Table I.

**Isolation of heart mitochondria and assessment of mitochondrial function**

Mitochondria were freshly isolated from perfused hearts before the induction of ischemia (Baseline) or at 3 min of reperfusion after 25 min of global ischemia as described previously. In brief, the heart tissue was washed in ice cold isolation buffer (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA) and minced with a razor blade. Trypsin was added to a concentration of 0.025% and the tissue further disrupted with a polytron tissue disruptor at moderate speed. After 10 min incubation on ice with intermittent gentle shaking, trypsin inhibitor (from soybean) was added to 0.25 mg/ml and the mixture homogenized with a motorized teflon/glass homogenizer. The homogenate was centrifuged for 5 min at 500 g at 4° C, the supernatant collected and centrifuged again for 5 min at 500g. The supernatant was then centrifuged for 10 min at 8000 g at 4° C and the pellet suspended in isolation buffer. Unless noted otherwise, all procedures were performed on ice. Protein concentration was determined using the BCA protein assay.

**Individual enzymatic activity of complexes I, II, III, and IV**

Individual enzymatic activity of complexes I, II, III, and IV was measured as described previously. Complex I activity was measured by following the oxidation of NADH at 340 nm (minus background of 425 nm). Rotenone was used to confirm Complex I-specific activity. Complex II activity was measured by following the reduction of 2,6-Dichlorophenolindophenol at 600nm. Complex III and IV activities were measured by monitoring the reduction or oxidation of cytochrome c at 550 nm (minus background of 580 nm), respectively.

**Mitochondrial oxygen consumption**

Mitochondrial oxygen consumption (mitochondrial respiration) was measured with a Clark-type O₂ electrode using the Oxygen Meter Model 781 and the Mitocell MT200
closed respiratory chamber (Strathkelvin Instruments) at 37 °C as described previously.\textsuperscript{4,6} Mitochondria were incubated at 0.25 mg/ml in respiration buffer (125 mM KCl, 1 mM K$_2$HPO$_4$, 5 mM MgCl$_2$, 25 mM HEPES, 0.2 mM EGTA, 20 mM mannitol, 0.2% defatted BSA). Mitochondrial state 2 respiration was assayed with pyruvate/malate (each 2.5 mM) or succinate (5 mM) with rotenone (0.5 µM) as substrates. State 3 respiration was measured by adding ADP (0.25 mM) to the assay buffer, followed by state 4 respiration by adding oligomycin (2 µg/ml).

**Mitochondrial hydrogen peroxide (H$_2$O$_2$) release**

Mitochondrial H$_2$O$_2$ production was assayed using the reaction of Amplex Red (Molecular Probes) with H$_2$O$_2$ in the presence of horseradish peroxidase.\textsuperscript{6} The increase in resorufin fluorescence was monitored at 37 °C in a Shimadzu RF-5300PC spectrophotometer (excitation: 530 nm and emission: 590 nm). Isolated mitochondria were incubated in respiration buffer (125 mM KCl, 1 mM K$_2$HPO$_4$, 5 mM MgCl$_2$, 25 mM HEPES, 0.2 mM EGTA, 20 mM mannitol) containing 1 U/ml horseradish peroxidase and 1 uM Amplex Red. Maximal H$_2$O$_2$ generation rates of complexes I and III were measured using pyruvate/malate (2.5 mM each) and then adding rotenone (2 µM) or succinate (5 mM) and then adding antimycin A (5 µM), respectively. The response was calibrated by adding known amounts of hydrogen peroxide.

**Ca$^{2+}$-induced opening of mPTP**

Mitochondrial swelling, which is the result of loss of mitochondrial membrane potential and opening of mPTP, was detected by the decrease in the absorbance at 520 nm when different doses of Ca$^{2+}$ were added to the mitochondrial suspension as described previously with a modification.\textsuperscript{7} Mitochondria were suspended at 0.25 mg protein/mL in 120 mM KCl, 10 mM Tris, 20 mM MOPS, 5 mM KH$_2$PO$_4$, pH 7.4 and the absorbance was measured at 20-s intervals for 10 min in the absence and presence of 200 µM CaCl$_2$. Ca$^{2+}$-induced decrease in the absorbance was almost inhibited by adding cyclosporine A (100 nM) to the mitochondria suspension 5 min before adding CaCl$_2$.

**Western immunoblotting**

Protein carbonyl content of heart homogenates was measured by western blotting by using the Oxyblot Kit (Millipore) with a modification, as described previously.\textsuperscript{1} Subcellular fractions, cytosolic and mitochondrial fractions, were prepared using a commercially available kit (Mitochondrial Isolation Kit, BioChain). Then, standard sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) western
immunoblotting technique was used to assess the expression levels of total acetylated proteins, Sirt1, Sirt3, MnSOD, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and voltage-dependent anion channel (VDAC) in subcellular fractions as described previously. Polyclonal antibodies against Sirt1 were purchased from Upstate; Sirt3, from Abcam; GAPDH, from Chemicon; MnSOD, from Millipore; and VDAC and proteins acetylated at lysine residues, from Cell Signaling. In densitomeric analysis, the expression levels of each protein in the subcellular fraction were normalized to the expression levels of GAPDH or VDAC in each sample.

**Measurement of NAD^+-dependent deacetylase activity**

NAD^+-dependent deacetylase activity in each fraction was measured using a commercially available kit (SIRT1/Sir2 Deacetylase Fluorometric Assay Kit, CycLex).

**PHASE II EXPERIMENTS**

**Ettan 2-DIGE**

Mitochondrial proteins from AL hearts and CR hearts were labeled with Cy2 dye (Molecular Probes) and Cy5 dye (Molecular Probes), respectively. Then, an identical amount of each sample (300 µg each) was mixed and separated by 2-D electrophoresis. The fluorescence of each spot was analyzed using the Ettan DIGE Imager (GE). In another set of experiments, with mitochondrial proteins from AL or CR hearts, 700 µg of unlabeled proteins were added to 150 µg of Cy5-labeled proteins and separated by 2-D electrophoresis. Western immunoblotting technique with Cy2-labeled 2nd antibodies (Molecular Probes) was used to assess acetylated proteins at lysine residues. The same experiments were performed using mitochondrial proteins from young control hearts (YC: 8-week-old Fischer344 male rat) and AL hearts (52-week-old Fischer344 male rat) to clarify the effect of aging per se on the differences with or without CR. After spots were cut out, protein identification was carried out using matrix-assisted laser desorption/ionization-time-of-flight/mass spectrometry (MALDI-TOF/MS) and the Mascot and ProFound search engines. We repeated the same experiments 3 times using different samples. We mixed equivalent amount of 2 heart extracts into a single sample. Finally, in the proteomics experiments, we used 6, 12, and 6 hearts in the YC, AL, and CR groups, respectively.
**Confirmation and quantification of modified mitochondrial proteins**

The results of MALDI-TOF/MS were confirmed and quantified by immunoprecipitation and western immunoblotting with specific antibodies against acetylated lysine and individual mitochondrial proteins. Among candidate proteins responsible for CR-induced cardioprotection, we focused on 2 mitochondrial proteins belonging to the ETC: NDUFS1 and cytochrome bc1 complex Rieske subunit. Polyclonal antibodies against NDUFS1 and Rieske subunit were purchased from Mitoscience.

**PHASE III EXPERIMENTS**

**Isolation of adult cardiomyocytes and western immunoblotting**

Adult ventricular cardiomyocytes were isolated from 26-week-old Fischer344 male rats as described previously. Briefly, hearts were mounted on a Langendorff apparatus and perfused with Tyrode solution containing 0.1 mM Ca$^{2+}$. Type II collagenase (Worthington) was then added to the perfusate. After 30 min, the hearts were taken down, ventricles minced, and myocytes dissociated by trituration. Subsequently, myocytes were filtered, centrifuged, and resuspended in a culture medium composed of 47.5% Medium 199 (Sigma-Aldrich), 5% fetal bovine serum (FBS), and 47.5% modified Tyrode solution containing (mM) 137 NaCl, 5 Hepes, 22 dextrose, 20 taurine, 5 creatine, 5.4 KCl, 1 MgCl2, and 5 sodium pyruvate (pH 7.4). After 4 h of isolation, medium was exchanged to a culture medium without FBS and adult cardiomyocytes were treated with either of vehicle (dimethyl sulfoxide: DMSO), Resveratrol (Orchid Chemicals), Resveratrol + nicotinamide (Sigma-Aldrich), or Kaempferol (Sigma-Aldrich) for 12 h. Deprivation of FBS caused cardiomyocyte apoptosis and cell viability was assessed by typan blue exclusion. Western immunoblotting with or without immunoprecipitation was performed to detect acetylated proteins at lysine residues, NDUFS1, and Rieske subunit by using mitochondrial proteins obtained from viable cardiomyocytes 12 h later.

**Cell culturing and western immunoblotting**

Neonatal ventricular myocytes from 1–2-day-old Sprague Dawley rats were subjected to Percoll gradient centrifugation and differential plating to enrich cardiac myocytes and deplete non-myocytes. After 3 days of culture for stabilization, neonatal cardiomyocytes were treated with either of vehicle (DMSO), Resveratrol, Resveratrol + nicotinamide, or Kaempferol for 24 h. Then, western immunoblotting with or without
immunoprecipitation was performed to detect acetylated proteins at lysine residues, NDUFS1, Rieske subunit, and MnSOD by using proteins extracted from the mitochondrial fraction.

**Glucose-free hypoxia and reoxygenation**

An anaerobic jar equipped with Anaero Pack (Mitsubishi Gas Chemical) was used to expose cells to hypoxic stress \(^\text{10}\). The medium for neonatal cardiomyocytes was replaced with glucose-free DMEM before the cells were exposed to hypoxic stress. After exposure to hypoxia for 5 h, the medium was replaced with 10% fetal bovine serum-containing Dulbecco’s modified Eagle’s medium (DMEM) (reoxygenation). Cell viability was determined by the LIVE/DEAD Viability/Cytotoxicity Assay Kit (Molecular Probes). In another set of experiments, cardiomyocytes were pre-incubated with 1 µM of 2',7'-dichlorodihydrofluorescin diacetate (DCFH-DA; Molecular Probes) for 30 min in the dark \(^\text{9}\). Then, cardiomyocytes were exposed to 5 h of hypoxia, followed by 1 h of reoxygenation. Produced ROS can oxidize DCFH-DA, yielding the fluorescent product, DCF. The imaging of fluorescence from these dyes was recorded with a fluorescence microscope (BZ-9000; Keyence).

**Statistical analysis**

Data are reported as mean ± SEM. Data were compared by unpaired Student’s *t*-test. *In vitro* experimental data were analyzed by a one-way ANOVA, followed by a Scheffe’s *post hoc* test. A *P* < 0.05 was considered statistically significant. Statistical analyses were performed using Stat-View 5.0 software (SAS Institute) for Windows.

**References**


Online Table I. Primers and probes for RT-PCR.

<table>
<thead>
<tr>
<th>Mitochondrial DNA (cytochrome c oxidase III gene region)</th>
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<tr>
<td><strong>F primer</strong></td>
<td>5'-CGAGATATCATCCGTGAAGGAAC-3'</td>
</tr>
<tr>
<td><strong>R primer</strong></td>
<td>5'-GATTATTCCGTATCGGAGGCCT-3'</td>
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<tr>
<td><strong>Probe</strong></td>
<td>5'-ACCAAGGCCACCACCCCTATTGTAC-3'</td>
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<table>
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<tr>
<th>Genomic DNA (chromosome 12, aldehyde dehydrogenase 2 gene region)</th>
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<tr>
<td><strong>F primer</strong></td>
<td>5'-CGCCCAAAAACCCAACAA-3'</td>
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<td><strong>R primer</strong></td>
<td>5'-TTCTCTTGGCAGGCGCA-3'</td>
</tr>
<tr>
<td><strong>probe</strong></td>
<td>5'-TCCTGAGAAAAGCCACCACCAAGCA-3'</td>
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Online Table II. Mitochondrial proteins altered by aging (A) and those acetylated by aging (B)

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<thead>
<tr>
<th>Name</th>
<th>Calculated MW</th>
<th>Calculated pl</th>
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<tr>
<td><strong>A-1. Increased proteins with aging</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Prohibitin</td>
<td>29802</td>
<td>5.57</td>
</tr>
<tr>
<td>2 Cytochrome bc-1 complex subunit 2</td>
<td>48366</td>
<td>9.16</td>
</tr>
<tr>
<td>3 Succinate dehydrogenase flavoprotein subunit</td>
<td>71570</td>
<td>6.75</td>
</tr>
<tr>
<td>4 Acetyl-CoA acetyltransferase</td>
<td>44666</td>
<td>8.92</td>
</tr>
<tr>
<td><strong>A-2. Decreased proteins with aging</strong></td>
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<td></td>
</tr>
<tr>
<td>1 Cytochrome bc-1 complex subunit 1</td>
<td>53815</td>
<td>5.57</td>
</tr>
<tr>
<td>2 Aconitate hydratase</td>
<td>85380</td>
<td>7.87</td>
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<tr>
<td><strong>B-1. Acetylated proteins with aging</strong></td>
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<tr>
<td>1 ATP synthase subunit β</td>
<td>56318</td>
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<tr>
<td>2 Cytochrome bc-1 complex subunit 1</td>
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<td>5.57</td>
</tr>
<tr>
<td>3 Succinate dehydrogenase flavoprotein subunit</td>
<td>71570</td>
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</tr>
<tr>
<td>4 Creatine kinase</td>
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<td>8.76</td>
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<tr>
<td>5 Acetyl-CoA acetyltransferase</td>
<td>44666</td>
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<tr>
<td>6 Voltage-dependent anion-selective channel protein 1</td>
<td>30737</td>
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<tr>
<td>7 Superoxide dismutase [Mn]</td>
<td>24659</td>
<td>8.96</td>
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Online Figure I. (A) Mitochondrial protein content, and mitochondrial DNA content (n = 8, each group). (B) Enzymatic activities of the electron transport chain (ETC) complexes I, II, III, and IV (n = 4, each group). AL: ad libitum, CR: caloric restriction, Data are represented as means ± SEM.
Online Figure II. (A) Representative overlay image showing both total proteins and acetylated proteins in the mitochondrial fraction from a young control heart. (B) Representative overlay image showing both total proteins and acetylated proteins in the mitochondrial fraction from an AL heart. The green spot indicates the acetylated form of each protein at lysine residues and the red spot, total expression levels of each protein. The number in Figure 2B corresponds to that in Online Table IIB.