Cell Metabolism
Short Article

Calorie Restriction Reduces Oxidative Stress by SIRT3-Mediated SOD2 Activation

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SUMMARY

A major cause of aging and numerous diseases is thought to be cumulative oxidative stress, resulting from the production of reactive oxygen species (ROS) during respiration. Calorie restriction (CR), the most robust intervention to extend life span and ameliorate various diseases in mammals, reduces oxidative stress and damage. However, the underlying mechanism is unknown. Here, we show that the protective effects of CR on oxidative stress and damage are diminished in mice lacking SIRT3, a mitochondrial deacetylase. SIRT3 reduces cellular ROS levels dependent on superoxide dismutase 2 (SOD2), a major mitochondrial antioxidant enzyme. SIRT3 deacetylates two critical lysine residues on SOD2 and promotes its antioxidative activity. Importantly, the ability of SOD2 to reduce cellular ROS and promote oxidative stress resistance is greatly enhanced by SIRT3. Our studies identify a defense program that CR provokes to reduce oxidative stress and suggest approaches to combat aging and oxidative stress-related diseases.

INTRODUCTION

The free radical theory of aging postulates that the production of reactive oxygen species (ROS) is the major determinant of life span (Balaban et al., 2005; Merry, 2004; Sohal and Weindruch, 1996). ROS are produced as a natural byproduct of cellular respiration. Antioxidant enzymes, such as superoxide dismutases (SODs), scavenge ROS and maintain a reducing environment in the cell. An imbalance between the production of ROS and the cell's ability to readily detoxify ROS disturbs the cellular reducing environment and results in oxidative stress. ROS can damage various components of the cell, including DNA, RNA, proteins, and lipids. Accumulated oxidative stress is thought to be a major cause of aging (Balaban et al., 2005; Merry, 2004). Consistent with this, animals with reduced levels of oxidative stress, such as animals fed a calorie restriction (CR) diet or overexpressing mitochondrially targeted catalase, have extended life spans, while animals with high levels of oxidative stress, such as mice lacking SODs, have shortened life spans (Merry, 2004; Schriner et al., 2005; Wallace and Fan, 2009).

CR is the most robust intervention to extend life span in mammals, and delays the onset of numerous age-associated diseases including cancer, diabetes, and neurodegenerative diseases (Colman et al., 2009; Van Remmen et al., 2001; Weindruch and Walford, 1988). CR was hypothesized to extend life span by slowing metabolism and reducing mitochondrial ROS (Sohal and Weindruch, 1996). However, metabolic rate normalized to body weight does not decrease in CR mice (Masoro et al., 1982). In fact, mitochondrial activity is increased during CR (Nisoli et al., 2005). Thus, the molecular mechanism underlying CR-induced reduction of oxidative stress remains elusive.

In the budding yeast *Saccharomyces cerevisiae* and the fruit fly *Drosophila melanogaster*, CR extends life span by increasing the activity of the Sir2 protein (Lin et al., 2000; Rogina and Helfand, 2004). Sir2p has nicotinamide adenine dinucleotide (NAD^+)-dependent deacetylase activity (Frye, 2000; Imai et al., 2000). An extra copy of SIR2 extends life span in yeast, flies, and worms (Kaeberlein et al., 1999; Rogina and Helfand, 2004; Tissenbaum and Guarente, 2001). Increasing evidence suggests that the function of SIR2 in mediating the CR response is conserved in mammals. SIRT1, the mammalian ortholog of SIR2, is upregulated in many tissues of CR mice and is an essential mediator of many aspects of the CR response, such as increased cell survival and physical activity, and decreased somatotropic signaling (Chen et al., 2005; Cohen et al., 2004, 2009; Li et al., 2008).

Given the role of SIR2 in mediating the CR response and the observation that animals fed a CR diet have reduced oxidative stress, we speculate that mammalian SIR2 homologs might play a role in regulating oxidative stress. Since 90% of cellular ROS are produced in the mitochondria (Balaban et al., 2005), we set out to investigate whether SIRT3, a mammalian SIR2 homolog localized in the mitochondria (Onyango et al., 2002; Schwer et al., 2002), mediates the CR response and reduces oxidative stress.

RESULTS

Reduction of Oxidative Stress and Damage by CR Requires SIRT3

CR induces an increase in the expression of SIRT3 (Palacios et al., 2009; Shi et al., 2005) and mitochondrial NAD^+ levels (Nakagawa et al., 2009), suggesting that SIRT3 activity is likely to be upregulated during CR. To investigate whether SIRT3 is required for CR to reduce oxidative stress, we fed SIRT3 knockout (KO) mice (Lombard et al., 2007) and wild-type (WT)

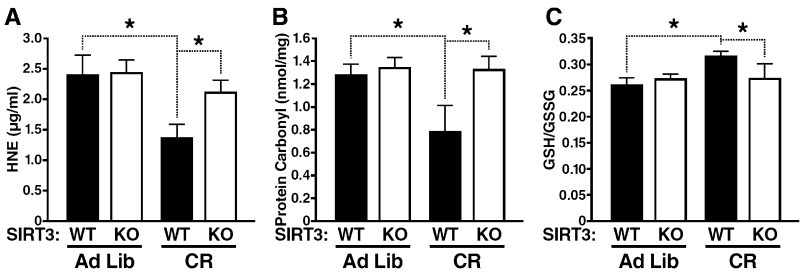

Figure 1. Reduction of Oxidative Stress and Damage by Calorie Restriction Requires SIRT3

(A–C) Liver lysates from WT and SIRT3 KO mice fed AL or CR diet were assayed for lipid peroxidation (A), protein carbonyl formation (B), and the GSH:GSSG ratio (C). *$p < 0.05$.

littermates a CR diet for 6 months. We compared oxidative stress and damage between WT and SIRT3 KO mice fed ad libitum (AL) or CR diets. Consistent with earlier reports, CR significantly reduced oxidative stress and damage in WT mice, as shown by levels of 4-hydroxy-2-nonenal (HNE), a marker for lipid peroxidation (Figure 1A), protein carbonyl content, a protein oxidative modification (Figure 1B), and the GSH:GSSG ratio, a common measure of oxidative stress (Figure 1C) (Merry, 2004; Rebrin et al., 2003). However, the reduction in oxidative stress and damage under CR was not observed in SIRT3 KO mice (Figures 1A–1C), suggesting that SIRT3 is required for reducing oxidative stress during CR.

SIRT3 Reduces Cellular ROS via SOD2

In mammals, there are three forms of SODs localized in various cellular compartments (Balaban et al., 2005). SOD2 is located in the mitochondria. To determine whether SOD2 plays a role in the reduction of cellular ROS mediated by SIRT3, we overexpressed SIRT3 in WT and SOD2 KO mouse embryonic fibroblasts (MEFs) via lentiviral transduction and assessed cellular ROS levels. Overexpression of SIRT3 in WT MEFs reduced cellular ROS by 40%. However, reduction of cellular ROS mediated by SIRT3 was blunted in SOD2 KO MEFs (Figure 2A), indicating that SOD2 is the major downstream mediator of SIRT3 in reducing cellular ROS.

SIRT3 Activates SOD2 via Deacetylation

SOD2 was identified in mass spectrometry-based screens for acetylated peptides (Choudhary et al., 2009; Kim et al., 2006, 2010; Schwer et al., 2009). We tested the possibility that SIRT3 regulates the acetylation state of SOD2. To test whether SIRT3 interacts with SOD2, we overexpressed Flag-tagged SIRT3 in 293T cells and SIRT3-associated proteins were immunopurified (anti-Flag). The association of SOD2 with SIRT3 was detected by western blotting with SOD2 antibody (Figure 2B). Additionally, we also transfected Flag-tagged SOD2 into 293T cells and the presence of SIRT3 in the immunopurified SOD2 complex was confirmed by western blotting with SIRT3 antibody (Figure 2C). Finally, we examined whether the interaction between SIRT3 and SOD2 is physiologically relevant by carrying out

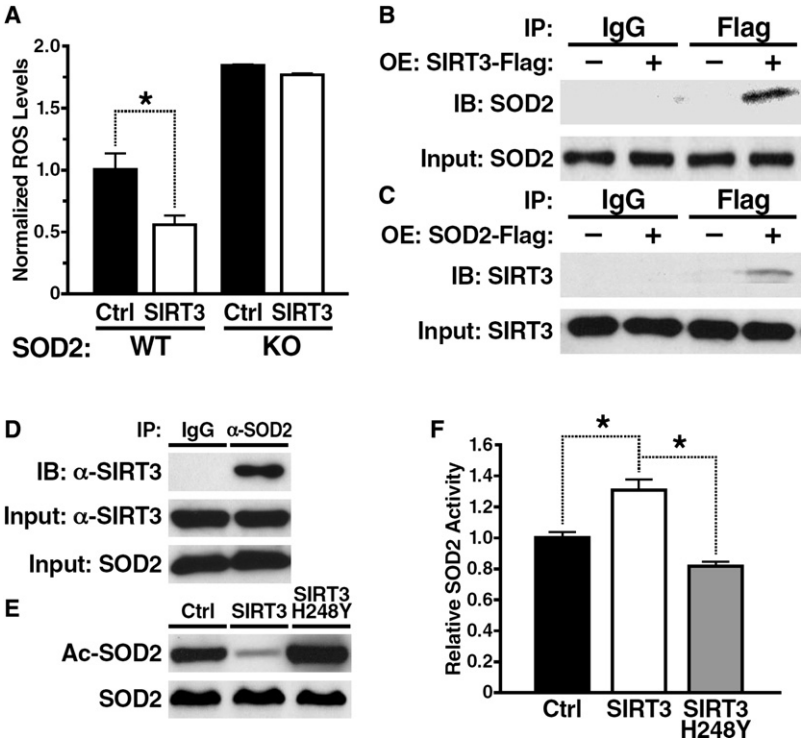

Figure 2. SIRT3 Deacetylates and Activates SOD2

(A) SIRT3 reduces cellular ROS via SOD2. SIRT3 was overexpressed in WT and SOD2 KO MEFs via lentiviral transduction and cellular ROS levels were quantified by MitoSox staining. *$p < 0.05$.

(B–D) SIRT3 physically interacts with SOD2 in vivo. Flag-tagged SIRT3 was transfected into 293T cells, immunopurified, followed by Western blotting with anti-SOD2 (B). Flag-tagged SOD2 was transfected into 293T cells. The association of SIRT3 with immunopurified Flag-SOD2 was detected by Western blotting with anti-SIRT3 antibody (C). Endogenous SOD2 was immunopurified from liver lysates with anti-SOD2 antibody, followed by western blotting with anti-SIRT3 antibody (D).

(E and F) SIRT3 deacetylates and activates SOD2. Flag-tagged SOD2 was transfected into 293T cells with a control vector, SIRT3, or enzymatically inactive SIRT3-H248Y. SOD2 was immunopurified. Its acetylation status was detected by western blotting with acetyl-lysine antibody (E), and its enzymatic activity was quantified by conversion of superoxide colormetrically as described (Schisler and Singh, 1985) (F). *$p < 0.05$.

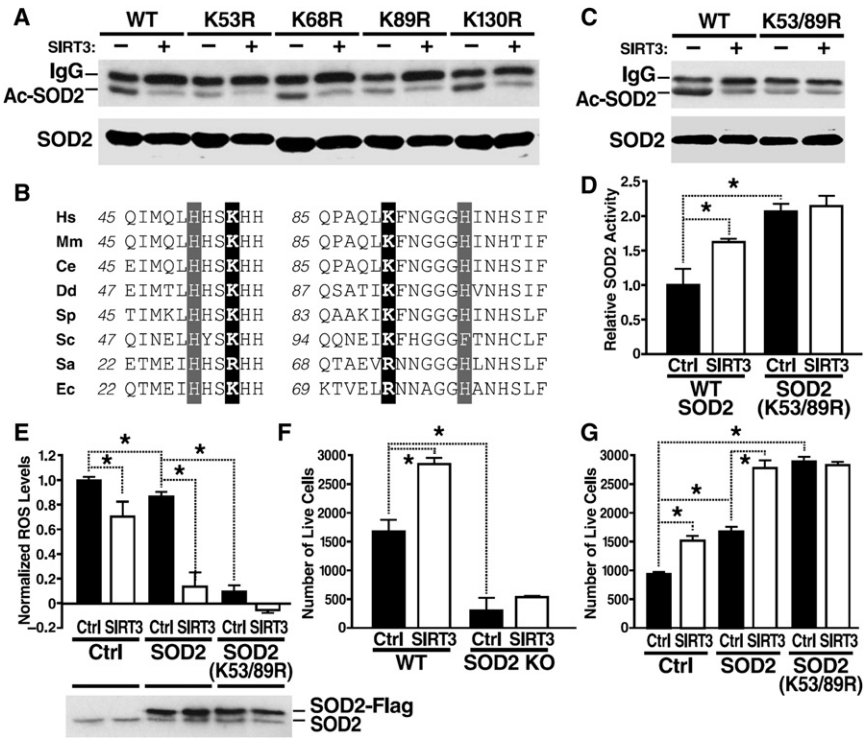

Figure 3. SIRT3 Reduces Cellular ROS Levels and Promotes Oxidative Stress Resistance by Deacetylating Two critical Lysine Residues on SOD2

(A and C) SIRT3 deacetylates two critical lysine residues on SOD2 in vivo. Flag-tagged SOD2 or SOD2 mutants were cotransfected with a control vector or SIRT3 into 293T cells. Immunopurified Flag-tagged SOD2 was examined for its acetylation levels by western blotting with anti-acetyl-lysine antibody. Sequence alignment of SOD2 from various species is shown in (B). Residues (H50 and H95) coordinating the metal center are shaded. Conserved lysines (K53 and K89) are in bold.

(D) SIRT3 activates SOD2 by deacetylating two critical lysine residues in vivo. Flag-tagged SOD2 or SOD2 K53/89R was cotransfected with a control vector or SIRT3 into 293T cells. The antioxidative activity of immunopurified SOD2 was determined. *$p < 0.05$.

(E) SIRT3 reduces cellular ROS by deacetylating SOD2. SOD2 or SOD2 K53/89R mutant was overexpressed with or without SIRT3 in SIRT3 KO MEFs, and cellular ROS levels were determined by MitoSox staining. *$p < 0.05$.

(F and G) SIRT3 promotes oxidative stress resistance by deacetylating SOD2. SIRT3 was overexpressed in WT or SOD2 KO MEFs (F). SOD2 or SOD2 K53/89R mutant was overexpressed with or without SIRT3 in SIRT3 KO MEFs (G). Ten thousand cells were treated with 200 μM paraquat and live cells were counted. See also Figure S1. *$p < 0.05$.

immunoprecipitation with liver extracts. SIRT3 was coimmunoprecipitated with SOD2 antibody (Figure 2D).

To test whether SIRT3 deacetylates SOD2, we cotransfected Flag-tagged SOD2 with SIRT3 or enzymatically inactive SIRT3-H248Y into 293T cells. Acetylation levels for SOD2 were measured after immunoprecipitation by western blotting with anti-acetyl-lysine antibody. SIRT3, but not SIRT3-H248Y, reduced acetylation levels of SOD2 (Figure 2E). To determine whether the acetylation state of SOD2 modifies its enzymatic activity, we overexpressed Flag-tagged SOD2 in 293T cells with SIRT3 or SIRT3-H248Y, immunopurified SOD2, and determined its enzymatic activity by measuring superoxide conversion colorimetrically (Schisler and Singh, 1985). Overexpression of WT SIRT3 decreased the acetylation of SOD2 (Figure 2E) and significantly increased its enzymatic activity (Figure 2F). In contrast, overexpression of SIRT3-H248Y had the opposite effect, suggesting that SIRT3 activates SOD2 via deacetylation.

To identify which lysine residue(s) on SOD2 are targeted by SIRT3 for deacetylation, we mutated lysines (K68 and K130) that have been shown to be acetylated in mass spectrometry-based acetylation proteomic surveys (Choudhary et al., 2009; Kim et al., 2006; Schwer et al., 2009). Surprisingly, mutating these lysine residues did not significantly reduce the overall acetylation levels of SOD2 (Figure 3A). Additionally, SIRT3 reduced acetylation levels of these SOD2 mutants, indicating that these lysines are unlikely to be the major acetylation sites on SOD2.

Protein sequence alignment studies showed that two lysines (K53 and K89) adjacent to the active site of SOD2 are highly conserved across species (Figure 3B). Acetylation levels of SOD2 were decreased when these two lysines were mutated individually (K53R and K89R) or simultaneously (K53/89R) (Figures 3A and 3C). SIRT3 further decreased acetylation levels of K53R and K89R, but not K53/89R. Collectively, these studies identified K53 and K89 as the acetylation sites on SOD2 targeted by SIRT3.

To determine how deacetylation of these two lysines on SOD2 affects its antioxidative activity, we determined the antioxidative activity of K53/89R, which mimics the constitutively deacetylated state. The enzymatic activity of SOD2 K53/89R was 100% higher than the WT control and SIRT3 did not further increase its enzymatic activity (Figure 3D). Thus, SIRT3 promotes the enzymatic activity of SOD2 by deacetylating two critical lysine residues adjacent to the active site. Conceivably, these two lysine residues, when exposed, increase the positive charge around the active site and improve the efficiency of trapping the negatively charged superoxide.

SIRT3 Reduces Cellular ROS by Deacetylating SOD2

To determine whether SIRT3 reduces cellular ROS levels by deacetylating SOD2, we overexpressed SOD2 or SOD2 K53/89R with or without SIRT3 into SIRT3 KO MEFs and assessed cellular ROS levels. We used SIRT3 KO MEFs in this assay to avoid the influence of endogenous SIRT3. Surprisingly, overexpression of SOD2 6-fold above the endogenous levels only marginally decreased cellular ROS (10%) (Figure 3E). However, coexpression of SIRT3 and SOD2 depleted 90% of cellular ROS.

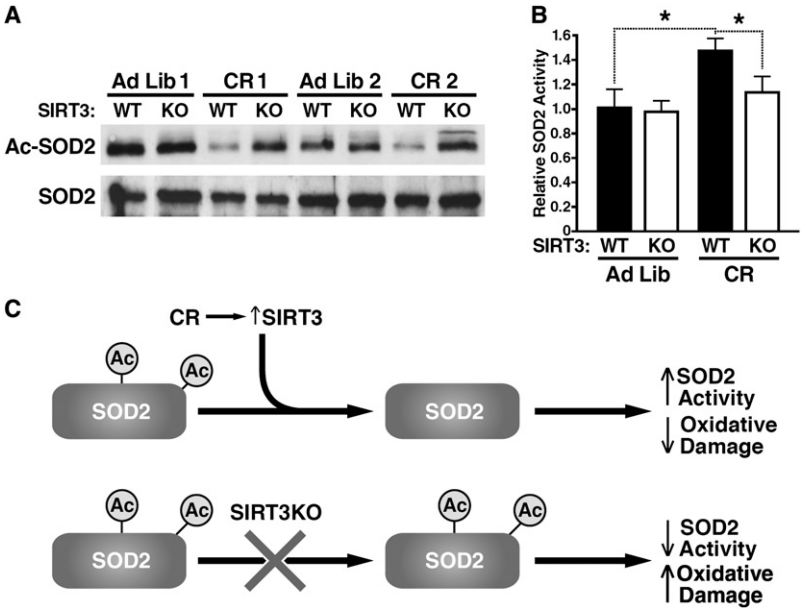

Figure 4. SIRT3 Activates SOD2 via Deacetylation during Calorie Restriction

(A) SIRT3 deacetylates SOD2 in CR mice. Acetylation levels of SOD2 in WT and SIRT3 KO mice fed an AL or CR diet were determined. Endogenous SOD2 was isolated by immunoprecipitation with anti-SOD2 antibody followed by western blotting with anti-acetyl-lysine antibody.

(B) SIRT3 increases the antioxidative activity of SOD2 in CR mice. The antioxidative activity of SOD2 in white adipose tissues of WT and SIRT3 KO mice fed an AL or CR diet were determined (Schisler and Singh, 1985). *$p < 0.05$.

(C) A model on SIRT3-mediated SOD2 activation.

Additionally, constitutively deacetylated SOD2 K53/89R alone also diminished cellular ROS by 90%. These results indicate that increasing SOD2 expression alone can only modestly reduce cellular ROS. SIRT3 deacetylation significantly enhances the ability of SOD2 to reduce cellular ROS.

SIRT3 Increases Oxidative Stress Resistance by Deacetylating SOD2

To determine whether SIRT3-mediated SOD2 activation results in increased oxidative stress resistance, we overexpressed SIRT3 in WT or SOD2 KO MEFs, treated the cells with paraquat, a superoxide-generating compound, and assayed cell survival. SIRT3 overexpression in WT MEFs doubled the number of surviving cells (Figures 3F and S1). However, SIRT3 overexpression in SOD2 KO MEFs had no effect on cell survival, indicating that SIRT3 promotes oxidative stress resistance and SOD2 is the mediator of this process. To determine whether SIRT3 increases oxidative stress resistance by deacetylating SOD2, we overexpressed WT SOD2 or SOD2 K53/89R with or without SIRT3 into SIRT3 KO MEFs, and assessed oxidative stress resistance of these cells. Although overexpression of SOD2 alone only modestly reduced cellular ROS (10%) (Figure 3E), it increased the cell survival rate by 50% upon paraquat treatment (Figure 3G). It is worth noting that the condition for measuring cellular ROS level (Figure 3E) is different from the condition for assessing oxidative stress resistance (Figures 3F and 3G). The former is for untreated cells, while the latter is for cells treated with paraquat when ROS levels are significantly higher than physiological levels. Importantly, coexpression of SOD2 and SIRT3 resulted in a nearly 3-fold increase in cell survival (Figure 3G). Moreover, constitutively deacetylated SOD2 K53/89R increased cell survival to the same level as coexpression of SOD2 and SIRT3. Coexpression of SIRT3 did not further increase cell survival mediated by SOD2 K53/89R. These results indicate that SIRT3 promotes oxidative stress resistance by deacetylating SOD2.

SOD2 Is Activated by SIRT3 via Deacetylation during Calorie Restriction

Given that SIRT3 is induced by CR and that SIRT3 activates SOD2 via deacetylation, we speculated that SIRT3 might deacetylate SOD2 and increase its antioxidative activity in CR animals. We compared acetylation levels of SOD2 and its antioxidative activity in WT and SIRT3 KO mice fed AL or CR diets. To assess acetylation levels of SOD2 in mouse tissues, endogenous proteins were immunoprecipitated with anti-SOD2 antibody and analyzed by western blotting using acetyl-lysine antibody. Endogenous SOD2 was acetylated and became deacetylated during CR in WT mice (Figure 4A). However, CR-induced SOD2 deacetylation was not observed in SIRT3 KO mice, demonstrating that SIRT3 is necessary for SOD2 deacetylation during CR. We next determined endogenous SOD2 activity using tissue lysates as described (Schisler and Singh, 1985). CR induced a 50% increase in SOD2 activity in the white adipose tissues of WT mice (Figure 4B). Importantly, this increase in SOD2 activity under CR was lost in SIRT3 KO mice. These results suggest that during CR, SIRT3 reduces oxidative stress by activating SOD2 and promoting the detoxification of ROS. It is worth noting that SIRT3-mediated SOD2 deacetylation and activation was not observed in mice fed AL (Figures 4A and 4B). Consistent with this, oxidative stress and damage were comparable in WT and SIRT3 KO mice fed AL (Figure 1), suggesting that SIRT3 is not active under AL conditions.

DISCUSSION

Our studies identify an active defense program CR provokes to reduce oxidative stress. CR induces an increase in SIRT3 expression (Palacios et al., 2009; Shi et al., 2005). Activation of SIRT3 during CR reduces oxidative stress by activating the mitochondrial antioxidant enzyme SOD2. SOD2 activation and increased oxidative stress resistance have been linked to numerous long-lived mouse models (Baba et al., 2005; Hauck et al., 2001; Taguchi et al., 2007; Yamamoto et al., 2005). We speculate that the loss of protection from oxidative stress in the SIRT3 KO mice could abrogate CR-induced life-span extension.

Despite the prevalence of the free radical theory of aging, it is intriguing that SOD2 transgenic mice do not have increased life

spans (Pérez et al., 2009). Our studies suggest that increasing SOD2 expression alone can only modestly reduce cellular ROS. The ability of SOD2 to reduce cellular ROS is greatly enhanced by SIRT3 deacetylation. Transgenic mice overexpressing both SIRT3 and SOD2 might have extended life spans.

Although mitochondrial activity is increased by CR (Nisoli et al., 2005), oxidative stress and damage are reduced in CR animals (Merry, 2004). Evidence is emerging to support the mitochondrial hormesis or "mitohormesis" concept, which hypothesizes that induction of mitochondrial metabolism and the increased formation of ROS trigger an active defense program, resulting in increased stress resistance and possibly extended life span (Schulz et al., 2007; Sinclair, 2005). Perhaps activation of SIRT3 in CR animals is a key step of this defense program against oxidative stress.

In light of the importance of mitochondrial ROS production in the onset and progression of diverse diseases associated with aging (Wallace and Fan, 2009), our findings have important implications for developing CR mimetics for treating the diseases of aging. Notably, SIRT3 has been shown to protect mice from developing cancer and cardiac hypertrophy (Kim et al., 2010; Sundaresan et al., 2009). Future studies will determine the impact of SIRT3 in other pathological conditions and explore therapeutic strategies to treat human diseases with SIRT3 activators or SOD2 variants.

EXPERIMENTAL PROCEDURES

Mice

SIRT3$^{-/-}$ mice have been described (Lombard et al., 2007). All mice were housed on a 12:12 hr light:dark cycle at 25°C. Six-month-old animals (n = 8) were either fed AL or a 30% CR diet, which was provided daily for six months. All animal procedures were in accordance with the animal care committee at the University of California, Berkeley.

Protein Preparation and Analysis

Proteins from mouse tissues were extracted in lysis buffer (50 mM Tris-Cl pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM $MgCl_2$, 1 mM DTT and 1% NP40) supplemented with a complete protease inhibitor cocktail (Roche), trichostatin A, and nicotinamide. Protein extracts were subjected to centrifugation at 14,000 rpm for 10 min. Protein lysates were precleared with protein A beads for 30 min before immunoprecipitation with specified antibodies for 2 hr or overnight. Immunoprecipitates were extensively washed with lysis buffer and eluted with 100 mM glycine, pH 3.0, or Flag peptides. Acetyl-lysine antibody was provided by Cell Signaling, BioLegend; SOD2 antibody was provided by Santa Cruz Biotechnology; Flag antibody was provided by Sigma.

Lentiviral Production and Transduction

SIRT3 and SOD2 were cloned into pFUGW lentiviral vector. Lentiviruses were produced by transient transfection of pFUGW and packaging vectors into 293T cells with lipofectamine. Lentiviruses were harvested 48 hr posttransfection and filtered through 0.45-μm-pore cellulose acetate filters. Virus containing media was mixed with fresh media (1:1) and added to MEF cells in the presence of 8 μg/ml polybrene.

Measurement of Mitochondrial Superoxide Levels

Cells were incubated with 3 μM of Mito-SOX at 37°C for 15 min prior to flow cytometry analysis.

Enzyme Assays

Superoxide dismutase activity was measured as the inhibition of nitroblue tetrazolium (NBT) reduction in a xanthine-xanthine oxidase system. The assay was performed as described (Schisler and Singh, 1985), and SOD2 specific activity was determined in the presence of 5 mM sodium cyanide.

Carbonyl Content Measurement

Protein carbonyls were spectrophotometrically quantified with a carbonyl specific reagent, 2,4-dinitrophenylhydrazine (DNPH) (Levine et al., 1994). Briefly, 1 ml of 0.5 mg protein was treated with 200 μl of 10 mM of DNPH (dissolved in 2M HCl) for 1 hr, and then precipitated by 10% trichloroacetic acid. The pellets were washed with 1:1 (v/v) ethanol:ethyl acetate for three times, and solublized in 0.5 ml 0.2% SDS, 20 mM Tris-Cl, pH 6.8. Protein concentration in the final solution was then determined with a BCA kit (Piercenet), and the absorbance at 360 nm was measured to calculate the carbonyl content. Protein samples treated with HCl but not with DNPH were used as blanks.

Glutathione Redox Measurement

Glutathione was measured in mitochondrial fractions isolated from liver (Merry, 2004; Rebrin et al., 2003). The GSH: GSSG ratio was determined by Glutathione Assay Kit (BioVision), following the manufacturer's instructions.

HNE Measurement

HNE levels were measured in indicated liver samples with an OxiSelect HNE-His Adduct ELISA Kit (Cell Biolabs, Inc. San Diego, CA) following the instructions.

Cell Survival Assay

Ten thousand cells were incubated with increasing doses of paraquat (Sigma) (0, 50, 100, 200, 400 μM) for 48 hr. Remaining adherent cells were trypsinized and counted using a hemocytometer. A dose-curve response was determined (Figure S1A).

Statistical Analysis

Student's t test was used for statistic analysis and null hypotheses were rejected at 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes one figure and can be found with this article online at doi:10.1016/j.cmet.2010.11.015.

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