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Differential Protein Acetylation Assists Import of Excess SOD2 into Mitochondria and Mediates SOD2 Aggregation Associated with Cardiac Hypertrophy in the Murine SOD2-tg Heart

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Abstract

SOD2 is the primary antioxidant enzyme neutralizing $\cdot\text{O}_2^-$ in mitochondria. Cardiac-specific SOD2 overexpression (SOD2-tg) induces supernormal function and cardiac hypertrophy in the mouse heart. However, the reductive stress imposed by SOD2 overexpression results in protein aggregation of SOD2 pentamers and differential hyperacetylation of SOD2 in the mitochondria and cytosol. Here, we studied SOD2 acetylation in SOD2-tg and wild-type mouse hearts. LC-MS/MS analysis indicated the presence of four acetylated lysines in matrix SOD2 and nine acetylated lysines in cytosolic SOD2 from the SOD2-tg heart. However, only one specific acetylated lysine residue was detected in the mitochondria of the wild-type heart, which was consistent with Sirt3 downregulation in the SOD2-tg heart. LC-MS/MS further detected hyperacetylated SOD2 with a signaling peptide in the mitochondrial inner membrane and matrix of the SOD2-tg heart, indicating partial arrest of the SOD2 precursor in the membrane during translocation into the mitochondria. Upregulation of HSP 70 and cytosolic HSP 60 enabled the translocation of excess SOD2 into mitochondria. In vitro acetylation of matrix SOD2 with Ac_2O deaggregated pentameric SOD2, restored the profile of cytosolic SOD2 hyperacetylation, and decreased matrix SOD2 activity. As revealed by 3D structure, acetylation of K89, K134, and K154

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Author Contributions: LZ and ZJ conducted the in-gel digestion LC-MS/MS experiments and data analysis. CLC prepared the cytosol, mitochondria, SMP, and matrix from the mouse myocardium and conducted the SDS-PAGE and immunoblotting experiments. PK conducted the EPR analysis and the activity assay of sirtuin deacetylase. YRC conceived the idea for the project and wrote the paper with LZ, ZJ, and PK.

Supporting Information: Three figure sets showing amino acid sequence coverage of human SOD2 based on LC-MS/MS analysis (Figure S1), detailed MS/MS spectra of identified acetylated lysine residues in the cytosolic and mitochondrial SOD2 from the SOD2-tg mouse heart (Figure S2 & Figure S3); additional figure showing protein staining of the inner membrane and matrix preparations from the mitochondria of the SOD2-tg mouse heart and studies of the matrix SOD2 by native PAGE (Figure S4); and one table presenting a summary of acetylated peptides induced by in vitro acetylation with Ac_2O (Table S).

of cytosolic SOD2 induces unfolding of the tertiary structure and breaking of the salt bridges that are important for the quaternary structure, suggesting that hyperacetylation and HSP 70 upregulation maintain the unfolded status of SOD2 in the cytosol and mediate the import of SOD2 across the membrane. Downregulation of Sirt3, HSP 60, and presequence protease in the mitochondria of the SOD2-tg heart promoted protein misfolding that led to pentameric aggregation.

Keywords

SOD2; mitochondria; protein acetylation; protein aggregation; mitochondrial translocation; cardiac-specific transgenic mouse

1. INTRODUCTION

Mitochondrial superoxide dismutase (MnSOD or SOD2) is one of the primary mitochondrial antioxidants in a network of detoxification enzymes that neutralize highly reactive superoxide ions (O_2^-) to less reactive hydrogen peroxide (H_2O_2), followed by its immediate conversion to H_2O by glutathione peroxidases or catalase. SOD2 deficiency is associated with human age-related cardiovascular disorders such as idiopathic cardiomyopathy [1].

In vivo and in vitro studies support the idea that physiological conditions of increased mitochondrial superoxide levels indirectly induce SOD2 activation. Studies with the sirtuin 3 knockout mouse ($\text{Sirt3}^{-/-}$) have proposed that SOD2 activation is directly regulated by Sirt3-mediated deacetylation in mitochondria. The above rationale has been validated under the physiological conditions of calorie restriction and stress conditions of ionizing radiation [2, 3]. Therefore, the reversible acetylation of lysine residues near the SOD2 active sites has been implicated in the suppression of SOD2 activity [4, 5].

In addition to directly regulating the catalytic activity of various enzymes, protein acetylation has been reported to regulate the subcellular localization of certain metabolic enzymes [4]. It has been documented that protein acetylation promotes the translocation of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) into the nucleus to regulate the cellular processes of transcription regulation, DNA repair, and telomere maintenance [6].

Mitochondrial SOD2 is encoded by nuclear DNA. As supported by experimental evidence using in vitro protein translation of human SOD2 [7], the SOD2 precursor synthesized in the cytosol contains an N-terminal signaling peptide ($^1\text{MLSRAVCGTSRQLAPALGYLGSRQ}^{24}$) that acts as an import sequence to mediate the mitochondrial localization of the matrix protein [7]. However, in vivo evidence showing that the N-terminal signaling peptide is used for import is still lacking because precursor proteins are either imported into the mitochondria for proteolytic cleavage of the signaling sequence by a matrix processing protease (MPP) or rapidly degraded by proteasomes in the cytosol [8].

Cardiac-specific overexpression of SOD2 (the SOD2 transgenic or SOD2-tg mouse model) induces supernormal cardiac function in the mouse heart via enhanced myocardial blood

flow and improved bioenergetic efficiency of mitochondria, as reported previously [9]. However, the reductive stress induced by SOD2 overexpression also induces a phenotypic switch to hypertrophy in the murine SOD2-tg heart [9]. This mouse model, which conditionally overexpresses SOD2 in cardiac mitochondria [9], provides an ideal system for investigating the mechanisms of SOD2 acetylation, the signaling peptide of the SOD2 precursor in vivo, translocation of SOD2 to mitochondria, and the role of protein acetylation in SOD2 aggregation associated with the phenotype of hypertrophy.

Here, we explore the fundamental mechanisms of SOD2 acetylation, mitochondrial localization, and aggregation relevant in the hypertrophic phenotype of the SOD2-tg mouse heart. We show evidence for SOD2 hyperacetylation induced by Sirt3 downregulation and the detection of differential acetylation present in the SOD2 of the cytosol, inner membrane (SMP), and matrix compartments in the SOD2-tg mouse heart. We further show arrest of the SOD2 precursor in the mitochondrial inner membrane and pentameric formation of SOD2 aggregates in the matrix, which was not observed in the wild-type mouse heart and likely contributes to the phenotype of hypertrophy in the SOD2-tg mouse heart. We then provide evidence indicating that in vitro acetylation can deaggregate SOD2 pentamer as well.

2. MATERIALS AND METHODS

2.1 Animals

The SOD2-tg mice were obtained from the Jackson Laboratory. All procedures were performed with the approval (protocol no. 15-028) of the Institutional Animal Care and Use Committee at Northeast Ohio Medical University (Rootstown, OH) and conformed to the Guide for the Care and Use of Laboratory Animals.

2.2 Reagents

Glutathione (GSH), diethylenetriaminepentaacetic acid (DTPA), ubiquinone-1 (Q_1), ubiquinone-2 (Q_2), DL-dithiothreitol, acetyl anhydride (Ac_2O), sodium cholate, deoxycholic acid, PEGSOD (polyethylene glycol-linked superoxide dismutase), and succinate were purchased from Sigma Chemical Company (St. Louis, MO) and used as received. Anti-acetylsine and anti-Sirt3 monoclonal antibodies were purchased from Cell Signaling Technology (CST, Danvers, MA). The polyclonal and monoclonal antibodies against SOD2 were purchased from Santa Cruz Biotechnology (Dallas, TX). The 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) spin trap was purchased from Dojindo Molecular Technologies, Inc. (Rockville, MD). The DEPMPO (5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide) spin trap was purchased from Enzo Life Sciences, Inc. (Farmingdale, NY) and stored under nitrogen at -80°C until needed.

2.3 Analytical Methods

Optical spectra were measured on a Shimadzu 2600 UV/VIS recording spectrophotometer. The protein concentrations of cytosolic, mitochondrial, SMP, and matrix preparations were determined by the Lowry method using BSA as a standard. The concentrations of Q_1 and Q_2 were determined by absorbance spectra from $NaBH_4$ reduction using a millimolar extinction coefficient $\epsilon_{(275\text{nm}-290\text{nm})} = 12.25 \text{ mM}^{-1} \text{ cm}^{-1}$ [10].

2.4 Preparation of mitochondria, SMP, the cytosolic fraction, and the matrix fraction from the hearts of SOD2-tg mice

Mitochondria were prepared from mouse hearts by differential centrifugation according to published methods [9, 11]. Mitochondria were precipitated by centrifugation at $20,000 \times g$ for 10 min in the final step and resuspended in a medium (M-buffer) containing the following agents: in mM, mannitol 230, sucrose 70, EDTA 1, Trizma 1; pH 7.4. The cytosolic compartment was collected from the supernatant after centrifugation at $20,000 \times g$. The mitochondrial and cytosolic fractions were evaluated by immunoblotting using a monoclonal antibody (1:500) against glyceraldehyde 3-phosphate dehydrogenase (GAPDH, a housekeeping cytosolic protein, Santa Cruz Biotechnology, Inc., Dallas, TX; catalog number: sc-32233 (6C5), blotting of cytosolic preparations served as a positive control) and a monoclonal antibody (1:1000, Santa Cruz Biotechnology, Inc. catalog number: sc-65237 (20E9),) against the ND1 subunit of complex I (a mitochondrial DNA-encoded protein) to ensure no cross contamination between mitochondria and cytosol [9, 11].

Preparation of submitochondrial particles (SMP) and the matrix was based on the published method with minor modifications [12]. Mitochondrial preparations were suspended in 30 mM phosphate buffer, pH 7.4, containing 10 mM NaCl, and then subjected to sonication 5 times at 0 °C (30-sec duration with an interval of 1 min, power: 20 W). The mixture was centrifuged at 8,800 rpm for 10 min to remove any intact mitochondria. The supernatant was centrifuged at $80,000 \times g$ for 1 h to separate the inner membrane preparation from the matrix preparation.

2.5 Measurement of mitochondrial $\bullet O_2^-$ production by EPR spin trapping

EPR measurements of $\bullet O_2^-$ generation within the SMP preparation were carried out on a Bruker EMX Micro spectrometer operating at 9.43 GHz with 100 kHz modulation frequency at room temperature [13]. The reaction mixture containing the NADH-linked respiration buffer supplemented with DTPA (1 mM), NADH (0.5 mM), and DMPO (90 mM) was mixed with the SMP preparation (to a final 1.0 mg protein/mL) at 30 °C for 4 min. The reaction mixture was then transferred into a 50- μ L capillary (Drummond Wiretrol, Broomall, PA), sealed, loaded into the EPR resonator (HS cavity, Bruker Instrument, Billerica, MA), equilibrated to 298 K, and tuned within 2 min. The scan of the EPR spectrum was started at exactly 6 min after the initial reaction. Parameters: center field 3360 G, sweep width 100 G, power 20 mW, receiver gain 1×10^5 , modulation amplitude 1 G, conversion time 40.96 ms, time constant 163.84 ms, number of scans: 5. The spectral simulations were performed using the WinSim program developed at NIEHS by Duling [14].

2.6 The assay of SOD2 activity as measured by EPR spin-trapping with DEPMPO

The mitochondrial supercomplex of isolated succinate-cytochrome *c* reductase (SCR) is known to mediate $\bullet O_2^-$ production in the presence of succinate (Suc) [15]. The nitron spin trap DEPMPO traps the $\bullet O_2^-$, resulting in a multiline EPR spectrum, which was simulated by the WinSim program and subsequently double-integrated for spin quantification. SOD and its isoforms in the mixture compete against DEPMPO and therefore reduce the signal intensity of the EPR spectrum of DEPMPO/ $\bullet OOH$.

EPR measurements of $\bullet\text{O}_2^-$ generation by SCR-Suc were carried out on a Bruker EMX Micro spectrometer operating at 9.43 GHz with 100 kHz modulation frequency at room temperature (RT). The reaction mixture containing HBSS buffer with DTPA (1 mM)/Suc (50 μM) was mixed with the mitochondrial matrix preparations (serial titration of 10 – 400 μg protein/mL) or cytosolic fractions at RT. To deactivate the SOD1 of the cytosolic fraction, 5 mM of KCN was preincubated with the stock cytosolic fraction (1 mg/mL) in an ice bath for 10 min. The reaction was initiated by the addition of SCR (0.32 mg/mL, heme $b = 1.2 \mu\text{M}$) and DEPMPO (50 mM). The reaction mixture was then transferred into a 50- μL capillary (Drummond Wiretrol, Broomall, PA), sealed, loaded into the EPR resonator (HS cavity, Bruker Instrument, Billerica, MA), equilibrated to 298 K, and tuned within 2 min. The scan of the EPR spectrum was started at exactly 2 min after the initial reaction. Parameters: center field 3360 G, sweep width 140 G, power 20 mW, receiver gain 1×10^5 , modulation amplitude 1 G, conversion time 81.92 ms, time constant 327.68 ms, resolution in $\times 1024$, number of scans: 3.

2.7 Measurement of NAD⁺-dependent deacetylase activity

The deacetylase activity assay was performed using the Fluor-de-Lys SIRT3 fluorometric drug discovery assay kit (Enzo Life Sciences Inc., Farmingdale, NY) following the manufacturer's protocol. Isolated mouse heart mitochondrial proteins were reacted with the Fluor-de-Lys deacetylase substrate (30 μM) in the presence of NAD⁺ (1 mM) at 37 °C for 40 min. Serial dilution of recombinant human Sirt3 (included in this assay kit) was used to establish a standard curve, and the heat-denatured (70 °C, 10 min) samples reacting with substrates were used to calculate background signal. The reaction was stopped by the addition of Fluor-de-Lys-developer II containing nicotinamide (5 mM), and the resulting fluorescence signal was detected by a Synergy 4 hybrid microplate reader (BioTek U.S., Winooski, VT) with excitation 360/40 nm and emission 460/40 nm filters.

2.8 Immunoblotting analysis

The reaction mixture was mixed with the Laemmli sample buffer at a ratio of 4:1 (v/v) under the reducing conditions in the presence of dithiothreitol (40 mM), incubated at 70 °C for 10 min, and then immediately loaded onto a 4–12% Bis-Tris polyacrylamide gradient gel according to previous published methods [9, 11]. Samples were run in the running buffer (in mM, MES 50, Tris Base 50, SDS 1.39, EDTA 1, DTT 0.31) at room temperature for 55 min at 190 V. Protein bands were electrophoretically transferred to a nitrocellulose membrane in 25 mM Bis-Tris, 25 mM Bicine, 0.029% (w/v) EDTA, and 10% methanol. Membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween-20 (TTBS) and 5% dry milk (BioRad, Hercules, CA). The blots were then incubated overnight with the primary antibody at 4 °C. Blots were then washed 3 times in TTBS and incubated for 1 h with horseradish peroxidase-conjugated anti-mouse IgG in TTBS at RT. The blots were again washed twice in TTBS and twice in TBS, and then visualized using ECL Western Blotting Detection Reagents (GE Healthcare Life Sciences, Fairfield, CT).

2.9 Trypsin in-gel digestion and LC-MS/MS Analysis

Gels were digested with sequencing-grade trypsin from Promega (Madison WI) as described in the previous publication [16]. Digested samples were analyzed either via capillary liquid

chromatography coupled with an LTQ-Orbitrap (Thermo Scientific) or via nanoflow liquid chromatography interfaced with a Q Exactive Plus mass spectrometer (Thermo Scientific).

The capillary-liquid chromatography-nanospray tandem mass spectrometry (Capillary-LC-MS/MS) was performed on an LTQ Orbitrap mass spectrometer equipped with a microspray source (Bruker Daltonics) operated in positive ion mode. Samples were separated on a capillary column (0.2×150 mm Magic C18AQ 3μ 200A, Bruker Daltonics) using an UltiMate™ 3000 HPLC system (Thermo Scientific). Each sample was injected into the μ-Precolumn Cartridge (Thermo Scientific) and desalted with 50 mM acetic acid for 5 min. The injector port was then switched to inject, and the peptides were eluted from the trap onto the column. Mobile phase A was 50 mM acetic acid in water, and acetonitrile was used as mobile phase B. The flow rate was set at 2 μL/min. Mobile phase B was increased from 2% to 35% in 30 min and then increased from 35–50% in 10 min. Mobile phase B was increased again to 90% in 1 min and then kept at 90% for another 1 min before being brought back quickly to 2% in 1 min. The column was equilibrated at 2% of mobile phase B (or 98% A) for 15 min before the next sample injection. MS/MS data were acquired with a spray voltage of 2.2 kV and a capillary temperature of 175 °C was used. The scan sequence of the mass spectrometer was based on the preview mode data-dependent TopTen™ method: the analysis was programmed for a full scan recorded between m/z 350 – 2000 and an MS/MS scan to generate product ion spectra to determine amino acid sequence in consecutive scans of the five most abundant peaks in the spectrum. To achieve high mass accuracy MS determination, the full scan was performed in FT mode and the resolution was set at 60,000. The AGC target ion number for the FT full scan was set at 1×10^6 ions, maximum ion injection time was set at 1000 ms, and micro scan number was set at 1. MSn was performed using ion trap mode to ensure the highest signal intensity of MSn spectra. The AGC Target ion number for the ion trap MSn scan was set at 10000 ions, maximum ion injection time was set at 50 ms, and microscan number was set at 1. The CID fragmentation energy was set to 35%. Dynamic exclusion was enabled with a repeat count of 1 within 12 s, a mass list size limit of 500, exclusion duration of 12–20 s (depends on the length of the gradient) and a low mass width and high mass width of 30 ppm. An exclusion list containing major trypsin autolysis peptides was applied so that these peaks would not be detected. The reject mass width window was set at 30 ppm.

The Q Exactive Plus was coupled to a Dionex UltiMate 3000 nanoflow LC system. The analytical column was a Dionex Acclaim PepMap RSLC 75 μm × 15 cm with a trapping column (C18 PepMap 100, 5 μm). Mobile phase A was water with 0.1% formic acid, and mobile phase B contained 80% acetonitrile with 0.1% formic acid. Samples were loaded onto the trap column, washed with solution containing 2% acetonitrile with 0.1% formic acid for 3 min, and back-flushed to the analytical column with 5% mobile phase B. For peptide separation, a 100-min gradient from 5% to 35% mobile phase B was followed by a 10-min wash with 90% mobile phase B. The flow rate was set at 300 nL/min. The Q Exactive Plus instrument was operated in positive and data-dependent mode. The survey scan was acquired at a resolution of 70,000 in the mass range of 350–1600 amu. Twelve MS/MS spectra were collected at a resolution of 17,500. The heated capillary was maintained at 250 °C, and the ESI spray voltage was kept at +2.0 kV. Sequence information from the MS/MS data were processed by converting the raw files into a merged file (mgf)

using an in-house program, RAW2MZXML_n_MGF_batch (merge.pl, a Perl script). Isotope distributions for the precursor ions of the MS/MS spectra were deconvoluted to obtain the charge states and monoisotopic m/z values of the precursor ions during the data conversion. The resulting mgf files were searched using Mascot Daemon by Matrix Science version 2.3.2 (Boston, MA), and the database was searched against the SwissProt human database. The mass accuracy of the precursor ions was set to 20 ppm, with an accidental pick of one ^{13}C peak also included in the search. The fragment mass tolerance was 0.5 Da for LTQ-Orbitrap and 0.02 Da for Q Exactive Plus. The variable modifications considered were oxidation (Met), deamidation (N and Q), carbamidomethylation (Cys) and acetylation (K). Four missed cleavages for the enzyme were permitted. A decoy database was also searched to determine the false discovery rate (FDR), and peptides were filtered according to the FDR. The significance threshold was set at $p < 0.05$, and bold red peptides were required for valid peptide identification. Any modifications or low-score peptide/protein identifications were manually checked for validation.

2.10. Statistical Analysis

All data were reported as group averages. Comparisons between two groups were assessed by student t -test to analyze the significance of differences. Comparisons among multiple groups were assessed by one-way ANOVA followed by the least significant difference, Tukey's honestly significant difference or Games-Howell post hoc tests. Results with $p < 0.05$ were considered statistically significant. Data were presented as mean $\pm$ SEM (Figure 3 and Figure 4) and mean $\pm$ SD (Figure 6).

3. Results

3.1 Detection of protein lysine acetylation in cytosolic SOD2 and matrix SOD2 in mitochondria from the SOD2-tg murine heart

The myocardium of the SOD2-tg mouse was fractionated into the cytosolic and mitochondrial fractions by differential centrifugation and then subjected to SDS-PAGE using 4–12% gradient polyacrylamide gel and immunoblotting with a polyclonal antibody against SOD2 (1:4,000, Santa Cruz Biotech. Inc. catalog number. sc-30080 (FL222)). As indicated in Figure 1A, both cytosolic and mitochondrial SOD2 were identified, with molecular weight ~ 22 kDa. However, an additional protein band with higher molecular weight caused by SOD2 polymerization or SOD2 aggregation was further identified in the mitochondrial fraction by both Western blotting and Coomassie staining (indicated by an arrow in Figures 1A and 1B).

Mitochondrial preparations were further subjected to fractionation to obtain the matrix and mitochondrial inner membrane (SMP) compartments. The cytosol and matrix preparations were subjected to SDS-PAGE, followed by immunoblotting with a monoclonal antibody against acetyllysine (1:1,000, anti-AcK Ab, Cell Signaling, catalog number:#9681 (Ac-K-103)). As indicated in Figure 1B, both cytosolic SOD2 and matrix SOD2 were acetylated.

3.2 LC-MS/MS analysis of protein lysine acetylation in cytosolic SOD2 from the SOD2-tg heart

The protein band of the cytosolic SOD2 was subjected to in-gel digestion with trypsin and analyzed by LC-MS/MS. With this technique, 99.5% of the amino acid sequence of the SOD2 precursor was identified (Figure S1). Fourteen of 15 lysine residues (excluding K25 in SOD2 precursor or K1 in mature matrix SOD2) were identified by LC-MS/MS under reducing conditions in the presence of DTT (Figure S1). Single acetylation of a lysine residue will increase the molecular weight by 42 Da. Therefore, the mass spectra from the proteolytic digest of cytosolic SOD2 were examined for the addition of $42 \times n$ Da (n is the number of lysine residues in the peptide). In the tryptic digest LC-MS/MS results, a mass difference of 42 Da was observed in 12 peptide fragments (Table 1), indicating that these 12 peptides were acetylated. Further analysis showed unequivocally that acetylation occurred at 9 specific lysine residues: K68, K75, K89, K114, K122, K130, K132, K134, and K154 in cytosolic SOD2 (shown in Table 1 and MS/MS spectra in Figure S2, $n=2$, data were confirmed by analysis of two batches of cytosolic preparation).

3.3 LC-MS/MS analysis of protein lysine acetylation in the SOD2 of the mitochondrial matrix from the SOD2-tg heart

The protein band of the SOD2 from the matrix preparation was further subjected to in-gel digestion with trypsin and analyzed by LC-MS/MS. Nearly the entire (99.5%) amino acid sequence of matrix SOD2 was identified. The MS/MS results of the tryptic digest revealed that a mass difference of 42 Da was identified in 4 peptide fragments as shown in Table 2 and MS/MS spectra in Figure S3. Further analysis explicitly identified the K68, K122, K130, and K202 residues of the SOD2 in the matrix preparation ($n=3$, data were confirmed by analysis of three batches of matrix preparation). It is worth noting that acetylation of K202 was not observed in the cytosolic SOD2 (MS/MS spectrum for K202 in Figure S3-d).

3.4 In vitro protein acetylation of the matrix SOD2

To determine whether the hyperacetylated SOD2 seen in the cytosolic protein can be duplicated by in vitro acetylation in the matrix, a matrix preparation containing SOD2 was incubated with acetyl anhydride (Ac_2O , 100–200 μM) at room temperature for 20 min. As indicated in Figure 2A, treatment of the matrix preparation with Ac_2O dramatically enhanced acetylation of matrix SOD2 as detected by immunoblotting with a monoclonal antibody against acetyllysine (AcK), while treatment of the matrix preparation with the same equivalent of acetic acid (AcOH) did not increase acetylation of SOD2, confirming Ac_2O -dependent in vitro acetylation of SOD2 in the matrix.

Hyperacetylated SOD2 (induced by 100 μM Ac_2O) was then subjected to in-gel tryptic digestion and LC-MS/MS analysis. The tryptic digest MS/MS results show that 10 specific lysine residues, K68, K75, K89, K114, K122, K130, K132, K134, K154, and K202, were involved in the acetylation (Table S, $n=2$, data were confirmed with two batches of matrix preparations), suggesting that in vitro acetylation of matrix SOD2 with Ac_2O restored the hyperacetylated profile observed in the cytosolic SOD2.

Since acetylation of mitochondrial proteins may be caused by a reaction of lysine residues with acetyl-CoA in a non-enzymatic process, in vitro acetylation of SOD2 was evaluated by incubating the matrix preparation with acetyl CoA (5 mM) at 37 °C for 20 min. As indicated in Figure 2B, acetylation of matrix SOD2 was modestly enhanced by acetyl CoA. In-gel tryptic digestion and LC-MS/MS analysis of acetyl-CoA-treated matrix SOD2 indicated additional acetylation at the residues of K75 and K134 (data not shown).

3.5 In vitro acetylation with Ac₂O decreases matrix SOD2 activity

The enzymatic activity of matrix SOD2 from the SOD2-tg heart was assayed by EPR spin trapping with DEPMPO and by measuring the ability of matrix SOD2 to dismutate the $\bullet\text{O}_2^-$ generated by mitochondrial SCR (succinate-cytochrome *c* reductase, a supercomplex hosting complex II and complex III). The generation of $\bullet\text{O}_2^-$ was mediated by mitochondrial SCR in the presence of succinate as the electron donor (Figure 3A), and the $\bullet\text{O}_2^-$ was trapped by DEPMPO as reported by our previous publications [15, 17]. A multiline EPR spectrum was produced that was characteristic of DEPMPO/ $\bullet\text{OOH}$ adduct (Figure 3A, *solid line*) based on the hyperfine coupling constants (isomer 1: $a^N=13.14$ G, $a^H=11.04$ G, $a^H=0.96$ G, $a^P=49.96$ G (80% relative concentration); isomer 2: $a^N=13.18$ G, $a^H=12.59$ G, $a^H=3.46$ G, $a^P=48.2$ G (20% relative concentration)) obtained from the computer simulation [15, 17, 18] (Figure 3A, *dashed line*). The activity of SOD2 was analyzed quantitatively by measuring the inhibition of SCR-mediated $\bullet\text{O}_2^-$ generation by DEPMPO trapping. In the presence of the matrix preparation (MxP in Figure 3), the formation of DEPMPO/ $\bullet\text{OOH}$ was inhibited by $50.1 \pm 3.3\%$ ($n=3$, data were collected from the average of three repeats) based on the spin quantitation of the simulated spectrum (Figure 3B, *dashed line*; Figure 3E; Figure 3F). Enhancing acetylation of matrix SOD2 with 100 μM Ac₂O inhibited the formation of DEPMPO/ $\bullet\text{OOH}$ by only $44.0 \pm 0.7\%$ ($n=3$, Figure 3C, Figures 3E–3F), indicating a decrease in SOD2 activity. Further augmenting the acetylation of matrix SOD2 with 1 mM Ac₂O inhibited the $\bullet\text{O}_2^-$ generation by only $13.9 \pm 1.1\%$ ($n=3$, Figure 3D, Figures 3E–3F), confirming that in vitro acetylation reduced SOD2 activity in converting $\bullet\text{O}_2^-$ to H₂O₂.

3.6 SOD2 in the wild-type murine heart

The cytosolic and matrix preparations from the myocardium of wild-type mice were further subjected to immunoblotting with anti-SOD2 and anti-AcK antibodies. As indicated in Figure 4A, negligible and insignificant Western signal from SOD2 in the cytosol could be detected by either anti-SOD2 or anti-AcK antibodies. However, substantial Western signal from acetylated SOD2 was detected in the matrix SOD2 of the wild-type murine heart. LC-MS/MS analysis indicated that only one specific lysine residue, K122, was acetylated (tryptic acetylated peptide, ¹¹⁵GELLEAIK_{Ac}R¹²³, $m/z = 535.8138^{2+}$, Table 3, and $n=2$, data were confirmed by analysis from two batches of matrix preparation).

3.7 Downregulation of sirtuin 3 (Sirt3) deacetylase in the mitochondria of the SOD2-tg murine heart

Protein expression of Sirt3 deacetylase from the mitochondria of wild-type and SOD2-tg hearts was probed by immunoblotting using a monoclonal antibody against Sirt3 (Cell Signaling Technology, Danvers, MA, 1:500, catalog number: #5490 (D22A3)). It was detected that Sirt3 deacetylase was present in the compartments of both inner membrane and

matrix (data not shown). It was further observed that Sirt3 expression in the mitochondria was significantly downregulated by transgenic overexpression of SOD2 (reduction by $46.6 \pm 2.6\%$, Figure 4B). The enzymatic activity of sirtuin deacetylase was assayed using an acetylated tetrapeptide (Gln-Pro-Lys-Lys(Ac), amino acids 317–320 of p53) as a substrate; a lower enzymatic activity of the deacetylase (77.5 ± 10.9 for SOD2-tg vs 129.7 ± 9.9 for wild type) was detected in the mitochondria of SOD2-tg murine hearts (Figure 4B). The results supported the idea that SOD2 acetylation is enhanced as a result of transgenic overexpression of SOD2.

3.8 Detection and protein acetylation of SOD2 from the mitochondrial inner membrane of the SOD2-tg murine heart

The inner membrane preparation of mitochondria (submitochondrial particles, SMP) was subjected to SDS-PAGE, followed by immunoblotting with anti-SOD2 and anti-acetyllysine antibodies. As indicated in Figure 4A, the detected SOD2 from the SMP of the SOD2-tg myocardium (tgSOD2-SMP) was acetylated. However, SOD2 was not detected in the SMP of the myocardium from wild-type controls (wt-SMP), indicating that SOD2 precursors were arrested within the mitochondrial inner membrane of the SOD2-tg murine heart.

3.9 Function of the SOD2 arrested in the mitochondrial inner membrane of the SOD2-tg murine heart

The level of function of the SMP was analyzed by EPR spin trapping with DMPO. As shown in Figure 4C, $\cdot\text{O}_2^-$ generation mediated by wt-SMP (1 mg/ml) was induced by NADH (0.5 mM) and was completely inhibited by external addition of SOD1 (spectra *b* and *f*). EPR spin trapping further indicated that the $\cdot\text{O}_2^-$ generation mediated by the SMP isolated from the SOD2-tg myocardium (tgSOD2-SMP in spectrum *d*) was significantly reduced, by $29.7 \pm 3.4\%$ ($n=4$, data were collected from the average of four batches of SMP preparation), as determined by spin quantitation.

The ratio of the amount of SOD2 in the tgSOD2-matrix to that in tgSOD2-SMP was 8.7 ± 0.6 to 1 ($n=4$) as measured by immunoblotting using an anti-SOD2 Ab (Figure 4D). The relative enzymatic activities of SOD2 from the SMP and the matrix were measured by the ability to dismutate SCR-mediated $\cdot\text{O}_2^-$ using EPR spin trapping with DEPMPO (Figure 4E). As indicated in Figure 4E, SCR-mediated $\cdot\text{O}_2^-$ production was diminished in the matrix preparation by up to $74.7 \pm 1.6\%$ ($n=4$), whereas SMP containing an equal amount of SOD2 only decreased SCR-mediated $\cdot\text{O}_2^-$ production by $10.8 \pm 1.5\%$ ($n=4$), suggesting that the specific activity of SOD2 in the matrix is approximately 7-fold higher than that of SOD2 in SMP.

3.10 LC-MS/MS analysis of protein lysine acetylation in the SMP SOD2

The protein band of the SOD2 from tgSOD2-SMP (indicated by an arrow in the left side of SDS-PAGE gel in Figure S4A) was subjected to in-gel digestion with trypsin and analyzed by LC-MS/MS. Nearly the entire amino acid sequence of SMP SOD2 was identified. The MS/MS results of the tryptic digest revealed 3 acetylated peptide fragments as indicated in Table 4. Further analysis explicitly confirmed that the residues of K68, K122, and K130 in the SOD2 from tgSOD2-SMP were involved in site-specific acetylation ($n=2$, data were

confirmed by analysis of two batches of SMP preparation). It is worth noting that acetylation of K68, K122, and K130 was also detected in the matrix SOD2 (Table 2).

3.11 Detection of the signaling peptide in the SMP SOD2 and matrix SOD2 from the SOD2-tg murine heart

We further detected a doubly protonated ion ($M+2H$)²⁺ with $m/z = 637.3672$ from the LC/MS of the tryptic digest of SOD2 from the tgSOD2-SMP. As indicated in Figure 4F, further MS/MS analysis indicated the amino acid sequence of the detected doubly protonated ion to be ¹²QLAPVLGYLGSR²³, which corresponds to a portion of the N-terminal signaling and import sequence (aa 1–24 in Figure S1) of the SOD2 precursor.

Matrix preparations from the SOD2-tg mouse heart were subjected to native PAGE analysis using 4–16% gradient Bis-Tris gel, followed by Coomassie blue staining. The major protein bands of the matrix preparation were confirmed by LC-MS/MS to be SOD2 (the gel bands used for LC-MS/MS analysis are indicated by the arrows of Figure S4B). LC-MS/MS also identified the N-terminal signaling peptide ($m/z = 637.3672^{2+}$) in the SOD2 bands of native gel as seen in the detected signaling peptide from the SMP preparation (Figure 4F).

3.12 Protein aggregation of matrix SOD2 in the heart of the SOD2-tg mouse

Mitochondrial preparations from the hearts of SOD2-tg and wild-type littermates were subjected to SDS-PAGE and subsequent Coomassie blue staining. As indicated in Figures 1A and 5A, a protein band with higher molecular weight (indicated by the arrow of Figure 5A) was present in the mitochondrial matrix of the SOD2-tg heart but not in the hearts of wild-type littermates (Figures 1A and 5A), nor was it present in the cytosol and inner membrane of the SOD2-tg heart. The detected protein band with higher molecular weight was subjected to in-gel tryptic digestion followed by LC-MS/MS analysis, revealing the involvement of SOD2 (amino acid sequence coverage 98%) in the detected protein aggregates. LC-MS/MS analysis also identified the E1 enzyme mitochondrial α -ketoglutarate dehydrogenase (α KGD-E1) present in the same protein band as the SOD2 aggregates. The calculated molecular weights of α KGD-E1 and matrix SOD2 are 115,861 Da and 22,204 Da, respectively, thus indicating involvement of pentamer formation in the SOD2 aggregates. Further evidence showed that pentameric SOD2 is localized in the matrix compartment and recognized by the antibodies against SOD2 and acetyllysine (Figures 1A, 5A and 5B).

3.13 In vitro protein acetylation mediates deaggregation of pentameric SOD2

Protein acetylation of SOD2 pentamer was further detected by immunoblotting, as indicated in Figure 5B. LC-MS/MS analysis of the pentameric SOD2 aggregate revealed that the acetylated residues were K68, K122, K130, and K202 (Table 5, $n=2$, data were confirmed by analysis of two batches of matrix preparation), identical to those seen in the matrix SOD2 (Table 2). To determine the effect of enhancing acetylation on the pentameric formation of SOD2, the matrix preparation was subjected to in vitro acetylation with Ac₂O. Treatment of the matrix preparation with an equivalent amount of acetic acid (AcOH) was used as a control. As indicated in Figure 5C, an increasing dose of Ac₂O (200 μ M – 1.0 mM) gradually diminished the pentameric form of SOD2. Complete deaggregation of the SOD2

pentamer by Ac₂O was detected at the 500 μ M dose as indicated by Coomassie blue staining (Figure 5C, upper and middle panels) and immunoblotting with anti-SOD2 Ab (Figure 5C, lower panel), suggesting that in vitro acetylation with Ac₂O mediates deaggregation of the SOD2 pentamer.

3.14 Regulation of HSP 70 (GRP 75), HSP 60, matrix processing peptidase (MPP), presequence protease (PREP), and lon protease (LONM) in the murine SOD2-tg heart

Mitochondrial matrix samples prepared from wild-type and SOD2-tg mouse hearts were subjected to stable isotope dimethyl labeling for quantitative proteomics [19, 20] of HSP 70 (GRP75, 75 kDa glucose-regulated protein), presequence protease, lon protease, HSP 60, and MPP, which proteins mediates mitochondrial protein translocation and folding. Since the D-isotopomer and H-isotopomer of formaldehyde were used to label the matrix preparations of wild type and SOD2-tg, respectively, quantitation was thus based on the ratio of specific heavy dimethylated peptide and light dimethylated peptide (H/L ratio in Figure 6A–6C). Measurements indicated upregulation of HSP 70 (Figure 6A) and lon protease (Figure 6C) and downregulation of presequence protease (Figure 6B) in the mitochondria of SOD2-tg murine hearts. However, stable isotope dimethyl labeling failed to reveal quantitative information on HSP 60 and MPP.

The HSP 60, HSP 70, and MPP from mitochondrial preparations and tissue homogenates were further subjected to probing with immunoblotting. Western blotting detected significant downregulation of HSP 60 (the signal intensity of anti-HSP60/anti-complex IV was decreased by $25.3 \pm 6.4\%$, average of three repeats from three different batches of mitochondrial preparation, $n=3$, $*p<0.05$) in the mitochondria and increased accumulation of HSP 60 (the signal intensity of anti-HSP60/anti-catalase was increased by $18.5 \pm 6.7\%$ in the tissue homogenates of SOD2-tg, average of three repeats from three tissue homogenates of three mouse hearts, $n=3$, $*p<0.05$) and HSP 70 (the signal intensity of anti-HSP70/anti-catalase was increased by $40.9 \pm 14.1\%$ in the tissue homogenates of SOD2-tg, $n=3$, $**p<0.01$) in the cytosol of SOD2-tg heart (Figure 6D). However, the blot also indicated a moderate upregulation of HSP 70 (the signal intensity of anti-HSP70/anti-complex IV was marginally increased by $14.1 \pm 1.2\%$, $n=3$, $*p<0.05$) (Figure 6D) and MPP in the mitochondria and decreased accumulation of cytosolic MPP precursor in the SOD2-tg mouse heart (the ratios of MPP precursor in cytosol to mature MPP in mitochondria are 58.9 ± 28.1 for wild type and 4.8 ± 1.1 for SOD2-tg, data were collected from the average of six tissue homogenates of six mouse hearts, $n=6$, Figure 6E).

4. DISCUSSION

4.1 Protein acetylation of SOD2 in the SOD2-tg mouse heart

As revealed by LC-MS/MS analysis, lysine 122 was identified as the specific exclusively acetylated site of myocardial SOD2 from the wild-type mouse, implicating it in the modulation of SOD2 activity in mitochondria. Lysine 122 has been reported as an evolutionarily conserved lysine involved in Sirt3-mediated deacetylation of liver mitochondrial SOD2 under the calorie-restricted conditions of 36-h fasting [2]. Acetylation of lysine 122 was detected in both cytosolic SOD2 and matrix SOD2 of the SOD2-tg heart.

Acetylation of lysine 130 is also preserved after mitochondrial localization of SOD2 in the SOD2-tg heart (Table 1 and Table 2). The catalytic activity of SOD2 was affected by the mutation of K122 to glutamine (which mimics acetylation) but was not affected by mutation to arginine, as shown in the cellular system of mouse embryonic fibroblasts (MEF) [2, 21]. However, mutation of lysine 130 to glutamine has no effect on SOD2 activity [5]. The protein structure of mammalian SOD2 revealed that K122 is situated on the outside of a tetramer complex, where it may be easily acetylated or deacetylated to regulate SOD2 activity [21]. Further evidence indicated that acetylation of K122 and K130 was preserved in the SOD2 of SMP from the SOD2-tg heart (Table 4). It is likely that acetylation of SOD2 at K122 and K130 can functionally mediate the importation of its cytosolic precursor into mitochondria.

Similar results were also observed in the mutation of K68 to glutamine or arginine [5]. Lysine 68 acetylation was identified in human cells (HEK 293T cell line), and it has been reported as the key site that is linked to Sirt3-mediated SOD2 deacetylation and activation in human cells [5]. K68 acetylation of matrix SOD2 was not detected in the wild-type mouse heart (Table 3). However, K68 acetylation was preserved in the SOD2 of the matrix and SMP (Tables 1, 2, 4) in the SOD2-tg heart, likely due to downregulation of Sirt3 expression. These results support the report that K68 regulates SOD2 activity via Sirt3-mediated deacetylation [22], presumably because K68 is found in the middle of the N-terminal helical hairpin domain that hosts histidine 50 and histidine 98; these two histidines are responsible for coordinating the Mn^{3+} required for SOD2 activity [22] (Figure 7A).

Furthermore, the K75 residue in the matrix SOD2 is surface exposed and positioned in the same α -helix that hosts the K68 residue (Figure 7A). Acetylation of both K68 and K75 impacts the coordination of H50 and H98 and can subsequently impair enzymatic activity. Deacetylation of K75 was detected in the matrix SOD2 of SOD2-tg mouse hearts, thus progressively enhancing matrix SOD2 activity.

Together with K75, complete deacetylation of the K89, K114, K132, K134, and K154 residues was also observed in the SOD2 from the SMP and matrix compartments of the SOD2-tg heart (Tables 1, 2, and 4), suggesting that these residues are potential targets of deacetylation and may be involved in SOD2 activation.

Acetylation of the K53, K194, K202, K221, and K222 residues was not detected in the cytosolic SOD2. However, acetylation of K202 was detected in the matrix SOD2 of SOD2-tg hearts. It is likely that K202 acetylation of SOD2 in mitochondria is promoted by Sirt3 downregulation and mediated non-enzymatically by acetyl CoA. In the MEF cellular model, mutation of K202 to alanine has no effect on the enzymatic activity of SOD2 [23].

4.2 Hyperacetylation, regulation of cytosolic HSP 60 and HSP 70, and mitochondrial localization of SOD2 in the SOD2-tg myocardium

Nuclear-encoded mitochondrial matrix proteins are imported into mitochondria as unfolded protein. Proteins destined for the mitochondrial matrix are synthesized on free ribosomes in the cytosol and maintain an unfolded conformation by binding to HSP 70 chaperonins [24–26]. HSP 70 (GRP75) and HSP 60 are constitutively expressed in the cytosol and

mitochondria and are involved in various chaperoning functions in aiding the translocation of nascent polypeptides and the correct refolding of proteins from the cytoplasm into the mitochondrial matrix [24–28]. Upregulation of HSP 70 and HSP 60 chaperones (Figure 6D) in the cytosol of the SOD2-tg mouse heart was likely induced by reductive stress imposed by SOD2 overexpression, and they helped to stabilize and maintain the unfolded conformation and facilitated translocation of the excess SOD2 precursor through the mitochondrial membrane. Upregulation of HSP 70 in the mitochondria (Figures 6A, 6D) may further stabilize excess incoming SOD2 precursors and ratchet them through the membrane. Together with MPP upregulation in the mitochondria, they may expedite folding and tetramer assembly to activate excess mature SOD2 in the SOD2-tg heart.

As indicated by LC-MS/MS analysis, a profile of differential acetylation is present in the SOD2 from the cytosol and mitochondria of the SOD2-tg mouse heart. Moreover, the import signaling sequence of the N-terminal extension has been consistently detected in the SOD2 of SMP and matrix compartments (Figure 4F). Protein acetylation involving 9 lysine residues was detected in the cytosolic SOD2, whereas in the SMP SOD2 and matrix SOD2, protein acetylation involved only 3 to 4 lysine residues. The formation of a tetramer complex is involved in the quaternary structure of matrix SOD2. Therefore, hyperacetylation of cytosolic SOD2 presumably maintains the cytosolic SOD2 in an unfolded status, prevents it from forming tetramers in the cytosol, and facilitates import of nuclear-encoded SOD2 to the mitochondria.

The K89 acetylation was unequivocally detected in the cytosolic SOD2 (MS/MS spectrum in Figure 8A and Table 1). Based on the 3D structure of matrix SOD2 (PDB 2P4K), the K89 residue is buried inside the protein (Figure 7A), which is consistent with its critical role in enzymatic functioning and stabilization of the tertiary structure. In vitro acetylation of matrix SOD2 with Ac₂O enhanced K89 acetylation (Tables 1 and 3), diminishing the enzymatic activity (Figure 3). The K89 acetylation observed in cytosolic SOD2 is thus expected to destabilize tertiary structure and impair the activity of the enzyme. In vitro acetylation of matrix SOD2 enhanced both Ac₂O dose-dependent K89 acetylation and inactivation of SOD2 (Tables 2–3 and Table S). Acetylation of K89 thus destroys the tertiary structure and quaternary structure.

The X-ray structure further reveals that the K134 residue forms an intermolecular salt bridge with E155 to stabilize the quaternary structure of SOD2 (Figure 7B). In vitro acetylation of matrix SOD2 with Ac₂O enhanced K134 acetylation (Table 2 and Table S), suggesting that breakage of the salt bridge between K134 and E155 was required for Ac₂O-induced K134 acetylation. Therefore, acetylation of K134 of cytosolic SOD2 likely precludes the salt bridge formation and subsequently blocks quaternary structure. K134 acetylation was unambiguously detected in the cytosolic SOD2 (MS/MS spectrum in Figure 8B and Table 1), thus confirming its role in unfolding cytosolic SOD2.

Acetylation of the K154 residue of cytosolic SOD2 was not detected in the SOD2 of either the matrix or the SMP (Tables 1, 2, 4). The K154 acetylation of the matrix protein was considerably enhanced by in vitro acetylation (Table S). The E155 residue (residue of glutamic acid 155) can further form an intramolecular charge interaction with K154 to

stabilize the loop required for SOD2 tertiary structure (Figure 7B). Acetylation of K154 (MS/MS spectrum in Figure S2-g) in cytosolic SOD2 presumably breaks the loop formation, destabilizing the tertiary structure required for folding, and inhibiting quaternary structure formation.

Since hyperacetylation of K89, K134, and K154 potentially alters the tertiary structure for protein folding and the quaternary structure of mature SOD2 in mitochondria, hyperacetylation of K89, K134, and K154 is thus expected to diminish the enzymatic activity of SOD2 and maintain its unfolded status in the cytosol. This conclusion was supported by the EPR assay in Figure 3, which showed that in vitro acetylation of the matrix preparation with Ac₂O partially diminished SOD2 activity in a dose-dependent manner, presumably due to acetylation-induced alteration of the structure and conformation of matrix SOD2 in vitro.

4.3 SOD2 hyperacetylation, Sirt 3 downregulation, mitochondrial HSP 60, presequence protease, lon protease, aggregation and deaggregation of SOD2 pentamer, and physiological relevance related to cardiac hypertrophy in the murine SOD2-tg heart

SOD2 overexpression down-regulates the Sirt 3 deacetylase in mitochondria was likely caused by a negative feedback mechanism involved in Foxo3a (a member of the family of forkhead transcription factors) downregulation [29] and a highly reductive status of the SOD2-tg heart [9]. It has been reported that Sirt 3-mediated deacetylation promotes nuclear localization of Foxo3a, leading to increased transcription of Foxo3a-dependent MnSOD2 gene, thus reducing ROS level in myocytes [29]. We have consistently detected the level of mRNA expression in the SOD2-tg mouse heart was decreased by $22.7 \pm 5.6\%$ for Foxo3a (average of six mouse hearts, $n=6$, $p<0.01$), and by $19.0 \pm 7.5\%$ for Sirt 3 (average of six mouse hearts, $n=6$, $p<0.05$). Thereby transgenic overexpression of SOD2 dramatically reduced ROS and subsequently negatively regulated Sirt 3 and Foxo3a. A highly reductive *milieu* with low NAD⁺/NADH ratio in mitochondria may further contribute to downregulation of the Sirt 3 activity in the SOD2-tg mouse heart.

The physiological relevance of Sirt 3 downregulation and mitochondrial amyloidosis associated with SOD2 aggregation is likely inferred as part of mechanistic insights for hypertrophic development in the mouse heart of SOD2-tg. The mouse model of Sirt 3 deficiency has been reported to develop cardiac hypertrophy at 8 weeks age, and transgenic overexpressing Sirt 3 in the mouse heart effectively blocked agonist-mediated cardiac hypertrophy [29, 30]. Cardiac amyloidosis is characterized by deposition of misfolded protein and related protein aggregates in the heart tissue, and progressive increase in the thickness of cardiac wall [31]. Observation of SOD2-related amyloidosis in mitochondria may further contribute to hypertrophic growth.

It is likely that SOD2 pentameric aggregation was caused by excess accumulation of misfolded protein in the matrix compartment. HSP 60 is an intramitochondrial molecule known to chaperone nascent polypeptides for their transport from the cytoplasm to the mitochondrial matrix [28, 32]. The mitochondrial localization of HSP 60 preserves its classical chaperone function as a refoldase and is critically involved in binding and catalysis of folding of newly synthesized proteins destined for the mitochondrial matrix [24, 28, 33,

34]. Presequence protease is an ATP-independent protease that degrades mitochondrial transit peptides and other unstructured polypeptides [35, 36]. Presequence protease is able to degrade amyloid A4 protein when it accumulates in the mitochondria of brain [37]. Downregulation of HSP 60 and presequence protease (Figures 6B and 6D) in mitochondria likely facilitated the formation of misfolded SOD2 and the process of SOD2 aggregation in the heart of the SOD2-tg mouse. Mouse lon protease (LONM) is an ATP-dependent serine protease that mediates the selective degradation of misfolded, unassembled or damaged proteins in the mitochondrial matrix [38, 39]. It has been documented that lon protease is highly inducible and upregulated to provide increased protection under conditions of acute stress [40]. Thereby, upregulation of mouse lon protease (Figure 6C) likely acts as a feedforward response to excess accumulation of misfolded SOD2 and reductive stress in the SOD2-tg mouse heart.

In vitro hyperacetylation with Ac₂O promoted deaggregation of the SOD2 pentamer (Figure 5). Protein aggregation of the SOD2 precursor was not observed in the cytosolic compartment in SOD2-tg animals, which correlates well with the results of hyperacetylation-induced deaggregation of SOD2 pentamer in vitro (Figure 5). In comparison with the myocardial SOD2 of wild-type mouse, downregulation of Sirt3 in the SOD2-tg mouse heart moderately increased lysine acetylation of SOD2, which may promote the formation of misfolded SOD2 as a seed for aggresome formation under the milieu of reductive stress, thus provoking physiological hypertrophic growth.

Hypertrophic growth is a primary mechanism through which the heart normalizes ventricular wall stress. It is characterized by enhanced protein synthesis and an increase in the size and organization of cardiomyocyte sarcomeres. Aggregates of misfolded proteins have been observed and marked in the phenotype of pathological hypertrophy as a result of pressure overload [41]. Furthermore, it has been shown that transgenic mice overexpressing the cardiac-specific human mutant α B-crystallin (hR120GCryAB) developed protein aggregation disease and pathological hypertrophy associated with the mechanism of imposed reductive stress [42, 43]. Current studies, together with a previous report [9], have revealed that the phenotype of hypertrophy in the SOD2-tg murine heart is linked to Sirt3 downregulation, aggresome formation, and reductive stress as imposed by SOD2 overexpression in the mouse heart. It is likely that SOD2-associated aggresomes in mitochondria are not cytotoxic, nor are they related to pathogenesis. SOD2 aggresomal formation may represent an attempt by the mitochondria to sequester potentially cytotoxic, misfolded proteins from the matrix compartment, as suggested by the publication of Robbins' group [44]. In support of this hypothesis, preventing aggresome formation actually resulted in increased cytotoxicity in myocytes expressing mutant α B-crystallin as reported by Sanbe et al. [45].

Together with the observations of differential acetylation, upregulation of cytosolic HSP60 and HSP70, the underlying mechanism of current studies facilitates mitochondrial localization of excess cytosolic SOD2, leading to enhanced bioenergetic efficiency, improved metabolic dilation, and consequent supernormal cardiac function in the SOD2-tg mouse heart [9].

5. CONCLUSION

The present studies provide insights regarding the differential acetylation of SOD2 in the cytosol and mitochondria resulting from cardiac-specific overexpression of SOD2. The data indicate a mechanism of differential acetylation with manifold regulations that mediates the importation of nuclear-encoded SOD2 into mitochondria and the formation of SOD2-derived pentameric aggregates in the matrix. As illustrated in Figure 9, the underlying process was uncovered through multiple observations, including hyperacetylation with HSP 70 upregulation helping to maintain an unfolded conformation in the cytosol, upregulation of both cytosolic HSP 60 and HSP 70 playing a role in translocation of unfolded protein through the inner membrane compartment, Sirt3 downregulation and partial deacetylation of the unfolded protein, protein folding for tetramer formation and activation, and misfolded SOD2 formation assisted by downregulation of mitochondrial HSP 60 and PREP for pentameric aggregates. Recognition of this mechanism is valuable in understanding the fundamental basis for how acetylation is involved in the importation of nuclear-encoded SOD2 into mitochondria and how deacetylation mediates the aggresomal formation of misfolded protein in the SOD2-tg mouse heart.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

SOD2	mitochondrial superoxide dismutase 2
MnSOD	manganese-dependent superoxide dismutase
AcK	acetyllysine
SCR	succinate cytochrome c reductase, a supercomplex hosting complex II and complex III
SQR	succinate ubiquinone reductase, or mitochondrial Complex II
QCR	ubiquinol-cytochrome c reductase, or complex III
SMP	submitochondrial particles or mitochondrial inner membrane preparation
$\cdot\text{O}_2^-$	superoxide anion radical
ROS	reactive oxygen species
ETC	electron transport chain
Sirt3	mitochondrial NAD ⁺ -dependent deacetylase sirtuin 3

DEPMPO	5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide
DMPO	5,5-dimethyl pyrroline N-oxide
Ab	antibody
SDS-PAGE	SDS polyacrylamide gel electrophoresis
EPR	electron paramagnetic resonance
Ac₂O	acetic anhydride
PBS	phosphate-buffered saline
HSP	heat shock protein
PREP	presequence protease

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Figure 1. Immunoblotting [with anti-SOD2 and anti-acetyllysine (anti-AcK) Abs] and Coomassie staining of the proteins from the mitochondrial, cytosolic and matrix preparations of the SOD2-tg mouse heart

Hearts were removed from cardiac-specific SOD2-tg and subjected to mitochondrial and cytosolic separation. The matrix was further isolated from the mitochondria according to the method described in the "Experimental Procedures". Samples (mitochondrial and cytosolic preparations) were subjected to SDS-PAGE under reducing conditions in the presence of DTT using 4–12% Bis-Tris polyacrylamide gradient gel followed by immunoblotting. **A**, In the left panel, mitochondria (from wild-type and SOD2-tg mouse hearts, 100 μ g loaded) and cytosol (100 μ g loaded) were immunoblotted with anti-SOD2 polyclonal Ab (1:4000, from Santa Cruz Biotechnology Inc.). In the right panel, both mitochondria and cytosol were stained with Coomassie blue. The SOD2 aggregate with a higher molecular weight is indicated by an arrow. **B**, In the left panel, cytosol (100 μ g loaded) and matrix (10 μ g loaded) were stained with Coomassie blue. In the right panel, both cytosol (50 μ g loaded) and matrix (50 μ g loaded) were immunoblotted using anti-AcK monoclonal Ab (1:1000, from Cell Signaling Technology). The exposure time of the X-ray film for ECL detection in **B** (for anti-AcK Ab) is approximately 9 min. (data represent six repeats from six preparations of six mouse hearts, $n=6$)

Figure 2. In vitro protein lysine acetylation of SOD2 by acetyl anhydride (in A) and acetyl CoA (in B)

Protein (2 mg/ml) from the matrix preparation was incubated with various concentrations (0.1–0.2 mM) of acetic anhydride (Ac₂O, dissolved in DMSO, in *A*) or 5 mM of acetyl CoA (in *B*) at room temperature for 20 min. The matrix preparation was treated with acetic acid (AcOH, 0.2 mM in *A*) as a control. The protein (15 µg loaded) was then subjected to SDS-PAGE (4–12% Bis-Tris polyacrylamide gradient gel and reducing conditions with DTT) and immunoblotting with anti-AcK monoclonal Ab ($n=3$, data were confirmed by three batches of matrix preparations). The exposure time of the X-ray film for ECL detection in *A* was less than 1 min, and approximately 3 min in *B*.

Figure 3. The effect of Ac₂O-enhanced SOD2 hyperacetylation in the matrix preparation on •O₂⁻ production by succinate-energized SCR as measured by EPR spin trapping with DEPMPO

The matrix preparation was treated with Ac₂O (0.1 mM and 1 mM, respectively) to enhance SOD2 acetylation as described in the legend of Figure 2. The mixture was dialyzed against 50 mM Tris-Cl buffer (pH 8.0) to remove excess Ac₂O at the end of incubation. The matrix preparation (5 µg/ml) was then subjected to the EPR assay of SOD2 activity in reducing the DEPMPO/•O₂⁻ spin adduct as described in the “Experimental Procedures”. **A-D**, the computer simulations (dashed lines) superimposed on the experimental spectra (solid lines); **E**, the spectra obtained from single integration of simulated spectra A-D; **F**, the spectra obtained from double integration of simulated spectra A-D. *SCR* denotes succinate-cytochrome *c* reductase; *Suc.* denotes succinate; *MxP* denotes matrix preparation; *AcMxP* denotes acetylated matrix preparation obtained from Ac₂O treatment (0.1 mM Ac₂O used in C and 1 mM Ac₂O in D). Inset, spin quantitation of DEPMPO/•OOH adduct generation mediated by SCR and the effect of MxP and AcMxP ($n=3$, data were collected from the average of three repeats of three matrix preparations. *** $p < 0.001$ and * $p < 0.05$).

F

m/z: 637.3672
 Charge: +2
 Theoretical m/z: 637.3668
 Error: +0.50 ppm

Figure 4. Detection and functional analysis of Sirt3 deacetylase and the SOD2 in the SMP from the mitochondria of wild type and SOD2-tg murine hearts

Hearts were removed from cardiac-specific SOD2-tg and wild type mice, and subjected to mitochondrial and cytosolic preparations. The fractions of matrix and SMP were further prepared from the mitochondria as described in the “Experimental Procedures”. **Panel A**, matrix (100 µg loaded), SMP (100 µg loaded), and cytosol (100 µg loaded) were immunoblotted with anti-SOD2 polyclonal antibody (1:1000) and anti-AcK monoclonal antibody (1:500) (data represent three repeats from three mouse groups, $n=3$). **Panel B**, mitochondrial preparations (100 µg loaded) were subjected to probing with a monoclonal antibody against Sirt3 (1:500, from Cell Signaling Technology), a monoclonal antibody against the subunit I of complex IV (anti-COX I, 1:2000, Santa Cruz biotechnology, catalog number: sc-58347 (1D6)), and a polyclonal antibody against SOD2. The ratio of signal intensity, anti-Sirt3/anti-COX I, was quantitated by NIH ImageJ software. The mitochondrial preparations were then subjected to an assay of Sirt3 deacetylase activity as described under Experimental Procedure (average of six mitochondrial preparations from six mouse groups,

$n=6$, *** $p<0.001$, ** $p<0.01$). **Panel C**, SMP preparations (final concentration at 1 mg/ml) of wild-type (wt-SMP) and SOD2-tg (tgSOD2-SMP) mouse hearts were incubated with a reaction mixture containing respiration buffer, DTPA (1 mM), NADH (0.5 mM), and DMPO (90 mM) to initiate $\bullet\text{O}_2^-$ generation at 30 °C for 4 min prior to EPR measurement. The produced $\bullet\text{O}_2^-$ was trapped by DMPO to give a four-line spectrum of the SOD1-inhibitable DMPO/ $\bullet\text{OH}$ adduct. **Panel D**, measurement of relative amount of SOD2 in the SMP (100 μg loaded) and matrix (15 μg loaded) prepared from isolated mitochondria of the SOD2-tg myocardium by immunoblotting using anti-SOD2 polyclonal antibody. The signal intensity of the blots was quantitated by NIH ImageJ software (data were collected from four batches of SMP and matrix preparations, $n=4$, *** $p<0.001$). **Panel E**, the effect of tgSOD2-SMP (20 $\mu\text{g}/\text{ml}$) and matrix preparation (tgSOD2-matrix, 2 $\mu\text{g}/\text{ml}$) on the $\bullet\text{O}_2^-$ production by succinate-energized SCR as measured by EPR spin trapping with DEPMPO. The upper panel is the spin quantitation based on double integration of simulated spectra (data were collected from four batches of SMP preparations, $n=4$, ** $p<0.01$ and *** $p<0.001$). **Panel F**, MS/MS of the doubly protonated molecular ion of the signaling peptide ($^{12}\text{QLAPVLGYLGSR}^{23}$) of SOD2. *The sequence-specific ions are labeled y and b ions on the spectrum, and the ions of a_2 and a_5 denote $b_2\text{-CO}$ and $b_5\text{-CO}$, respectively.* Note: SDS-PAGE in **A**, **B**, **D** was running under reducing conditions in the presence of DTT.

Figure 5. Detection of the pentameric aggregates of SOD2 in the mitochondria of the SOD2-tg murine heart and hyperacetylation-induced deaggregation of SOD2 pentamer

Panel A, left panel: protein staining of mitochondria isolated from wild type and SOD2-tg mice hearts with Coomassie blue (100 μg protein loaded). Middle panel: protein staining of matrix (100 μg protein loaded) and SMP preparations (100 μg protein loaded) from the mitochondria of SOD2-tg myocardium and of recombinant human SOD2 (hrSOD2, purchased from Abcam, 4 μg loaded) from *E. coli* with Ponceau S; the monomer and pentameric aggregates of SOD2 are identified by arrows. Right panel: immunoblotting of

hrSOD2 and matrix preparation of SOD2-tg with anti-SOD2 antibody. **Panel B**, immunoblotting of mitochondrial preparations (100 µg protein loaded) from wild type and SOD2-tg mouse myocardium with anti-AcK monoclonal antibody. **Panel C**, Dose-dependent Ac₂O-induced deaggregation of SOD2 pentamer. Matrix preparations were incubated with various doses (0.2 mM–1 mM) of Ac₂O and AcOH (as the control for Ac₂O treatment, 0.4 mM–2 mM) at 25 °C for 20 min. Samples (60 µg protein loaded for each lane) were subjected to SDS-PAGE (4–12% Bis-Tris polyacrylamide gradient gel under reducing conditions in the presence of DTT) followed by Coomassie blue staining (data represent two repeats from two batches of matrix preparation, *n*=2). Lower panel shows immunoblotting of the SOD2 pentamer protein band using anti-SOD2 antibody. *Note, no protein acetylation detected in the recombinant human SOD2.*

Figure 6. Regulation of HSP 60, HSP 70 (GRP 75), presequence protease, lon protease, and MPP in the SOD2-tg mouse heart

A–C, Stable-isotope dimethyl labeling (SIDML) for quantitative proteomics of HSP 70, presequence protease, and lon protease: mitochondrial matrix preparations (30 µg) from the wild-type and SOD2-tg mice were subjected to in-solution trypsin digestion at 37 °C for 12 h. Mixtures of tryptic peptides were then globally dimethyl labeled at the N-terminus and ε-amino group of lysine by formaldehyde (HCHO for wild type and DCDO for SOD2-tg) and subjected to reductive amination with sodium cyanoborohydride (NaBH₃CN) prior to

LC-MS/MS and MaxQuant software analysis [19, 20, 46]. The labeling strategy produced peaks differing by 28 mass units for each derivatized site relative to its non-derivatized counterpart and 4 mass units for each derivatized isotopic pair. *The H/L ratio indicated in each spectrum represents the average from duplicate SIDML experiments from two batches of matrix preparation and MS measurements (n=2).* **D-E**, mitochondria (100 µg) and tissue homogenates (100 µg) of wild-type and SOD2-tg mouse myocardium were subjected to SDS-PAGE under reducing conditions in the presence of DTT, followed by immunoblotting with the monoclonal antibodies against HSP 70 and HSP 60 (1:2000, Santa Cruz Biotechnology, catalog number: sc-133137 (D-9) and sc-376261 (F-9)) in **D** and a polyclonal antibody against MPP (1:20, Santa Cruz Biotechnology, catalog number: sc-160672 (T-15)) in **E**. Blotting with anti-catalase antibodies was used as a loading control for tissue homogenates, and blotting with anti-complex IV (in **D**) and anti-complex II (in **E**) was used as a loading control for mitochondria.

Figure 7. The three-dimensional structure of human SOD2 tetramer (PDB: 2P4K) showing the location of the manganese center (Mn, blue balls), the substrate of O_2^- (in dotted red ball); K68, K75, K89, K114, K122, K130, K132, K134, K154, K202, E155, and three of four Mn coordinates: H50, H98, D183

A, Structural portion displaying N-terminal hairpin domain (cyan ribbon) hosting K68, K75, K89, H50, H98, and the manganese center. **B**, Structural portion showing (i) the salt bridge formation between K134 in one subunit (cyan ribbon) and E155 in the adjacent identical subunit (magenta ribbon), (ii) charge interaction between K154 and E155 in the same subunit to stabilize loop conformation (magenta ribbon).

underline indicates Lys-89) of SOD2 from the cytosolic preparation of SOD2-tg murine heart mitochondria; *B*, MS/MS of the triply protonated molecular ion of the acetylated peptide ($^{133}\text{E}\underline{\text{K}}(\text{Ac})\text{LTAASVGVQGSGWGLGFNK}^{154}$, where the underline indicates Lys134). The sequence-specific ions are labeled *y* and *b* ions on the spectrum. The amino acid involved in acetylation is identified by Ac in parentheses.

Figure 9. Diagram showing the effect of differential SOD2 acetylation, Sirt 3 downregulation, regulation of HSP 60, HSP 70, presequence protease (PREP), lon protease (LONM), and matrix processing peptidase (MPP) in mediating import of nuclear-encoded SOD2 into mitochondria, unfolded/folded status of SOD2, and protein aggregation of SOD2 in the hearts of SOD2-tg mice

An unfolded conformation of SOD2 is maintained in the cytosol through protein hyperacetylation involving nine specific lysine residues and upregulation of HSP 70 and HSP 60. Together with HSP 60 and HSP 70, the hyperacetylation-dependent unfolding conformation further mediates translocation of cytosolic SOD2 across the outer and inner membranes (*route 1*). It is suggested that deacetylation of K75, K89, K114, K132, K134, and K154 of SOD2 in the inner membrane is initiated on the matrix side of the inner membrane and acetylation of K202 occurs in the matrix compartment (*routes 2 and 3*). The majority of deacetylated/unfolded protein is properly folded *in situ* (*route 3 and enclosed red box*) and assembled into a tetramer to activate SOD2 (*route 4*). The above process (*routes 3 and 4*) is facilitated by upregulation of both MPP and HSP 70. A minority of unfolded protein (*enclosed blue box*) in the matrix is misfolded, leading to aggregate formation (*routes 5 and 6*). Formation of misfolded SOD2 (*route 2 and 5*) can be aided by downregulation of HSP 60 and PREP, which may further promote pentameric aggregation (*route 6*). Increased formation of misfolded SOD2 stimulates upregulation of lon protease, which may improve selective degradation of misfolded protein and decrease further aggresomal formation.

Table 1

Summary of the peptide sequences and corresponding acetylated lysines obtained from MS/MS analysis of tryptic digest of cytosolic SOD2 precursor from the SOD2-tg mouse heart

Amino Acid Sequence	Theoretical m/z	Observed m/z	Acetylated lysine
54HHAAYVNNLNVTEEK(Ac)YQEALAK75	862.0979 ³⁺	862.1002 ³⁺	K68
69YQEALAK(Ac)GDVTAQIALQPALK89	757.4179 ³⁺	757.4195 ³⁺	K75
54HHAAYVNNLNVTEEKYQEALAK(Ac)GDVTAQIALQPALK89	798.8240 ⁵⁺	798.8240 ⁵⁺	
76GDVTAQIALQPALK(Ac)FNGGGHINHSIFWTNLSPNGGGEPK114	1022.0212 ⁴⁺	1022.0246 ⁴⁺	K89
90FNGGGHINHSIFWTNLSPNGGGEPK(Ac)GELLEAIK122	883.9457 ⁴⁺	883.9499 ⁴⁺	K114
90FNGGGHINHSIFWTNLSPNGGGEPK(Ac)GELLEAIKR123	738.5782 ⁵⁺	738.5817 ⁵⁺	
115GELLEAIK(Ac)R123	535.8139 ²⁺	535.8147 ²⁺	K122
123RDFGSFDK(Ac)FK132	566.7691 ²⁺	566.7709 ²⁺	K130
124DFGSFDK(Ac)FKEK134	695.3379 ²⁺	695.3389 ²⁺	
124DFGSFDKFK(Ac)EK134	695.3379 ²⁺	695.3388 ²⁺	K132
133EK(Ac)LTAASVGVQGSGWGWLGFNK154	778.7341 ³⁺	778.7370 ³⁺	K134
135LTAASVGVQGSGWGWLGFNK(Ac)ER156	788.0695 ³⁺	787.0720 ³⁺	K154

Table 2

Summary of the peptide sequences and corresponding acetylated lysines obtained from MS/MS analysis of tryptic matrix SOD2 from the SOD2-tg mouse heart

Amino Acid Sequence	Theoretical m/z	Observed m/z	Acetylated lysine
54HHAAYVNNLNVTEEK(Ac)YQEALAK75	862.0979 ³⁺	862.1008 ³⁺	K68
	646.8252 ⁴⁺	646.8282 ⁴⁺	
115GELLEAIK(Ac)R123	535.8139 ²⁺	535.8136 ²⁺	K122
	357.5450 ³⁺	357.5449 ³⁺	
124DFGSFDK(Ac)FKEK134	695.3379 ²⁺	695.3375 ²⁺	K130
	463.8944 ³⁺	463.8933 ³⁺	
195NVRPDYLK(Ac)AIWNVINWENVTER216	924.4803 ³⁺	924.4834 ³⁺	K202

Table 3

A single peptide sequence and corresponding acetylated lysine (K122) obtained from MS/MS analysis of tryptic digest of mitochondrial SOD2 from wild-type mouse heart

Amino Acid Sequence	Theoretical m/z	Observed m/z	Acetylated lysine
115GELLEAIK(Ac)R123	535.8139 ²⁺	535.8138 ²⁺	K122

Table 4

Summary of the peptide sequences and corresponding acetylated lysines obtained from MS/MS analysis of tryptic SMP SOD2 from the SOD2-tg mouse heart

Amino Acid Sequence	Theoretical m/z	Observed m/z	Acetylated lysine
54HHAAYVNNLNVTEEK(Ac)YQEALAK75	862.0979 ³⁺	862.0961 ³⁺	K68
	1292.6432 ²⁺	1292.6397 ²⁺	
54HHAAYVNNLNVTEEK(Ac)YQEALAK75 (Deamidation on N)	862.4310 ³⁺	862.4317 ³⁺	
	1293.1437 ²⁺	1293.1396 ²⁺	
115GELLEAIK(Ac)R123	535.8139 ²⁺	535.8129 ²⁺	K122
124DFGSFDK(Ac)FK132	566.7709 ²⁺	566.7687 ²⁺	K130
124DFGSFDK(Ac)FKEK134	695.3379 ²⁺	695.3370 ²⁺	

Table 5

Summary of the peptide sequences and corresponding acetylated lysines obtained from MS/MS analysis of tryptic SOD2-Derived pentameric aggregate from the SOD2-tg mouse heart

Amino Acid Sequence	Theoretical m/z	Observed m/z	Acetylated lysine
54HHAAYVNNLNVTEEK(Ac)YQEALAK75	646.8252 ⁴⁺	646.8258 ⁴⁺	K68
115GELLEAIK(Ac)R123	535.8139 ²⁺	535.8140 ²⁺	K122
124DFGSFDK(Ac)FKEK134	463.8944 ³⁺	463.8945 ³⁺	K130
195NVRPDYLK(Ac)AIWNVINWENVTER216	924.4803 ³⁺	924.4829 ³⁺	K202