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## Role of Copper,Zinc-Superoxide Dismutase in Catalyzing Nitrotyrosine Formation in Murine Liver

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### Abstract

The solely known function of Cu,Zn-superoxide dismutase (SOD1) is to catalyze the dismutation of superoxide anion into hydrogen peroxide. Our objective was to determine if SOD1 catalyzed murine liver protein nitration induced by acetaminophen (APAP) and lipopolysaccharide (LPS). Liver and plasma samples were collected from young adult SOD1 knockout mice (SOD1<sup>-/-</sup>) and wild-type (WT) mice at 5 or 6 h after an ip injection of saline, APAP, or LPS. Hepatic nitrotyrosine formation was induced by APAP and LPS only in the WT mice. The diminished hepatic protein nitration in the SOD1<sup>-/-</sup> mice was not directly related to plasma nitrite and nitrate concentrations. Similar genotype differences were seen in liver homogenates treated with a bolus of peroxynitrite. Adding only the holo, but not the apo-SOD1 enzyme into the liver homogenates enhanced the reaction in an activity-dependent fashion and nearly eliminated the genotype difference at the high doses. Mass Spectrometry showed 4 more nitrotyrosine residues in bovine serum albumin and 10 more nitrated protein candidates in the SOD1<sup>-/-</sup> liver homogenates by peroxynitrite with added SOD1. In conclusion, the diminished hepatic protein nitration mediated by APAP or LPS in the SOD1<sup>-/-</sup> mice was due to the lack of SOD1 activity *per se*.

### Keywords

SOD1; Protein nitration; Acetaminophen; Peroxynitrite; Lipopolysaccharide; Mouse

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Although copper,zinc-superoxide dismutase (SOD1) has been widely considered a major intracellular antioxidant enzyme in mammals, its solely known function is to catalyze the conversion of superoxide anion into less active hydrogen peroxide. Indeed, SOD1 is protective against oxidative stress associated with acute paraquat toxicity, myocardial ischemia/reperfusion injury, and N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced dopaminergic neurodegeneration [1-3]. Mice deficient in SOD1 develop

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hepatocarcinogenesis [4] and retinal degeneration [5] in late life. However, overexpression of human SOD1 accelerated mitochondrial vacuolization, axonal degeneration, and premature motor neuron death in mice [6]. Knockout of SOD1 rendered mice resistant to acetaminophen (APAP)-mediated hepatotoxicity [7,8]. These paradoxical findings strongly suggest that SOD1 may exert functions more than just superoxide dismutation. In fact, SOD1 was shown to promote peroxynitrite-mediated nitrotyrosine formation *in vitro* [9,10].

Peroxyntirite is formed by a rapid diffusion-limited, radical-radical coupling reaction between nitric oxide (NO) and superoxide anion [11,12]. Peroxyntirite can directly react with target molecules such as glutathione via one or two-electron oxidations [13], or yield radical NO<sub>2</sub> via homolysis that nitrates tyrosine residuals of proteins into nitrotyrosine [14]. This particular chemical modification of tyrosine residues is called protein nitration [12,15]. With a potential in inactivating or altering functions of the modified proteins [16-18], protein nitration has been suggested as an early biomarker for the degenerations of motor neurons in amyotrophic lateral sclerosis (ALS) and of brains in Alzheimer disease [19-22]. It has also been proposed as a mediator of APAP-induced hepatotoxicity [23-28]. Coincidentally, alterations of SOD1 expression are related to Down Syndrome [29], ALS [30], and body resistance to acetaminophen toxicity [7,31].

Despite these pathological implications, the earlier observed *in vitro* catalytic role of SOD1 in nitrotyrosine formation [9,10] has been largely dismissed or neglected. There has been no experiment to test a cause-effect relationship between SOD1 and protein nitration *in vivo*. The extremely short biological half-life of peroxynitrite [32] and the lack of specific *in vivo* models have made such a test very difficult, if not impossible. The development of SOD1 knockout (SOD1<sup>-/-</sup>) mice [1] and the demonstration of several drugs such as APAP to induce protein nitration in tissues [24,25,33,34] provide us with an unprecedented opportunity to study the *in vivo* role of SOD1 in nitrotyrosine formation. Furthermore, the application of mass spectrometry allows identification of specific nitrated protein candidates in tissue homogenates and particular residues in selected proteins. Therefore, we used SOD1<sup>-/-</sup> and their wild-type (WT) mice and conducted a series of *in vivo* and *in vitro* experiments to determine: 1) if SOD1 knockout diminished hepatic protein nitration induced by different types and doses of stimuli; 2) if such impact of SOD1 was independent of NO production; 3) if the changes in protein nitration due to SOD1 knockout could be reversed by the holo- or apo-SOD1 enzyme; and 4) if SOD1 catalyzed nitrotyrosine formation selectively in target proteins of liver homogenates and(or) certain tyrosine residues in bovine serum albumin (BSA).

## Materials and methods

### Chemicals and Antibodies

All chemicals including bovine SOD1 protein were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise indicated. Peroxyntirite was purchased from Calbiochem (San Diego, CA). Mouse anti-nitrotyrosine antibody was from Upstate Biotechnology (Lake Placid, NY), and anti-mouse IgG was from Pierce (Rockford, IL). Human holo- and apo-SOD1 protein (Cu, Zn-depleted) [35] were kindly provided by Dr. John Hart, University of Texas Health Science Center at San Antonio.

### Animals and Treatments

Our experiments were approved by the Institutional Animal Care and Use Committee at Cornell University and conducted in accordance with the NIH guidelines for animal care. The SOD1<sup>-/-</sup> and WT mice were generated from 129SVJ × C57BL/6 mice [1]. All used mice were 8-10 weeks old and were fed a diet containing adequate levels of all required

nutrients. To determine if SOD1 knockout exerted a general impact on hepatic protein nitration, we injected (intraperitoneal) mice with two different stimuli: APAP and lipopolysaccharide (LPS). The former is a widely used analgesic and antipyretic drug that raises plasma nitrate/nitrite concentrations and produces nitrotyrosine adducts in centrilobular areas of liver [24,25,27]. The latter induces the release of NO and superoxide in targeted tissues, leading to the formation of peroxynitrite and protein nitration [33,36]. Based on preliminary experiments, we selected injections of APAP at 300 and 600 mg/kg of body weight and of LPS at 5 mg/kg of body weight. The two compounds were prepared in phosphate-buffered saline (PBS). The WT and SOD1<sup>-/-</sup> mice (n = 4-8 for each treatment by genotype) were euthanized for blood and liver sample collection at 5 and 6 h post the injection of APAP and LPS, respectively. For both stimuli, the same number of WT and SOD1<sup>-/-</sup> mice were injected with PBS and euthanized as the respective controls.

### Hepatic Protein Nitration, Antibody Verification, and Plasma NO Production

Both immunohistochemistry and Western blot were used to characterize the impact of SOD1 knockout on liver nitrotyrosine formation. The Histomouse<sup>TM</sup>-MAX kit (Zymed, South San Francisco, CA) was used for immunostaining of liver sections, and 12% gel was run to separate liver homogenates (50 µg protein/lane) for Western analysis. Specificity of the antibody against nitrotyrosine was verified by using 10 mM sodium dithionite to reduce nitrotyrosine on the blotting membrane and incubating the antibody with 20 µM 3-nitrotyrosine overnight for a prior block [37]. Protein concentration was measured by the Lowry method [38]. As an indicator of NO production, plasma nitrate and nitrite concentrations were measured [39,40] to test if the impact of SOD1 knockout on hepatic protein nitration was related to NO formation.

### In Vitro Analysis of SOD1-catalyzed Protein Nitration

A series of experiments with mouse liver homogenates or BSA were conducted to determine: 1) if the observed genotype difference in the induced hepatic protein nitration was actually due to the SOD1 activity, but not other changes associated with the gene ablation; 2) if the hepatic protein nitration in the SOD1<sup>-/-</sup> mice was limited by formation of peroxynitrite; and 3) if the catalysis of SOD1 enzyme in protein nitration was dependent on its activity (the presence of metal Cu). Liver samples of WT and SOD1<sup>-/-</sup> mice treated with PBS or APAP were homogenized in 50 mM potassium phosphate buffer, pH 7.8, 0.1% Triton X-100, 1.34 mM diethylenetriaminepentaacetic acid. The homogenates were centrifuged at 14,000 g for 20 min at 4°C, and the resulting supernatants were adjusted to the same protein concentration (7.5 mg/ml). The same buffer was also used to prepare BSA solution (7.5 mg/ml). For each reaction, 16.4 nmol of peroxynitrite [41] was mixed with 150 µg of liver homogenate protein (20 µl) or 300 µg of BSA (40 µl) in the presence or absence of different amounts of SOD1 enzyme. The added SOD activities ranged from 1 to 4-times of the WT activity, and the mixture volumes were controlled to ensure identical peroxynitrite concentrations between different treatments. The mixture was incubated for 5 min at 37°C, and then put on ice. The SOD activities of bovine SOD1 and human holo-SOD1 were  $3.4 \times 10^4$  and  $9.6 \times 10^4$  units/mg protein, respectively. Little SOD activity was detected with human apo-SOD1 protein. Total SOD activity was measured using a water-soluble formazan dye kit (Dojindo Molecular Technologies, Gaithersburg, MD), following the manufacturer's instructions. After the peroxynitrite treatment, 50 µg of protein from the liver homogenate mixture or 5 µg of protein from the BSA mixture was used for analysis of nitrotyrosine formation by Western blot (as described above) and mass spectrometry (see below).

## Nitrotyrosine Characterization by Mass Spectrometry

After the peroxynitrite-mediated nitrotyrosine formation was verified by Western blot, a portion of BSA solution (containing 76 pmol of protein) was reduced, alkylated, and digested with trypsin as described previously [42]. The peroxynitrite-treated WT and SOD1<sup>-/-</sup> liver homogenates (200 µg of protein) were pre-incubated with 2 µg of the anti-nitrotyrosine antibody for 1 h before adding 20 µl of protein G agarose beads for overnight incubation at 4°C with constant agitation. The mixture was then centrifuged at 3,000 g for 1 min and the precipitate was washed for 5 times with 1 ml of the homogenization buffer. After being boiled for 5 min in the sample buffer, the resulting supernatants were loaded onto two lanes (10 vs 90% of sample) of SDS-PAGE (12% gel). The lane with the 10% sample was used for Western blot analysis, while the other lane was stained with deep purple (Amersham, Piscataway, NJ). Bands corresponding to positive Western blot bands were excised, digested *in situ*, and extracted [42]. The in-gel extracted tryptic peptides were reconstituted in 15 µl of 0.5% formic acid containing 2% acetonitrile, and 6.4 µl of this solution were used for nanoLC-MS/MS analysis as described previously [43]. The solution-based BSA digests were treated in a similar manner. The MS/MS data generated from nanoLC/ESI-based information dependent acquisition (IDA) analysis were submitted to Mascot 1.9 for database searching using in-house licensed Mascot local server and the search was performed to query to NCBI database (taxonomy: Mammals) with one missed cleavage site by trypsin allowed. The peptide tolerance was set to 2 Da and MS/MS tolerance was set to 0.8 Da. Only significant scores for the peptides defined by Mascot probability analysis ([www.matrixscience.com/help/scoring\\_help.html#PBM](http://www.matrixscience.com/help/scoring_help.html#PBM)) greater than “identity” were considered for the peptide identification.

## Computer Modeling

A three-dimensional model for the sequence of BSA was generated from comparative protein modeling using the program MODELLER [44,45]. The input data for MODELLER were: (a) the template structure, corresponding to the experimentally-determined structure of human serum albumin, monomer A, obtained from the Protein Data Bank code 1A06, and (b) a sequence alignment of BSA and human serum albumin, obtained by using the program BLAST [46,47].

## Statistical Analysis

Numerical data were analyzed using the general linear model procedure in SAS (release 6.11, SAS Institute, Cary, NC). Statistical significance was defined as  $P < 0.05$ .

## Results

### Knockout of SOD1 Diminished both APAP and LPS-induced Liver Protein Nitration

Little hepatic SOD activity was detected in the SOD1<sup>-/-</sup> mice, and the treatment of 600 mg APAP/kg resulted in a decrease ( $P < 0.05$ ) of the enzyme activity in the WT mice (Table 1). Western blot analysis showed strong bands of hepatic nitrotyrosine formation in the WT mice treated with 300 mg of APAP/kg (Fig. 1A), 600 mg of APAP/kg (Fig. 1B), and LPS (Fig. 1C). The same treatments produced little hepatic protein nitration in the SOD1<sup>-/-</sup> mice under the present condition. Immunostaining was performed to further confirm this genotype difference in mice treated with 300 mg APAP/kg (Fig. 1D). While the PBS-treated WT and SOD1<sup>-/-</sup> mice showed little background, there was intense staining (brown color) scattered in the hepatic lobules of the WT mice treated with APAP. The stained positive fine granules appeared in cytoplasm within the degenerated hepatocytes in the central lobular regions. The intravascular erythrocytes, fibroblast-like cells in the triads and endothelial

cells were also stained positive. In contrast, very little hepatocellular nitrotyrosine staining was detected in the APAP-treated SOD1<sup>-/-</sup> mice.

### Plasma NO Concentration Varied with Treatments and Genotypes

Compared with the PBS-injected controls, both APAP doses of 300 (Fig. 2A) and 600 mg/kg (Fig. 2B) led to increase ( $P < 0.05$ ) in plasma NO concentrations in the WT mice. The increases in the SOD1<sup>-/-</sup> were significant ( $P < 0.05$ ) only at the dose of 600 mg/kg. Plasma NO concentrations in the SOD1<sup>-/-</sup> were 79 and 49% lower ( $P < 0.05$ ) than those in the WT mice treated with 300 and 600 mg APAP/kg, respectively. The LPS treatment caused similar increases ( $P < 0.05$ ) in plasma NO concentrations in the two genotypes (Fig. 2C).

### Bovine SOD1 Rescued the Peroxynitrite-mediated Protein Nitration in Liver Homogenates of SOD1<sup>-/-</sup> Mice

Compared with the respective controls, the peroxynitrite treatment produced much stronger formation of nitrotyrosine in the liver homogenates of WT mice than that in the SOD1<sup>-/-</sup> mice (Fig. 3 & 4A). Adding the SOD1 enzyme into the liver homogenates greatly enhanced the reactions in both genotypes, and the enhancements were SOD activity-dependent (Fig. 3). Accordingly, the dose of 4-times of WT activity was selected for further analyses. There was little total SOD activity in the liver homogenates of the SOD1<sup>-/-</sup> mice, and the activity was decreased by 27% ( $P < 0.05$ ) in the WT mice after the peroxynitrite treatment (Fig. 4B, upper graph). When bovine SOD1 was added into the liver homogenates, total SOD activities in both genotypes were increased, and were not significantly decreased by the peroxynitrite treatment (Fig. 4B, lower graph). Likewise, the peroxynitrite-mediated nitration of BSA was greatly enhanced by the addition of bovine SOD1 enzyme (Fig. 4C). The added bovine SOD1 activity in the BSA reaction mixture was decreased ( $P < 0.05$ ) after the peroxynitrite treatment (Fig. 4D).

### Apo-SOD1 Was Unable to Catalyze the Peroxynitrite-mediated Nitrotyrosine Formation

Adding the apo-human SOD1 protein into the liver homogenates of WT and SOD1<sup>-/-</sup> mice or the BSA mixture failed to enhance the peroxynitrite-mediated nitrotyrosine formation (Fig. 5). In contrast, the addition of human holo-SOD1 enzyme greatly enhanced the formation in all three cases. The SOD activity in the mixture with the human holo-SOD1 protein was  $4,860 \pm 205$  units/mg of protein and was decreased by 21% in the BSA mixture after the peroxynitrite treatment.

### The SOD1-Catalyzed Nitrotyrosine Formation Was Residue and Protein Specific

After BSA was incubated with peroxynitrite alone, three tyrosine residues (Y161, Y357, and Y424) were found to be nitrated. Four additional nitrotyrosine residues: Y108, Y179, Y184, and Y475 were detected in BSA incubated with peroxynitrite with added bovine SOD1 enzyme. A representative MS/MS-based identification of nitrotyrosine in BSA (Supplemental Fig. 1) showed that Y475 was nitrated by peroxynitrite in the presence of bovine SOD1 enzyme, and all the ions in the y ion series starting at  $y_8$  and higher were shifted by 45.0 amu when compared to the corresponding native peptide of BSA treated with peroxynitrite alone. This shift confirmed nitration at tyrosine residue. No nitrotyrosine was found in the bovine SOD1 protein on the basis of 60% sequence coverage including both Y108 and Q131 that were previously suspected to be the nitrated targets [10]. When the liver homogenates of SOD1<sup>-/-</sup> mice were incubated with peroxynitrite alone, only 10 proteins were identified from the reaction mixture immuno-precipitated by the nitrotyrosine antibody. In contrast, there were 20 proteins identified from that of the liver precipitates treated with peroxynitrite and added bovine SOD1 enzyme. The 10 candidate proteins uniquely associated with bovine SOD1 catalysis are listed in Table 2.

## Discussion

The most significant finding from this study is that knockout of SOD1 attenuated or diminished hepatic protein nitration in mice induced by two types/doses of stimuli. Although the two stimuli APAP and LPS display different chemical properties, metabolic fates, target organs, and abilities to induce NO or peroxynitrite formation [48,49], impacts of SOD1 knockout on their induced hepatic protein nitration remained similar. The inability of either compound to induce strong hepatic protein nitration in the SOD1<sup>-/-</sup> mice as in the WT indicates a general need for SOD1 to catalyze the process *in vivo*. Because the catalytic role of SOD1 in protein nitration has been shown *in vitro* [9,10,40,50], our results provide direct evidence for such a role of SOD1 *in vivo*.

The diminished hepatic protein nitration in the SOD1<sup>-/-</sup> mice was unlikely due to a limited NO formation or secondary changes associated with SOD1 knockout. Both treatments of APAP and LPS resulted in similar differences in hepatic protein nitration between the SOD1<sup>-/-</sup> and WT mice, despite a large variation (0 to 79%) of genotype differences in plasma NO concentrations. Although NO is needed for the formation of peroxynitrite and inhibition of NO synthesis attenuates protein nitration in some instances [28,51], protein nitration may take place at baseline of NO production, and thereby is not strictly dependent on elevated NO concentrations [24,26,28]. In fact, expression of inducible nitric oxide synthase in liver was not different between WT and the SOD1<sup>-/-</sup> mice (data not shown). More convincingly, adding the same amount of peroxynitrite in the liver homogenates produced remarkably less nitrotyrosine in the SOD1<sup>-/-</sup> mice than that in the WT mice. This *in vitro* genotype difference resembled the *in vivo* case. Thus, the lack of SOD1 activity, instead of peroxynitrite supply, was the primary or limiting factor responsible for the attenuated hepatic protein nitration in the SOD1<sup>-/-</sup> mice. Furthermore, adding bovine SOD1 enzyme into the liver homogenates of both genotypes resulted in activity-dependent increases in the peroxynitrite-induced nitrotyrosine formation. The genotype difference was nearly eliminated at the high level of SOD1 enzyme addition. Only the holo-SOD1 enzyme, but not the Cu,Zn-depleted apo-SOD1 protein, was able to enhance the peroxynitrite-mediated nitrotyrosine formation in BSA and liver homogenates. These types of rescues strongly suggest that the lack of SOD1 activity *per se*, instead of compensatory changes or an accelerated nitrotyrosine degradation [52,53] caused by the SOD1 knockout, was responsible for the greatly attenuated hepatic protein nitration in the APAP or LPS-treated SOD1<sup>-/-</sup> mice.

The catalytic or promoting role of SOD1 in *in vivo* hepatic protein nitration demonstrated in the present study is in contrast to observations from several previous experiments. Overexpression of SOD1 in mice prevented nitration of tyrosine hydroxylase [54] and APAP toxicity [31]. Those impacts by the elevated SOD1 activity represented pharmacological rather than the actual metabolic roles of SOD1. That protection was likely to decrease the steady-state levels of superoxide and consequently the formation of peroxynitrite. Meanwhile, copper deficient mouse embryos with low SOD1 activity showed an increased protein nitration [55], and a 3-wk ethanol feeding enhanced liver nitrotyrosine formation in SOD1<sup>-/-</sup> mice [56]. In these two cases, the elevated protein nitration was probably caused by secondary metabolic changes [57,58] resulting from complex changes in body other than just SOD1 activity decrease by copper deficiency, and the interaction between long-term ethanol feeding and SOD1 ablation. Because the above-described impacts of SOD1 overexpression and knockout on protein nitration seem to be asymmetric, we compared the responses of SOD1 heterozygous mice (SOD1<sup>+/-</sup>) to the APAP-induced hepatic protein nitration and the related toxicity with those of the WT and SOD1<sup>-/-</sup> (Supplemental Fig. 2 & 3 and Supplemental Table 1). Our data show that liver protein nitration, mouse survival time, and mouse resistance to hepatotoxicity of APAP were SOD1

activity dose-dependent. All responses of the SOD1<sup>+/-</sup> mice were between the WT and SOD1<sup>-/-</sup> mice.

Our proteomic analysis provides several new leads to the catalytic mechanism by SOD1 in protein nitration. While the inability of the apo-SOD1 protein to enhance the peroxynitrite-mediated nitrotyrosine formation confirms the earlier view that Cu in SOD1 protein is involved in the reaction between peroxynitrite and the enzyme to form an intermediate with the reactivity of nitronium ion that nitrates tyrosine residues of proteins [10,15], nitrotyrosine was not detected in the two tryptic peptides of the added bovine SOD1 protein covering two previously-suspected target residues, Y108 and Q131 [10]. Thus, other residues in the enzyme might be served as the intermediate transfer for passing the nitro-group to the target proteins. In addition, other types of modifications of the SOD1 protein by the peroxynitrite treatment might contribute to the post-reaction decrease in SOD1 activity in the mixture. Based on the modeled crystal structure of BSA (Fig. 6), locations of the three tyrosine residues nitrated by peroxynitrite alone were as follows: Y161 is buried, Y357 is situated in small cleft, and Y424 is on the surface. Two (Y108 and Y184) of the four additional tyrosine residues nitrated by peroxynitrite with added bovine SOD1 enzyme are buried in the inside of structure. Although six of the seven nitrated tyrosine residues by the latter treatment lie in regions with highly positive values of the electrostatic potential, surrounded by a number of positively charged residues (Supplemental Fig. 4), significant structural rearrangement of BSA may be required for the nitration of the buried tyrosine residues. Conceivably, the peroxynitrite treatment could cause BSA to undergo a local conformation change to render certain buried regions available, and SOD1 enzyme might facilitate the process. Based on the genome annotation database DAVID [59], the 10 unique proteins that were presumably nitrated by peroxynitrite under the catalysis by SOD1 enzyme bear the following functional annotation: 4 in mitochondrion, 8 in cytoplasm, 3 in endoplasmic reticulum or peroxisome, 8 related to metabolism, 7 with catalytic activity (4 with transferase activity and 3 with oxidoreductase activity), and 4 dealing with generation of precursor metabolites and energy. More interestingly, at least 4 out of the 10 proteins have been previously shown to be related to APAP toxicity [60,61]. It is our future interest to find out if and how nitration of these proteins in mouse liver affects their resistance to APAP toxicity.

All together, our data show that the SOD1 activity was important for the catalysis of APAP and LPS-induced hepatic protein nitration in mice. The catalysis was not directly related to plasma NO concentrations, peroxynitrite availability, or secondary alternations associated with the SOD1 knockout. More than a decade after the demonstration of SOD1 in catalyzing nitrotyrosine formation *in vitro* [9,10,14], we have provided direct evidence for such a role of SOD1 *in vivo*. The application of mass spectrometry and proteomic analysis helps elucidate the structural mechanism for that catalytic function of SOD1 and the metabolic implications.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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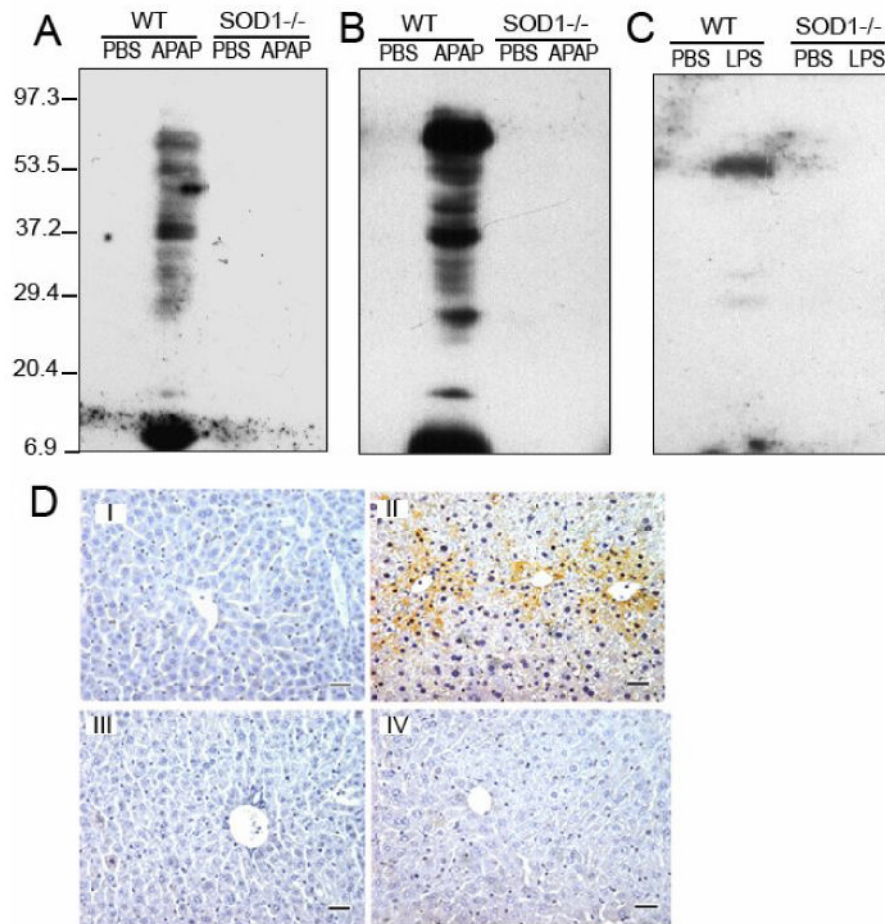
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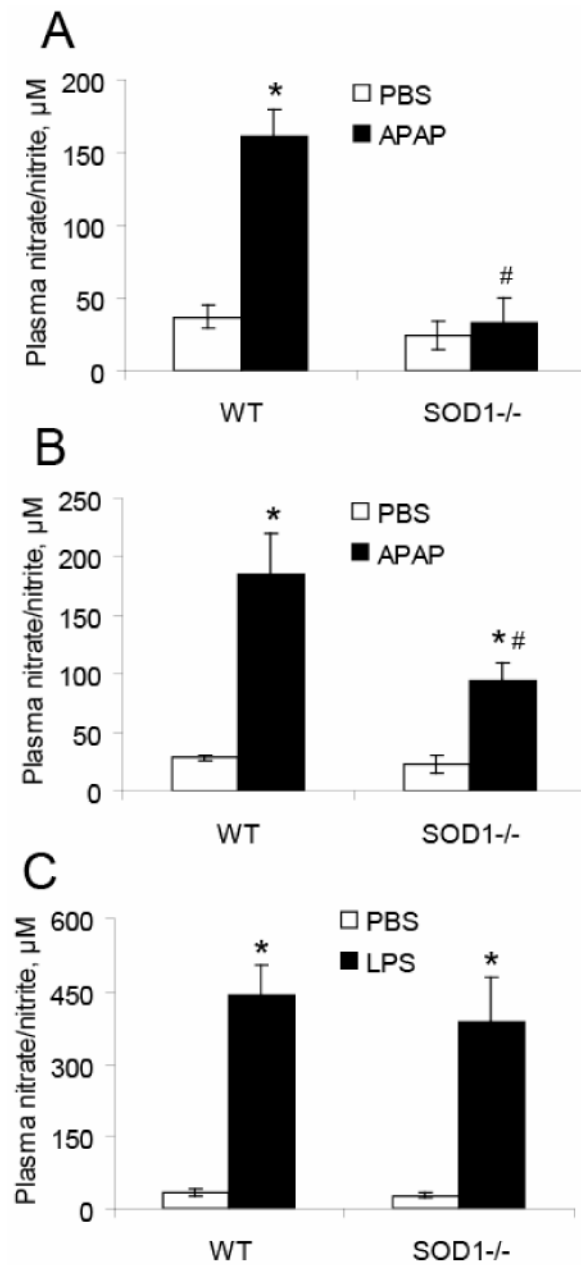
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## Abbreviations used

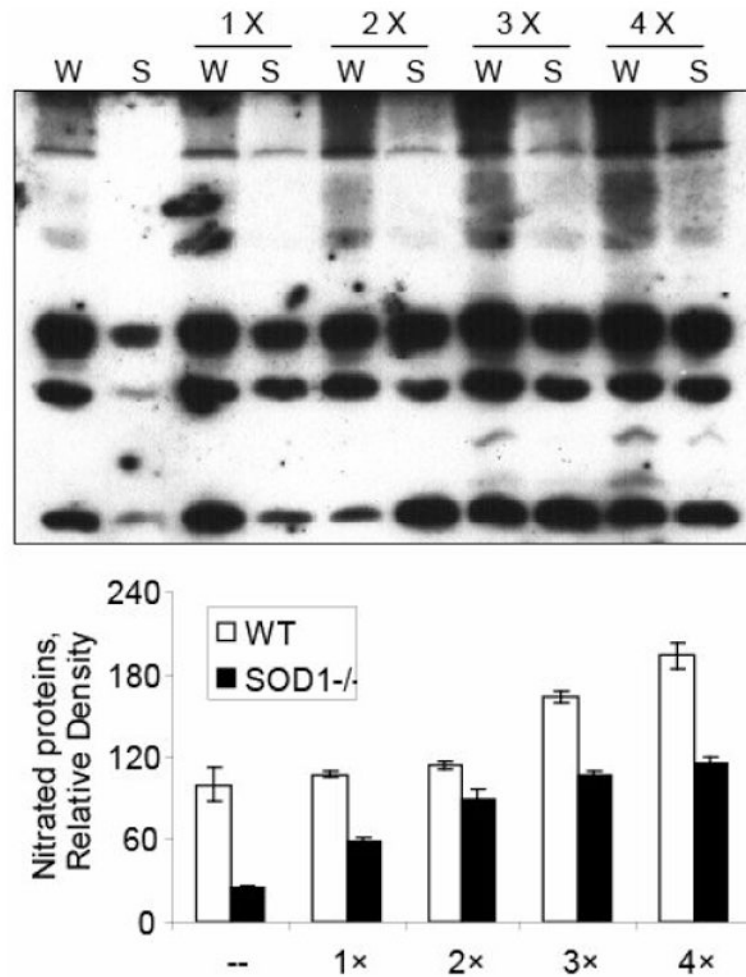
|                |                                  |
|----------------|----------------------------------|
| <b>ALS</b>     | amyotrophic lateral sclerosis    |
| <b>APAP</b>    | acetaminophen                    |
| <b>BSA</b>     | bovine serum albumin             |
| <b>LPS</b>     | lipopolysaccharide               |
| <b>NO</b>      | nitric oxide                     |
| <b>PBS</b>     | phosphate-buffered saline        |
| <b>SOD1</b>    | copper,zinc-superoxide dismutase |
| <b>SOD1-/-</b> | SOD1 knockout mice               |
| <b>WT</b>      | wild-type mice                   |



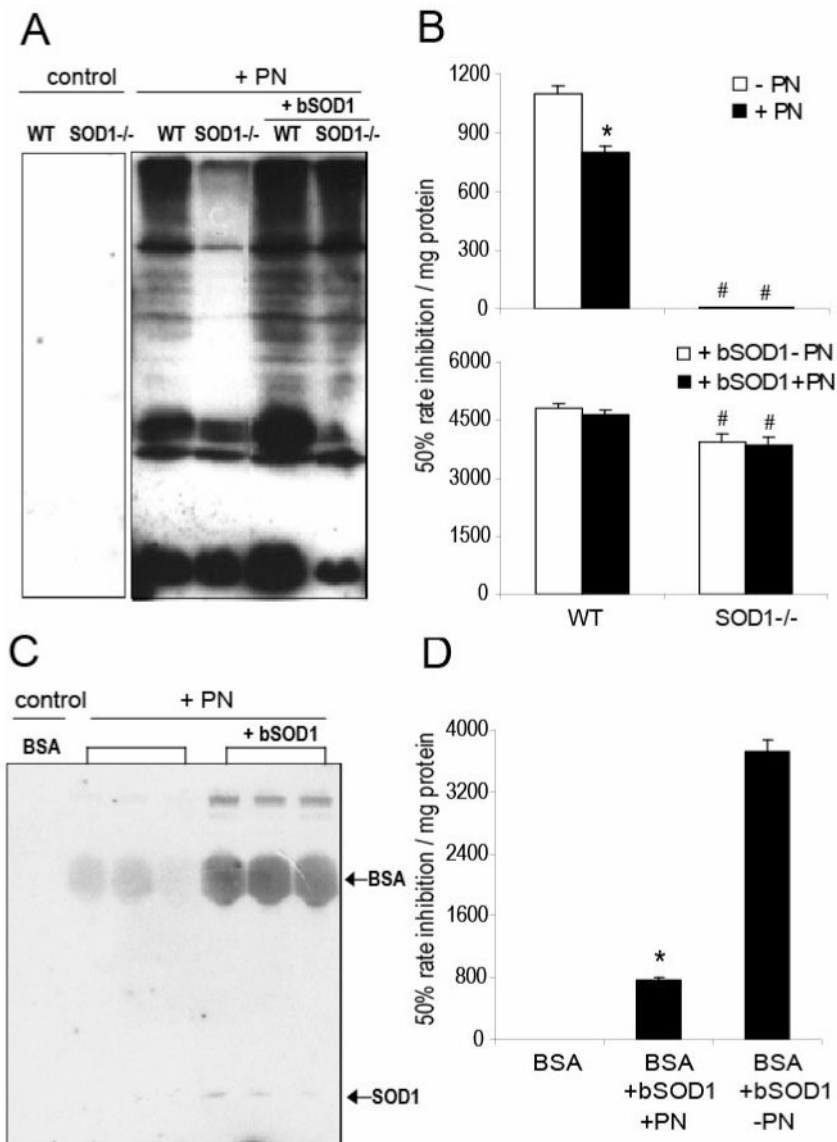
**Fig. 1.** *A, B, and C.* Effect of SOD1 knockout on the APAP and LPS-induced liver nitrotyrosine protein adducts by Western blot analysis in mice treated with APAP (300 mg/kg, 5 h; *A*), APAP (600 mg/kg, 5 h; *B*), or LPS (5 mg/kg, 6 h; *C*). Each lane was loaded with 50  $\mu$ g protein. *D.* Effect of SOD1 knockout on the APAP-induced liver nitrotyrosine adducts by immunostaining. Liver samples were collected from WT or SOD1<sup>-/-</sup> mice at 5 h after the injection of PBS (*I* and *III*) or APAP (*II* and *IV*; 300 mg/kg), and were formalin-fixed, paraffin-embedded, and sectioned. The nitrotyrosine formation was assessed with the Histomouse<sup>TM</sup>-MAX kit (Zymed Inc.) using the mouse anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY). There was intense staining (brown color) scattered in the hepatic lobules of the WT mice treated with APAP, representing the induced nitrotyrosine formation (Original magnification 100 $\times$ ). In contrast, little hepatocellular nitrotyrosine staining was detected in the APAP-treated SOD1<sup>-/-</sup> mice or the PBS-treated mice. The images are representatives of four independent analyses.



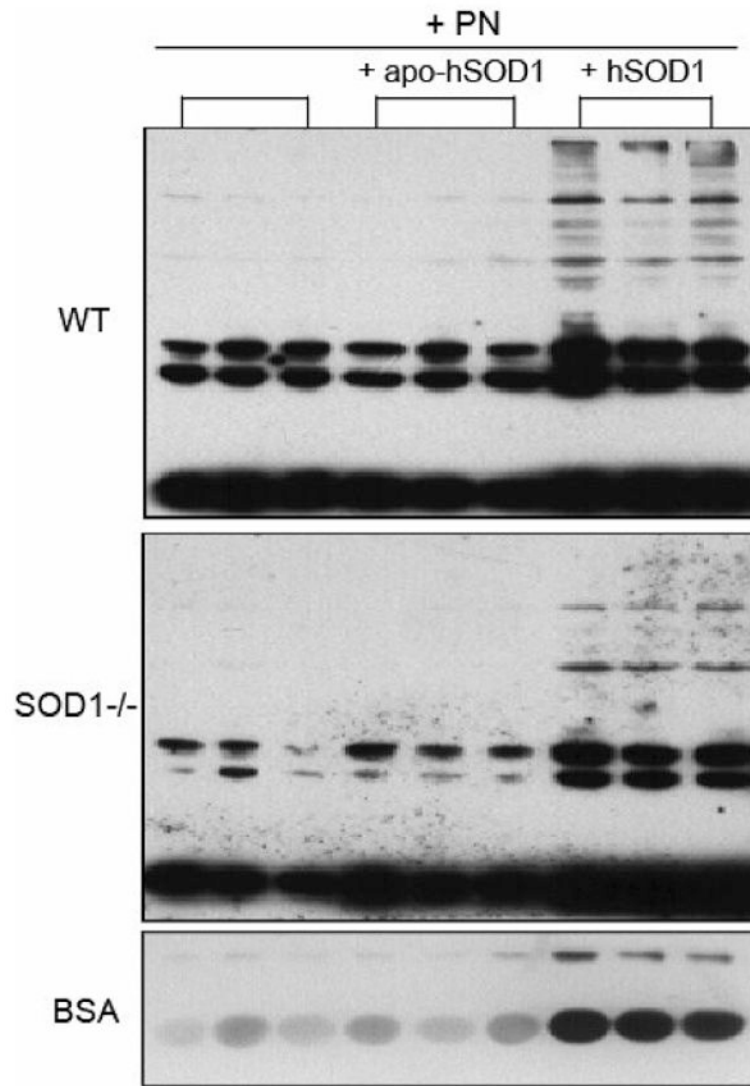
**Fig. 2.** Effects of SOD1 knockout on the APAP or LPS-induced NO formation as measured by plasma nitrate and nitrite concentrations. **A.** Mice treated with PBS or APAP (300 mg/kg, 5 h, n = 4 - 6). **B.** Mice treated with PBS or APAP (600 mg/kg, 5 h, n = 4 - 6). **C.** Mice treated with PBS or LPS (5 mg/kg, 6 h, n = 6 - 8). \*  $P < 0.05$  vs. the PBS-treated mice within genotypes; #  $P < 0.05$  vs. WT within the same treatment.



**Fig. 3.** Effects of different doses of bovine SOD1 enzyme addition on the peroxynitrite-mediated protein nitration in liver homogenates. A total of 150 µg of protein was treated with 0.8 mM peroxynitrite in the absence or presence of added SOD1 protein. The upper panel shows representative Western blot of protein nitration (50 µg protein/lane). The lower panel (bar graphs) shows the relative density of the nitrated proteins (n = 2). W, WT; S, SOD1<sup>-/-</sup>; 1 ×, 2 ×, 3 × and 4 ×, times of SOD activity added as the WT level.

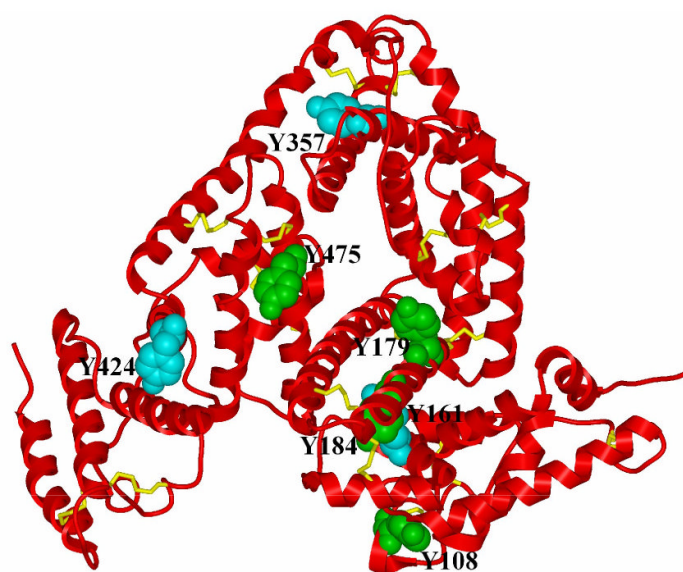


**Fig. 4.** Effects of bovine SOD1 enzyme (bSOD1) addition on the peroxynitrite-mediated protein nitration in liver homogenates and BSA. For liver homogenates, a total of 150  $\mu$ g of protein was treated with 0.8 mM peroxynitrite in the absence or presence of added SOD1 protein (20  $\mu$ g). For BSA, a total of 300  $\mu$ g of protein was treated with 0.4 mM peroxynitrite in the absence or presence of added SOD1 protein (40  $\mu$ g). **A.** Western blot analysis of protein nitration of liver homogenates mixture (50  $\mu$ g protein/lane). **B.** SOD activity of liver homogenates mixture measured before and after peroxynitrite treatment ( $n = 3 - 6$ ). **C.** Western blot analysis of BSA nitration (5  $\mu$ g protein/lane). **D.** SOD activity in the BSA reaction mixture measured before and after the peroxynitrite treatment in the presence of added SOD1 protein ( $n = 3$ ). \*  $P < 0.05$  vs. before the peroxynitrite treatment. #  $P < 0.05$  vs. WT.



**Fig. 5.** Effect of adding the human holo-SOD1 (hSOD1) and the metal-depleted apo-SOD1 (apo-hSOD1) enzymes on the peroxynitrite-mediated protein nitration in liver homogenates and BSA. For liver homogenates, a total of 150  $\mu\text{g}$  of protein was treated with 0.8 mM peroxynitrite in the absence or presence of added holo-SOD1 or apo-SOD1 protein (6.5  $\mu\text{g}$ ). For BSA, a total of 300  $\mu\text{g}$  of BSA was treated with 0.4 mM peroxynitrite in the absence or presence of added holo-SOD1 or apo-SOD1 protein (13  $\mu\text{g}$ ). After the reaction, 50  $\mu\text{g}$  of liver homogenate protein or 5  $\mu\text{g}$  of BSA was loaded per lane for Western blot analysis.





**Fig. 6.**

A simplified representation of a three-dimensional model of the bovine serum albumin (BSA) structure describing the position of nitrated tyrosine residues. The backbone of the structure is shown by using a ribbon representation colored red. Nitrated tyrosine residues are shown using a CPK representation. Residues in cyan correspond to those nitrated when BSA was treated with peroxynitrite alone. Residues in green were found in BSA after treatment with peroxynitrite in the presence of added bovine SOD1 enzyme. All other side chains in the structure were omitted except for cysteine residues involved in S-S bonds which are shown in yellow using a stick representation.

**Table 1**

Effect of APAP or LPS on total SOD activity in mouse liver

|                  | WT                   | SOD1 <sup>-/-</sup>   |
|------------------|----------------------|-----------------------|
| PBS              | 1194±131             | 16.5±5.3 <sup>b</sup> |
| APAP (300 mg/kg) | 1180±40              | 20.6±5.5 <sup>b</sup> |
| PBS              | 1266±61              | 10.8±0.6 <sup>b</sup> |
| APAP (600 mg/kg) | 1021±56 <sup>a</sup> | 12.2±1.2 <sup>b</sup> |
| PBS              | 1315±97              | 12.9±0.8 <sup>b</sup> |
| LPS (5 mg/kg)    | 1360±73              | 14.5±3.3 <sup>b</sup> |

Mice were treated with APAP for 5 h or for 6 h. Values are means ± S.E. (n=3-5).

<sup>a</sup> indicates P < 0.05 compared to PBS treatment within genotypes;

<sup>b</sup> indicates P < 0.05 compared to WT within the same treatment. PBS, phosphate-buffered saline; APAP, acetaminophen; LPS, lipopolysaccharide; WT, wild-type.

**Table 2**Candidate nitrated proteins in liver homogenate associated with SOD1 catalysis<sup>a</sup>

| GI #     | Protein name  | Function  | Location     |
|----------|---|---|--------------|
| 200246   | Pyruvate carboxylase                                  | Catalyzes the initial reactions of glucose and lipid synthesis from pyruvate in a tissue specific manner            | Mito<br>Cyto |
| 22477996 | Glutathione s-transferase, mu 2                       | Conjugation of reduced glutathione to a wide number of exogenous and endogenous hydrophobic electrophiles           | Cyto         |
| 19354269 | Methyltransferase like 7b                             | Probable methyltransferase, highly expressed in liver and kidney  | ER           |
| 74141423 | Glyceraldehyde-3-phosphate dehydrogenase              | Involved in carbohydrate degradation, glycolysis and pyruvate from D-glyceraldehyde 3-phosphate                     | Mito<br>Cyto |
| 13097441 | Sarcosine dehydrogenase                               | Involved in amine and polyamine degradation, sarcosine degradation, and formaldehyde and glycine from sarcosine     | Mito<br>Cyto |
| 18044708 | Fibrinogen, gamma polypeptide                         | Yields monomers that polymerize into fibrin and acts as a cofactor in platelet aggregation                          | Sec          |
| 1304157  | Heat shock 70kd protein 5 (glucose-regulated protein) | Probably plays a role in facilitating the assembly of multimeric protein complexes inside the endoplasmic reticulum | Cyto<br>ER   |
| 18314540 | Dihydroxyacetone kinase 2 homolog (yeast)             | Catalyzes the phosphorylation of dihydroxyacetone, involved in lipid metabolism                                     | Cyto         |
| 6753272  | Catalase  | Protect cells from the toxic effects of hydrogen peroxide   | Mito<br>Cyto |
| 82958328 | Ribosomal protein s14                                 | Structural constituent of ribosome  | Cyto         |

<sup>a</sup>Liver homogenates of SOD1<sup>-/-</sup> mice were treated with peroxynitrite in the presence or absence of bovine SOD1 enzyme. The homogenates were then immuno-precipitated using the mouse antibody against nitrotyrosine, and processed for Mass Spectrometry. Listed are proteins uniquely resulted from the presence of SOD1 enzyme. Proteins are sorted based on <http://david.abcc.ncifcrf.gov/tools.jsp> and <http://ca.expasy.org/>. Cyto, cytoplasm; ER, endoplasmic reticulum; Mito, mitochondrion; Pero, peroxisome; and Sec, secreted.