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## Oxidative Inactivation of Key Mitochondrial Proteins Leads to Dysfunction and Injury in Hepatic Ischemia Reperfusion

KWAN-HOON MOON<sup>1</sup>, BRIAN L. HOOD<sup>2,@</sup>, PARTHA MUKHOPADHYAY<sup>3</sup>, RAJESH MOHANRAJ<sup>3</sup>, MOHAMED A. ABDELMEGEED<sup>1</sup>, YONG-IL KWON<sup>1,†</sup>, THOMAS P. CONRADS<sup>2,#</sup>, TIMOTHY D. VEENSTRA<sup>2</sup>, BYOUNG-JOON SONG<sup>1,\*</sup>, and PAL PACHER<sup>3,\*</sup>

<sup>1</sup>Laboratories of Membrane Biochemistry and Biophysics National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD 20892-9410

<sup>2</sup>Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, Inc., Frederick, MD 21702.

<sup>3</sup>Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, Bethesda, MD 20892-9410

### Summary

**Background & Aims**—Ischemia-reperfusion (I/R) is a major mechanism of liver injury following hepatic surgery or transplantation. Despite numerous reports on the role of oxidative/nitrosative stress and mitochondrial dysfunction in hepatic I/R injury, the proteins that are oxidatively-modified during I/R damage are poorly characterized. This study was aimed at investigating the oxidatively-modified proteins underlying the mechanism for mitochondrial dysfunction in hepatic I/R injury. We also studied the effects of a superoxide dismutase mimetic/peroxynitrite scavenger metalloporphyrin MnTMPyP on oxidatively-modified proteins and their functions.

**Methods**—The oxidized and/or *S*-nitrosylated mitochondrial proteins from I/R-injured mouse livers with or without MnTMPyP pretreatment, were labeled with biotin-*N*-maleimide, purified with streptavidin-agarose and resolved by two-dimensional gel electrophoresis. The identities of the oxidatively-modified proteins were determined using mass-spectrometric analysis. Liver histopathology, serum transaminase levels, nitrosative stress markers and the activities of oxidatively-modified mitochondrial proteins were measured.

**Results**—Comparative two-dimensional gel analysis revealed markedly increased numbers of oxidized and *S*-nitrosylated mitochondrial proteins following hepatic I/R injury. Many key mitochondrial enzymes involved in cellular defense, fat metabolism, energy supply, and chaperones were identified as being oxidatively-modified proteins. Pretreatment with MnTMPyP attenuated the I/R-induced increased serum transaminase levels, histological damage, increased *i*NOS expression, and *S*-nitrosylation and/or nitration of various key mitochondrial proteins. MnTMPyP pretreatment

\*To whom correspondence should be addressed: Dr. B. J. Song, (e-mail) bjs@mail.nih.gov, Laboratory of Membrane Biochemistry and Biophysics; and Dr. Pal Pacher, (e-mail) pacher@mail.nih.gov, Section on Oxidative Stress and Tissue Injury, Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, 9000 Rockville Pike, Bethesda, MD 20892-9410 USA.

@Present address: Clinical Proteomics Facility, University of Pittsburgh Cancer Institute University of Pittsburgh, Pittsburgh School of Medicine, PA 15213

#Present address: Department of Pharmacology and Chemical Biology, University of Pittsburgh, Pittsburgh School of Medicine, PA 15213

†Present address: Department of Obstetrics/Gynecology, Kangdong Sacred Heart Hospital, Hallym University Medical College, Seoul, Korea 134-701.

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E-mail addresses of other co-authors are: Kwan-Hoon Moon: kwanhoonm@mail.nih.gov Brian L. Hood: hoodb@upmc.edu Partha Mukhopadhyay: mpartha@mail.nih.gov Rajesh Mohanraj: mohanrajr@mail.nih.gov Mohamed A. Abdelmegeed: abdelmegeedm@mail.nih.gov Yong-Il Kwon: kbg@hallym.or.kr Thomas P. Conrads: conradstp@upmc.edu Timothy D. Veenstra: veenstra@ncifcrf.gov.

also restored I/R-induced suppressed activities of mitochondrial aldehyde dehydrogenase, 3-ketoacyl-CoA thiolases, and ATP synthase.

**Conclusions**—These results suggest that increased nitrosative stress is critically important in promoting S-nitrosylation and nitration of various mitochondrial proteins, leading to mitochondrial dysfunction with decreased energy supply and hepatic injury.

### Keywords

Ischemia-reperfusion injury; liver mitochondria dysfunction; redox biology; Cysteine oxidation; S-nitrosylation; metalloporphyrin peroxynitrite scavenger

## Introduction

Organ injury, caused by transient ischemia followed by reperfusion (I/R), is a pivotal mechanism of tissue damage in diseases such as stroke, myocardial infarction, organ transplantation and vascular surgeries. Hepatic I/R injury can develop during liver transplantation, surgical removal of hepatic tumors or traumas, circulation shock, and acute exposure to toxic substances, etc. Ischemia (hypoxia) followed by return of oxygenated blood flow (reperfusion) to the liver can eventually lead to hepatic failure with increased mortality and morbidity.<sup>1</sup> Reactive oxygen/nitrogen species (ROS/RNS) and pro-inflammatory cytokines play an important role in liver injury depending on the duration of the reperfusion period.<sup>1</sup>

At earlier stages, increased superoxide and other ROS, derived from the activation of various sources [e.g. xanthine oxidoreductases<sup>2,3</sup>] play a critical role in tissue damage. The activities of these complex enzymes in the mitochondrial respiratory chain are inhibited,<sup>4-6</sup> allowing more ROS to leak out of the respiratory chain. The increased superoxide and NO generation during I/R (the latter being mostly derived from increased iNOS induction) favors the formation of the more potent oxidant peroxynitrite (ONOO<sup>-</sup>),<sup>7</sup> causing impaired cellular functions.<sup>8</sup>

After a longer period of reperfusion, increased amounts of pro-inflammatory cytokines produced from resident macrophage-Kupffer cells and infiltrating leukocytes are critical in inflicting tissue damage.<sup>9</sup> Both elevated ROS/RNS and pro-inflammatory cytokines such as TNF $\alpha$  also activate various cell death signaling pathways via c-Jun, N-terminal kinase (JNK) 10-12 and p38 kinase,<sup>12,13</sup> leading to apoptosis and/or necrosis.<sup>10,11,14-18</sup> Consistent with the importance of these pathways, a variety of pharmacological agents such as specific JNK inhibitors<sup>11</sup> and various antioxidants (e.g. ascorbate, glutathione, S-adenosylmethionine, vitamin E, etc.) attenuate the severity of I/R-mediated liver injury.<sup>3,8,19-21</sup> Accumulation of oxidatively-modified proteins, lipid peroxides, and nucleic acids may lead to mitochondrial dysfunction and tissue damage.<sup>8,9,21-25</sup> Despite numerous reports on the role of oxidative stress and mitochondrial dysfunction in hepatic I/R injury,<sup>4-9,26-28</sup> the proteins that are oxidatively-modified during I/R injury are poorly characterized. We hypothesized that oxidative modifications of key mitochondrial proteins lead to their inactivation and mitochondrial dysfunction with decreased energy supply, ultimately contributing to cellular damage. To test this hypothesis, we employed a method that uses biotin-N-maleimide (biotin-NM) to detect oxidatively-modified proteins.<sup>23,29,30</sup> In this report, we examined the mechanism of inactivation of oxidatively-modified mitochondrial proteins in a murine model of hepatic I/R injury. Furthermore, by using the superoxide dismutase (SOD) mimetic and peroxynitrite decomposition catalyst (termed “peroxynitrite scavenger”) MnTMPyP,<sup>20,31,32</sup> we demonstrated that elevated oxidative/nitrosative stress plays a critical role in I/R-mediated mitochondrial dysfunction and hepatic damage.

## Materials and Methods

### Materials

Biotin-NM, anti- $\beta$ -ATP synthase antibody, dithiothreitol (DTT), MnTMPyP, and L-sodium ascorbate (Asc) were obtained from Sigma Chemical. Specific antibodies to prohibitin or horse radish peroxidase-conjugated MAb-biotin were purchased from Oncogene Science and Cell Signaling, respectively. Specific antibodies to inducible nitric oxide synthase (iNOS) and 3-nitrotyrosine (3-NT) were purchased from Santa Cruz Biotechnologies and Upstate Biotechnologies, respectively.

### Animals, Hepatic I/R Protocol and Histological Analysis

All animal experiments were conducted in accordance with the NIH guidelines and were approved by the Institutional Animal Care and Use Committee. Young male C57BL/6J mice from Jackson Laboratory were anesthetized with a single dose of pentobarbital (65 mg/kg i.p.) and a midline laparotomy incision was performed to expose the liver of anesthetized mice as described.<sup>33,34</sup> The hepatic artery and the portal vein were clamped using microaneurysm clamps. This model results in segmental (70%) hepatic ischemia. This method of partial ischemia prevents mesenteric venous congestion by allowing portal decompression throughout the right and caudate lobes of the liver. The liver was kept moist at 37 °C with gauzes soaked with 0.9% saline. Body temperature, monitored with a rectal temperature probe, was maintained at 37 °C using a thermoregulatory heating blanket. Some of the mice were pretreated with MnTMPyP (20 mg/kg i.p.) 1 hour prior to hepatic I/R procedure. Mice injected with the same volume of saline were used as negative controls. Sham surgeries were conducted in a similar manner except that hepatic blood flow was not reduced with a microaneurysm clamp. The duration of warm hepatic ischemia was 1-h, after which the microaneurysm clamps were removed. The duration of reperfusion was 2-h (or 10-h and 24-h shown in Supplementary Figures). After reperfusion, blood was collected and liver tissues were removed, weighed, and snap-frozen in liquid nitrogen. A small frontal piece of the largest lobe of each mouse was fixed in 4 % buffered formalin for histopathological evaluation. After embedding formalin-fixed liver tissues and cutting 5  $\mu$ m slices, all sections were stained with hematoxylin and eosin (H&E). Histological evaluation was performed in a blinded manner.<sup>33,34</sup>

### Determination of the Levels of Transaminases, Nitrotyrosine, Malondialdehyde, and Enzyme Activities

The activities of alanine aminotransferase (ALT) and aminotransferase (AST) were measured in serum samples using a clinical chemistry analyzer system (PROCHEM-V; Drew Scientific, Oxford, CT).<sup>23,33,34</sup> The level of 3-NT was quantified using the HBT Nitrotyrosine ELISA Kit as per manufacturer's instructions (Cell Sciences, Sharon, MA). Hepatic levels of malondialdehyde (MDA) were measured using a lipid peroxidation assay kit (EMD-Calbiochem, San Diego, CA) as per manufacturer's instructions. ATP synthase activity was determined using an ATP bioluminescence assay kit (Roche, Mannheim, Germany) following the manufacturer's instructions. One unit of ATP synthesis activity represents 1 nM ATP produced/min/mg protein at ambient temperature. NADH-ubiquinone oxidoreductase (complex I) activity was determined by measuring OD at 340 nm and subtracting the OD values in the presence of rotenone, an inhibitor of NADH-ubiquinone oxidoreductase.<sup>35</sup> One unit represents a reduction of 1 nmol NADH/min/mg protein.

### Identification of Oxidatively-Modified Proteins Using Mass Spectrometry

Mitochondrial fractions were prepared from pooled mice livers ( $n \geq 5$  per group) treated differently using differential centrifugation followed by two separate washing steps, as described.<sup>22,23,29</sup> Relative purities of the mitochondrial fractions were determined by

immunoblot analysis using a specific antibody against mitochondrial  $\alpha$ -ATP synthase or cytosolic peroxiredoxin-II. Little contamination with cytosolic proteins in our mitochondrial fractions and *vice versa* was observed (Supplementary Fig. 1). Labeling of oxidatively-modified proteins with biotin-NM was performed concurrently for three different groups including the sham-operated mice (Supplementary Figure 2).<sup>23,29,30</sup> Purified biotin-NM labeled oxidized and S-nitrosylated proteins bound to the streptavidin-agarose beads were washed twice to remove non-specifically bound proteins before being resolved using 2-dimensional polyacrylamide gel electrophoresis (2-DE), silver-stained, scanned, and analyzed. In-gel digestion of protein gel spots, nanoflow reversed-phase liquid chromatography (nanoRPLC)—tandem mass spectrometry and bioinformatic analyses were performed as described.<sup>23,29,30</sup>

### Data Processing and Statistical Analysis

All data in this report represent results from at least three separate experiments, unless stated otherwise. Statistical analyses were performed using the Student's *t* test, with  $p < 0.05$  being considered statistically significant. Other materials and methods not described here were performed as previously described.<sup>23,29,30,33,34</sup>

## Results

### Serum Transaminase Levels and Hepatic Histopathology

To assess hepatocellular damage following I/R in mouse liver, serum transaminase ALT/AST activities and hepatic histopathology were evaluated. After 1-h of warm ischemia followed by reperfusion for 2-h, serum ALT and AST activities were increased ~33-fold and 8-fold, respectively, compared to sham-operated controls (Fig. 1A,B). Pretreatment with the peroxynitrite scavenger MnTMPyP significantly reduced the levels of serum ALT and AST following I/R injury. Serum ALT and AST levels were markedly elevated after reperfusion for 10-h and 24-h compared to sham controls, although much higher ALT and AST levels were detected in mice reperfused for 10-h than 24-h (Supplementary Fig. 3). However, MnTMPyP significantly suppressed the elevated ALT and AST levels in I/R-injured mice. Sham-operated mice showed normal hepatic histology (Fig. 1C, top). In contrast, increased signs of blood cell infiltration with sinusoidal congestion and some necrotic hepatocytes were observed in mouse livers reperfused for 2-h (Fig. 1C, middle). These initial signs of I/R-induced tissue injury were markedly attenuated in mice pretreated with MnTMPyP (Fig. 1C, bottom). Liver histology data also showed marked necrosis after reperfusion for 10-h (Supplementary Figs. 4 and 5) or 24-h (Supplementary Figs. 6 and 7). MnTMPyP significantly attenuated the histopathological damage under these conditions (Supplementary Figs 4-7).

### Increased Amounts of Nitrite, iNOS, and Nitrotyrosine in I/R-Injured Mouse Livers

By using knockout mice,<sup>36,37</sup> it was demonstrated that local *i*NOS contributes to hepatic I/R injury, while endothelial NOS protects against I/R injury. Furthermore, induction of *i*NOS produces large quantities of NO for extended periods, affecting liver physiology.<sup>38,39</sup> To demonstrate elevated nitrosative stress in I/R injured mouse liver (2-h reperfusion), the levels of nitrite, *i*NOS, and nitrotyrosine were measured. Nitrite levels were significantly elevated from  $2.07 \pm 0.70 \mu\text{M}$  to  $7.28 \pm 1.45 \mu\text{M}$  after I/R injury (Fig. 2A). Pretreatment with MnTMPyP reduced the nitrite level to  $3.50 \pm 0.76 \mu\text{M}$ . I/R injury increased hepatic *i*NOS expression (Fig. 2B, top), a source for increased nitrosative stress. Increased *i*NOS expression was attenuated by MnTMPyP pretreatment. In contrast, the amounts of prohibitin were similar in all samples (Fig. 2B, bottom). I/R also increased hepatic 3-NT levels [footprint of peroxynitrite generation and/or nitritative stress].<sup>8</sup> Pretreatment with MnTMPyP significantly reduced hepatic 3-NT levels (Fig. 2C).

## Identification of Oxidatively-Modified Mitochondrial Proteins in I/R Injury

Increased ROS/RNS, which can be effectively prevented using various anti-oxidants, plays an important role in hepatic I/R injury.<sup>7,8,18-21</sup> Due to elevated levels of nitrite, *i*NOS, and nitrotyrosine, we hypothesized that many mitochondrial proteins are oxidized and/or *S*-nitrosylated in the livers of I/R injured mice. To test this hypothesis and selectively identify oxidized proteins versus *S*-nitrosylated (and/or *S*-glutathionylated) proteins, *N*-ethylmaleimide-modified proteins were treated with DTT and Asc, respectively, to specifically reduce oxidized (disulfides and sulfenic acids of Cys residues) versus *S*-nitrosylated (and/or *S*-glutathionylated) proteins (see Supplementary Fig. 2 for overview).<sup>23</sup> DTT- or Asc-treated proteins were subsequently labeled with biotin-NM. Figure 3 (left panel) depicts a pattern of Coomassie blue staining of mitochondrial proteins, showing that similar levels of proteins were analyzed for all samples. Only a small number of biotin-NM labeled, oxidatively-modified proteins were detected in sham control animals (right panel, lane 1). The levels of biotin-NM labeled oxidized proteins (identified after DTT treatment: lane 2) or *S*-nitrosylated proteins (identified after Asc treatment: lane 4) were markedly elevated in I/R injured mice (2-h reperfusion) compared to their sham controls, when their relative levels were determined by immunoblot analysis by using MAb-biotin-HRP as a detection probe. Pretreatment with MnTMPyP, however, markedly decreased the intensity of *S*-nitrosylated proteins (right panel, lane 5). In contrast, MnTMPyP did not decrease the intensity of oxidized (disulfides and/or sulfenic acids) proteins (lane 3).

## Summary of Protein Sequencing Analyses of Oxidized and/or *S*-Nitrosylated Mitochondrial Proteins

Because of the elevated levels of oxidized and/or *S*-nitrosylated mitochondrial proteins in I/R injured mouse livers, we used mass spectrometry to identify each biotin-NM labeled oxidatively-modified protein. Once again, DTT and Asc were used, respectively, to selectively identify oxidized versus *S*-nitrosylated (and/or *S*-glutathionylated) proteins (Fig. 4).<sup>23</sup> Consistent with the results shown in Figure 3, only a few biotin-NM labeled oxidized and *S*-nitrosylated proteins were detected in the sham control mouse livers (Fig. 4A and 4D, respectively). The number and abundance of oxidized (Fig. 4B) and *S*-nitrosylated proteins (Fig. 4E) found in mouse livers reperfused for 2-h, however, were markedly increased compared to sham controls. Our results showed that MnTMPyP did not decrease the levels of oxidized proteins (Fig. 4C), however, it drastically decreased the levels of *S*-nitrosylated proteins when mouse livers were reperfused for 2-h (Fig. 4F). More oxidized and *S*-nitrosylated proteins (Supplementary Figs. 8 and 9, respectively) were observed after reperfusion for 10-h and 24-h without MnTMPyP. Under these conditions, we observed a time-dependent effect of MnTMPyP, which also seems to have affected the oxidized proteins (ie, DTT-treated samples in Supplementary Fig. 8) although a greater effect of MnTMPyP seems to be observed with the *S*-nitrosylated proteins (i.e. Asc-treated samples in Supplementary Fig. 9). However, it is unclear why MnTMPyP reversed the oxidized proteins after reperfusion for 10-h but not for 2-h.

Thirty-one oxidized and nineteen *S*-nitrosylated proteins acquired from mouse liver mitochondria after I/R injury (2-h reperfusion) that showed an increased staining intensity (Fig. 4B and 4E) were subjected to MS analysis. The protein sequence data acquired for each spot are provided in Table 1 and summarized in Fig. 5. Protein sequence analysis revealed that many mitochondrial proteins are identified as being oxidized as well as *S*-nitrosylated, suggesting that these two modifications occur concurrently after I/R injury. These proteins include: oxidative phosphorylation [NADH-ubiquinone oxidoreductase (complex I), ubiquinol-cytochrom-c-reductase (complex III), and  $\alpha$ - and  $\beta$ -ATP synthase (complex V)]; anti-oxidative defense system [aldehyde dehydrogenase (ALDH2), methylmalonate-semialdehyde dehydrogenase (ALDH6), 4-trimethylaminobutyraldehyde dehydrogenase (ALDH9),

glutathione transferase, glutathione peroxidase (GPx), etc]; fatty acid  $\beta$ -oxidation and intermediary metabolism (acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, acetyl-CoA acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA lyase, isovaleryl-CoA dehydrogenase, carbamoyl-phosphate synthase, etc); TCA cycle and energy supply (pyruvate dehydrogenase, dihydrolipoamide succinyltransferase, glutamate dehydrogenase, pyruvate carboxylase, etc); molecular chaperon proteins [heat shock protein (Hsp) 60, Hsp 70.2, Hsp 70.3, Hsp 70.8, glucose-regulated protein (GRP) 75, GRP78, etc]; mitochondrial electron transport and ion channeling proteins [ $\alpha$ - or  $\beta$ -subunit of electron transfer flavoprotein ( $\alpha$ - or  $\beta$ -ETF) and voltage-dependent anion channel protein1 (VDAC1)].

Many proteins such as ATP synthase (spots 12,13,37,38), ALDH2 (spots 7-11), ALDH6 (spots 12,13,34-38), acetyl-CoA acetyltransferase (spots 20-24,40-44), acyl-CoA dehydrogenase (spots 21,22,40-43), 3-ketoacyl-CoA thiolase (spots 16-24,39-44), etc. were repeatedly detected from spots with different pI values, suggesting they were also covalently modified by either phosphorylation or hyper-oxidation to sulfinic and/or sulfonic acids. Several proteins were also identified from spots with apparently lower molecular weights of the parent protein (e.g. spots 27-28,45,48-50 for  $\alpha$ -ATP synthase). These spots likely represent fragments of the full-length proteins (e.g. 58 kDa for  $\alpha$ -ATP synthase). Increased degradation of oxidized proteins was also observed with  $\beta$ -ATP synthase, 3-ketoacyl-CoA thiolase and other proteins even after 2-h reperfusion.

### Reversible inactivation of oxidized and/or S-nitrosylated mitochondrial enzymes in I/R injured mice without and with MnTMPyP

We recently reported that the activities of many mitochondrial proteins are inhibited through S-nitrosylation of Cys residue(s) and nitration of Tyr residue(s).<sup>22,23,29</sup> Indeed, several proteins such as ALDH2<sup>40</sup> and 3-ketoacyl-CoA thiolase<sup>41</sup> are known to contain Cys residue(s) within their catalytic sites. Therefore, we evaluated whether the catalytic activities of ALDH2, 3-ketoacyl-CoA thiolase, and ATP synthase were altered in I/R injured mice and if this activity could be restored in the presence of MnTMPyP. Hepatic ALDH2 activity in sham control mice was  $3.30 \pm 0.13$  units compared to  $1.52 \pm 0.58$  and  $2.74 \pm 0.17$  units in 2-h reperused mice without and with MnTMPyP, respectively. These data suggest that mouse ALDH2 activity was markedly reduced in the absence of MnTMPyP and that its activity recovered significantly with MnTMPyP treatment (Fig. 6A). Significant suppression of ALDH2 activity was also observed after reperfusion for 10-h or 24-h. However, MnTMPyP treatment fully restored the ALDH2 activity (Supplementary Fig. 10A). Since ALDH2 is known to metabolize toxic lipid aldehydes such as malondialdehyde (MDA),<sup>42</sup> we also measured the level of MDA, as an indicator of lipid peroxidation, in different groups. The level of MDA, which was low in sham control mice, was increased by 45% in mouse livers reperused for 2-h (Fig. 6B). The increased MDA level in I/R injured mice, was abolished by pretreatment with MnTMPyP. MDA levels were also elevated in 10-h or 24-h reperused mice and significantly attenuated by MnTMPyP pretreatment (Supplementary Fig. 10B).

One His (His<sup>352</sup>) and two Cys residues (Cys<sup>92</sup> and Cys<sup>382</sup>) are essential for the activity of 3-ketoacyl-CoA thiolase.<sup>41</sup> These residues are likely targets of oxidative/nitrosative modification, thereby inactivating the protein. Indeed, mitochondrial 3-ketoacyl-CoA thiolase showed an approximately 61% decrease in activity in I/R injured mice compared to sham control mice ( $5.82 \pm 0.01$  unit). The suppressed activity of 3-ketoacyl-CoA thiolase was restored to 92.3% of control animals when I/R-injured mice were pretreated with MnTMPyP (data not shown).

Mitochondrial dysfunction with decreased activities of the complex enzymes in the respiratory chain has been reported in I/R injury.<sup>4-6,43</sup> It is possible that I/R injury results in S-

nitrosylation, nitration or other modification of critical Cys or Tyr residues in the active site of ATP synthase and other components of the mitochondrial respiratory chain under elevated levels of nitrite and/or peroxynitrite. In fact, Tomkins *et al* recently reported that ROS can be generated through thiol modification of mitochondrial complex I.<sup>43</sup> Therefore, we measured the activity of ATP synthase (complex V) in mice with or without MnTMPyP pretreatment. The ATP synthase activity was  $472.8 \pm 7.5$  units in sham control mice, and  $265.1 \pm 41.8$  and  $444.4 \pm 10.2$  units, respectively, in 2-h reperfused mice without and with MnTMPyP treatment (Fig. 7A). Inhibition of ATP synthase activity was also observed after reperfusion for 10-h or 24-h, however, this activity was restored following MnTMPyP treatment (Supplementary Fig. 11A). These data indicate that mitochondrial ATP synthase activities are significantly inhibited in I/R injured mouse livers, and this suppression can be restored by MnTMPyP. To further elucidate the mechanism for I/R-mediated inhibition of ATP synthase activity, we determined the levels of 3-NT in immunoprecipitated  $\beta$ -ATP synthase from mitochondria of sham controls and I/R-injured mice with or without MnTMPyP. Immunoblot analysis showed that similar levels of  $\beta$ -ATP synthase exist in all conditions (Fig. 7B, left panel). However, 3-NT was only detected in mouse livers reperfused for 2-h and not in the sham controls (right panel). Furthermore, the 3-NT immunoreactive band was absent in mice pretreated with MnTMPyP. These data support the hypothesis that ATP synthase is inactivated in I/R injured mice through nitration of its Tyr residue(s), including the Tyr residues within the catalytic site,<sup>23</sup> and that its inactivation could be prevented with the peroxynitrite scavenger MnTMPyP. Furthermore, the hepatic activity of NADH-ubiquinone oxidoreductase (complex I) was also markedly inhibited in 2-h reperfused mice compared to sham controls (Fig. 7C); an effect that was prevented by MnTMPyP pretreatment ( $p < 0.007$ ). Inhibition of complex I activity was also observed after reperfusion for 10-h or 24-h, while MnTMPyP significantly restored its activity (Supplementary Fig. 11B).

## Discussion

Despite the well-established role of ROS/RNS in I/R injury, the protein targets that are oxidatively-modified by elevated ROS/RNS remain elusive. Virtually no systematic approaches to characterize oxidatively-modified proteins (especially for mitochondrial proteins) during hepatic I/R injury have previously been taken. Recent reports show that a few proteins are altered in humans undergoing liver transplantation.<sup>25,44,45</sup> In addition, some other proteins were also altered in rabbit hearts following I/R injury.<sup>24,46</sup> However, these studies were conducted using whole cell homogenate without subcellular fractionation and functional characterization of those proteins altered during I/R injury.<sup>25,44,46</sup> In this study, we specifically aimed at identifying the oxidatively-modified target proteins by focusing on redox modulation of Cys residues and evaluating their functional roles in acute hepatic I/R injury, in the presence or absence of a SOD mimetic/peroxynitrite scavenger. The early time point of reperfusion (2-h) was intentionally chosen to identify early signs of oxidative modifications, which may lead to mitochondrial dysfunction prior to a secondary wave of severe liver damage inflicted by infiltrating immune cells during reperfusion for longer times.<sup>1,9,33,34</sup> Because of the involvement of neutrophils in the longer reperfusion periods, neutrophil-derived oxidative modifications and chlorination of proteins may take place and provide extra insult to the body system, contributing to more severe hepatotoxicity. In fact, our results in liver tissues reperfused for 10-h or 24-h showed fulminant liver damage (Supplementary Figures 4-7), despite similar degrees of inhibition of the mitochondrial enzymes measured in this study following reperfusion for 2-h versus 10-h or 24-h. These data support our hypothesis that mitochondrial dysfunction with decreased energy supply occurs prior to necrotic liver damage. These results also suggest that other events such as activation of the cell death-related JNK and/or p38 kinase likely occur and accelerate severe tissue damage.<sup>10-13</sup>

Numerous mitochondrial proteins involved in energy supply and electron transport system, molecular chaperone activity, anti-oxidant defense, the urea cycle, degradation of fatty acids, etc, have been simultaneously oxidized and *S*-nitrosylated (Fig. 4 and Table 1) during I/R injury, as observed in ethanol-exposed animals,<sup>23</sup> often leading to decreased activity/function. These results suggest that oxidative and nitrosative stress can simultaneously affect numerous proteins during the I/R injury process. Thus, it is extremely difficult to clearly separate oxidized proteins from *S*-nitrosylated or nitrated proteins especially for the liver tissues reperfused for longer periods. Although the activities of all of modified proteins have not been studied, their activities may have been attenuated as a result of oxidative modifications. For instance, ALDH2, ALDH5, ALDH6, and ALDH9 isozymes also have a highly conserved Cys residue within their active sites.<sup>40</sup> Although we did not show *S*-nitrosylation of Cys residue(s) within these ALDH isozymes, it is likely that these enzymes undergo *S*-nitrosylation, as recently demonstrated.<sup>22,23,40</sup> It was also reported that Cys323 is critical for the catalytic activity of glutamate dehydrogenase<sup>47</sup> and its activity is significantly inhibited during hepatic I/R injury.<sup>21</sup> Based on the inactivation of these critical enzymes (e.g. ALDH2 and ATP synthase) and possibly many others, we believe that oxidative modifications of the proteins listed in Table 1 likely contribute to mitochondrial dysfunction, leading to I/R-related cellular damage at later stage. Furthermore, inactivation of the mitochondrial respiratory chain complex enzymes through oxidative/nitrosative modifications lead to more ROS leakage with reduced efficiency of oxidative phosphorylation and decreased ATP synthesis.<sup>4-6,18,43</sup> Based on the positive correlation between the pattern of *S*-nitrosylated and nitrated proteins and hepatic injury (including ALT/AST levels, histology, nitrite and 3-NT levels, etc) and on the beneficial effects of MnTMPyP pre-treatment against hepatic damage, increased *i*NOS-derived NO coupled with superoxide formation and consequent generation of peroxynitrite play a critical role in causing I/R-mediated hepatic injury.<sup>7-9</sup>

Our current results conducted with mouse liver reperfused for 2-h show that many oxidatively-modified mitochondrial proteins exhibited multiple pI values on 2-DE. These multiple pI shifts may represent phosphorylations of Ser/Thr/Tyr residues by JNK and p38 kinase, which are known to be activated during the process of I/R injury.<sup>10-13</sup> Alternatively, multiple pI values of these proteins may represent hyper-oxidation of Cys residues to sulfinic and/or sulfonic acids that are not easily reduced by DTT or Asc.<sup>23</sup> We also identified smaller fragments of many mitochondrial proteins such as ATP synthase and 3-ketoacyl-CoA thiolase following I/R injury. These smaller protein fragments may increase the susceptibility of oxidized mitochondrial proteins to ubiquitin-dependent and -independent proteases activated under oxidative/nitrosative stress.<sup>30,48,49</sup> Alternatively, they may simply represent spontaneous fragmentation of oxidized proteins, as observed with cytosolic proteins in alcohol-exposed mouse livers.<sup>23,30</sup> Increased degradation of oxidatively-modified proteins may contribute to the reduced levels of several mitochondrial proteins, as recently reported using rabbit hearts exposed to I/R injury.<sup>24,46</sup> Furthermore, we expect that similar covalent modifications (e.g. phosphorylation) and/or fragmentations of mitochondrial proteins occur in 10-h or 24-h reperfused mice, based on the similar 2-DE patterns of oxidatively-modified proteins despite appearance of additional protein spots (Supplementary Figs. 8 and 9) compared to those in 2-h reperfused tissues (Fig. 4).

Oxidative modification of mitochondrial enzymes involved in the oxidation of fatty acids was also detected in this study. The inhibition of the mitochondrial fat oxidation pathway, as evidenced by inactivation of 3-ketoacyl-CoA thiolase, may explain the underlying mechanism for the low success rate in liver transplantation when steatotic livers are used as donor organs.<sup>11,50</sup> These results not only represent new information, but also imply possible limited energy supply derived from the  $\beta$ -oxidation pathway of fatty acids during I/R. In addition to blocking the fat oxidation pathway, inactivation of ATP synthase (Fig. 7) may explain the decreased level of ATP and thus favoring necrosis in I/R injury.<sup>15,17,19</sup> Furthermore, inactivation of



complex I of the respiratory chain and other enzymes such as ALDH isoenzymes, glutathione peroxidase and others listed in Table 1 may cause a marked imbalance between increased ROS/RNS during I/R and cellular antioxidant defense systems. Therefore, our current data supports the pivotal role of ROS/RNS in mitochondrial dysfunction and cell damage during hepatic I/R injury, and provides a possible underlying molecular mechanism(s).

In conclusion, numerous oxidatively-modified mitochondrial proteins from the livers of mice exposed to acute I/R injury have been identified. Inactivation of some of these oxidized/S-nitrosylated and nitrated proteins and others not tested herein, likely contribute to mitochondrial dysfunction and ultimate hepatic damage following acute hepatic ischemia-reperfusion.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgements

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## Abbreviations

I/R, ischemia-reperfusion  
 ALDH2, mitochondrial aldehyde dehydrogenase 2  
 ROS/RNS, reactive oxygen/nitrogen species  
 MnTMPyP, metalloporphyrin peroxynitrite scavenger/SOD mimetics  
 NO, nitric oxide  
*i*NOS, inducible nitric oxide synthase  
 Asc, sodium ascorbate  
 biotin-NM, biotin-*N*-maleimide  
 DTT, dithiothreitol  
 3-NT, 3-nitrotyrosine  
 2-DE, two-dimensional polyacrylamide gel electrophoresis  
 $\alpha$ -ATP synthase, ATP synthase  $\alpha$ -subunit  
 $\beta$ -ATP synthase, ATP synthase  $\beta$ -subunit  
 HSP, heat shock protein  
 GRP, glucose-regulated protein  
 MDA, malondialdehyde  
 AST, aspartate aminotransferase  
 ALT, alanine aminotransferase  
 Complex I, NADH—ubiquinone oxidoreductase  
 SOD, superoxide dismutase  
 H&E, hematoxylin and eosin  
 GPx, glutathione peroxidase

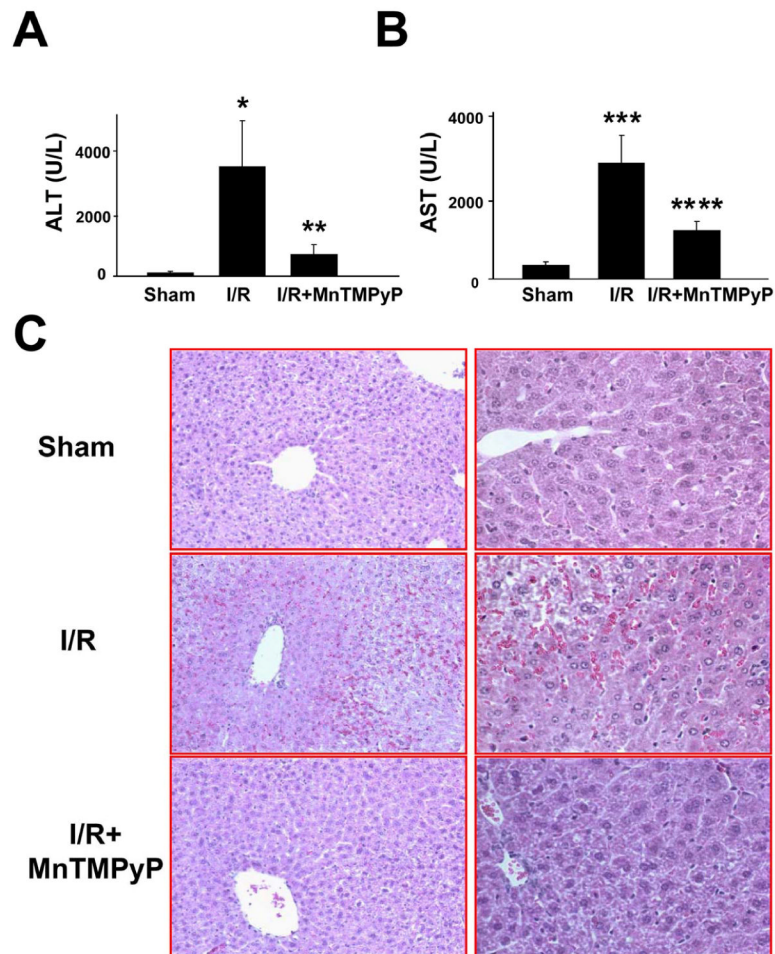
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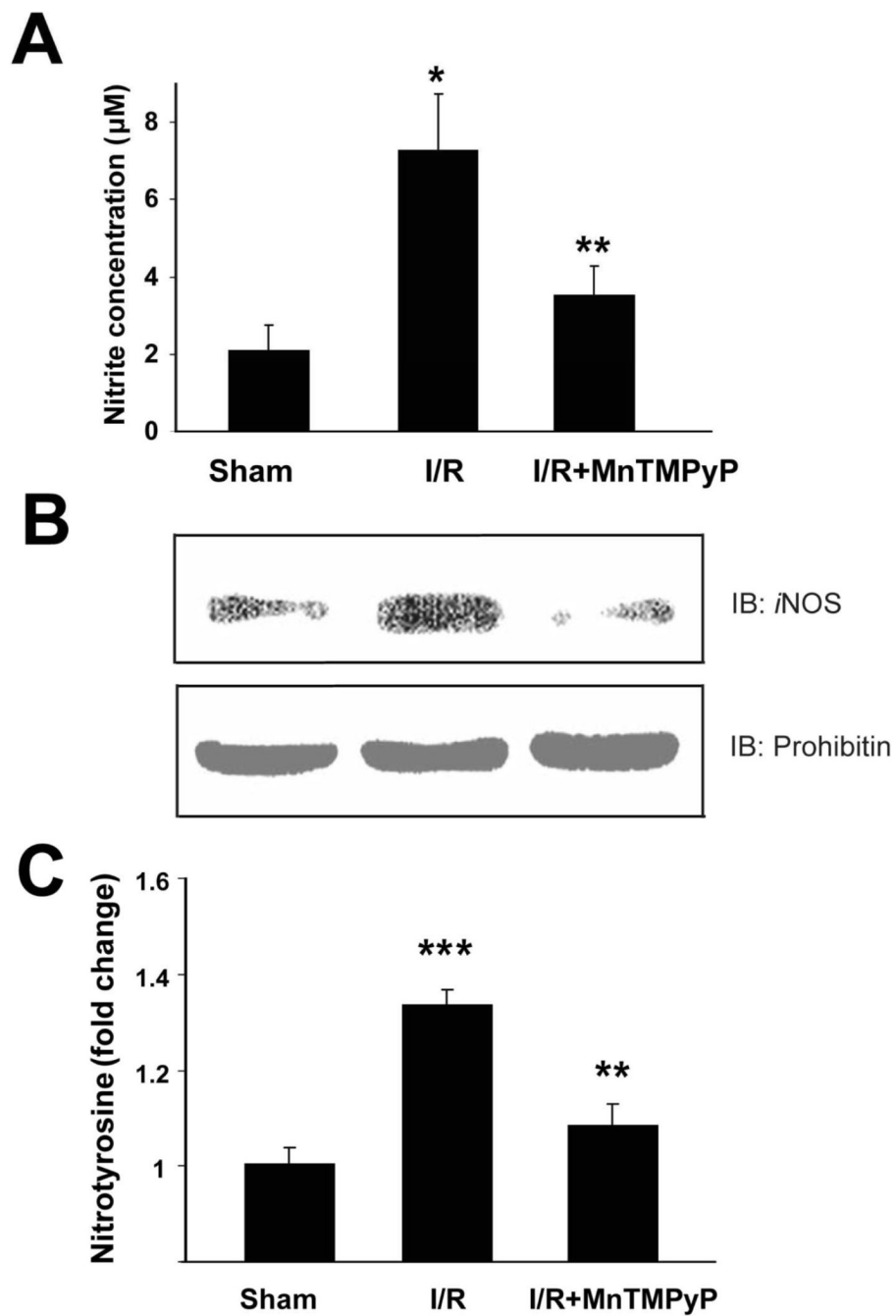
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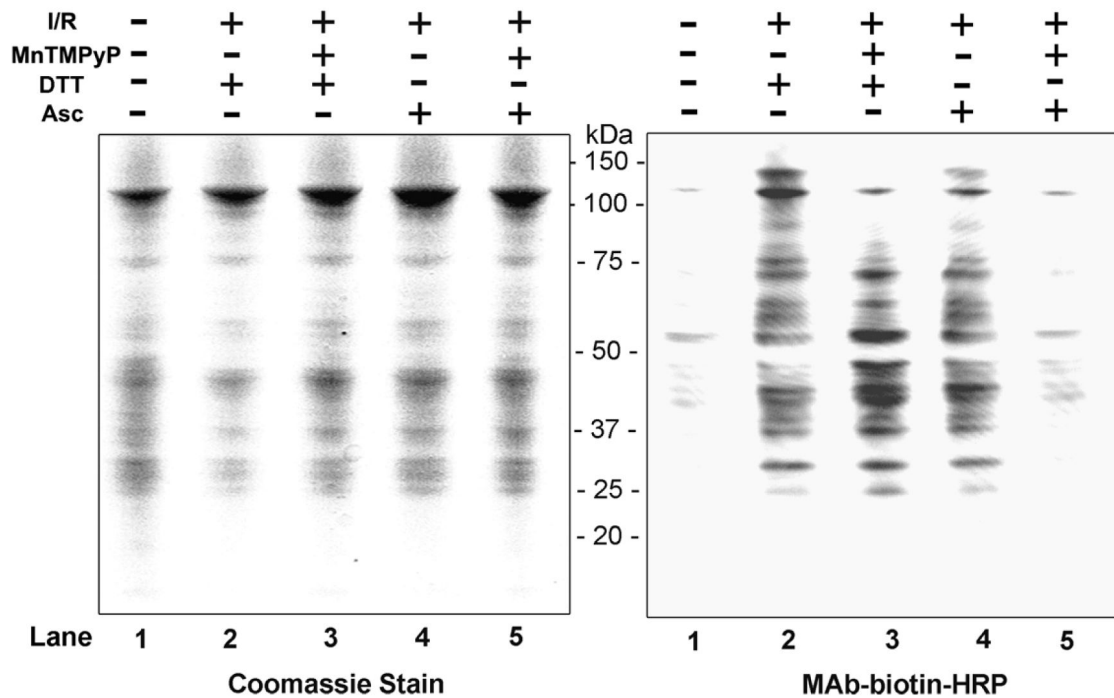


**Figure 1.**

The levels of serum transaminases and histological evaluation following I/R injury in the absence and presence of MnTMPyP. (A and B) Serum levels of ALT (A), and AST (B) following I/R injury (2-h reperfusion) without or with MnTMPyP are shown. (C) Typical H&E stained liver slides for different groups are also presented. Magnification: left panel, 100x; right panel, 400x. \*significantly different from the sham controls at  $p < 0.001$ ; \*\*significantly different from the I/R injured mice at  $p < 0.005$ ; \*\*\*significantly different from the sham controls at  $p < 0.0001$ ; \*\*\*\*significantly different from the I/R subjected samples at  $p < 0.0005$ .

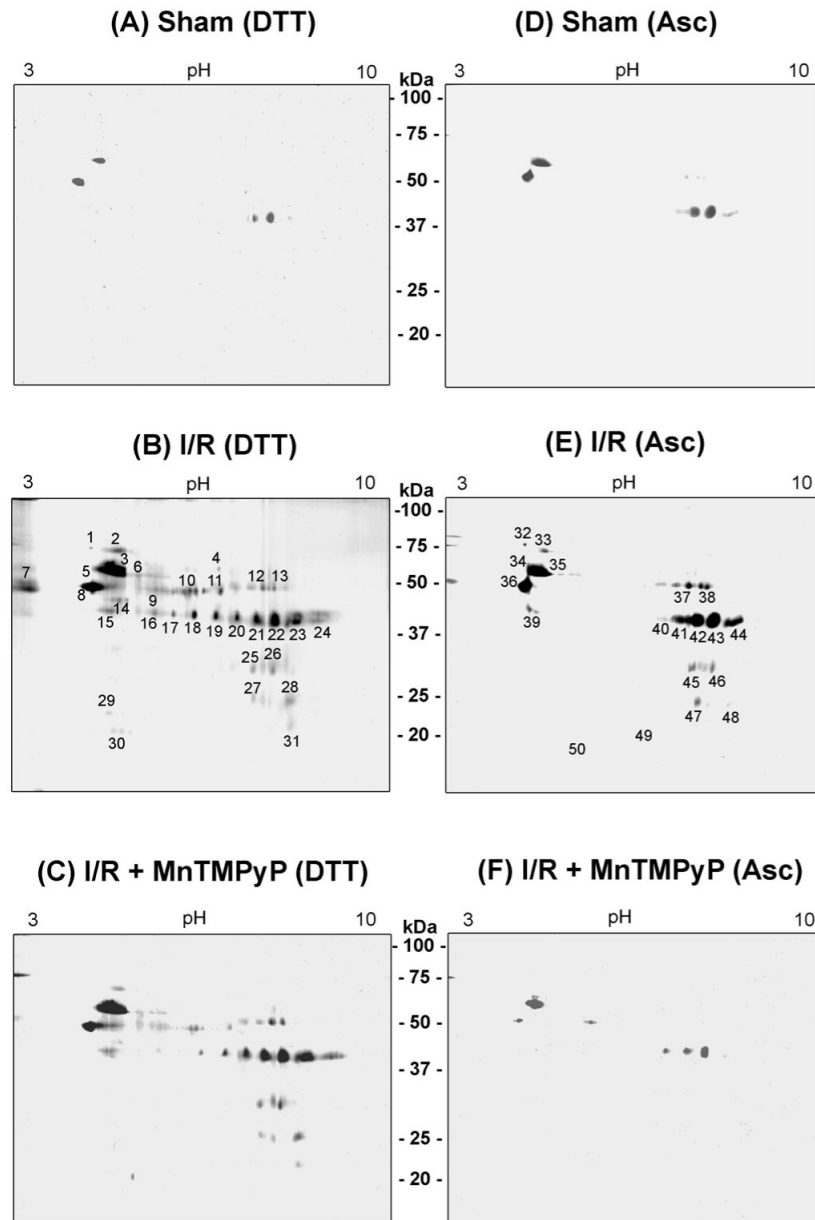


**Figure 2.** The hepatic levels of nitrite, *i*NOS, and nitrotyrosine following of I/R injury in the absence and presence of MnTMPyP. The hepatic levels of nitrite (A), *i*NOS (B), and nitrotyrosine (C) without or with MnTMPyP pretreatment are presented. \*significantly different from the sham control samples at  $p < 0.05$ ; \*\*significantly different from the I/R subjected samples (2-h reperfusion) at  $p < 0.05$ ; \*\*\*significantly different from the sham control samples at  $p < 0.005$ .



**Figure 3.**

Alteration of the levels of oxidatively-modified proteins in mouse livers following I/R injury. (Left panel) Equal amounts of mitochondrial proteins (20  $\mu\text{g}/\text{well}$ ) were isolated from sham-control or I/R injured mouse livers (2-h reperfusion) with and without MnTMPyP and analyzed by 12% SDS-PAGE subjected to Coomassie blue staining. (Right panel) Biotin-NM labeled oxidized- or S-nitrosylated-mitochondrial proteins (20  $\mu\text{g}/\text{well}$ ) were separated on 12 % SDS-PAGE, transferred to a PVDF-Immobilon membrane, and subjected to immunoblot analysis using HRP-conjugated monoclonal antibody against biotin (MAB-biotin-HRP). This figure represents a typical result from two separate experiments.

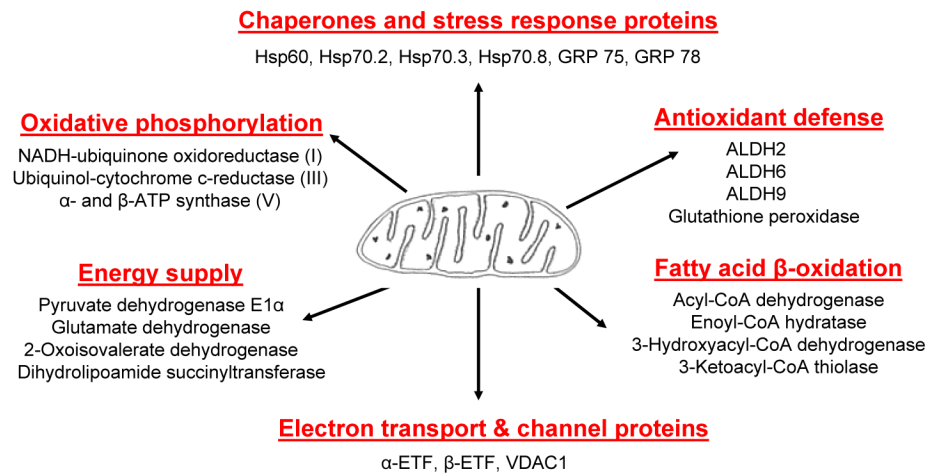


**Figure 4.**

Comparison of oxidized and S-nitrosylated mitochondrial proteins by 2-DE in hepatic I/R injury without or with MnTMPyP. Oxidized mitochondrial proteins (10 mg/sample) from sham-control (A) and I/R injured mice (2-h reperfusion) without (B) or with MnTMPyP (C) (I/R + MnTMPyP) were processed concurrently processed, labeled with biotin-NM in the presence of DTT, and then purified with streptavidin-agarose. S-nitrosylated mitochondrial proteins (10 mg/sample) from sham-control (D) and I/R injured mice (2-h reperfusion) without (E) or with MnTMPyP (F) were labeled with biotin-NM in the presence of Asc and then purified with streptavidin-agarose. Purified biotin-NM labeled oxidized (spots 1-31) and S-nitrosylated (spots 32-50) proteins (0.25 mg/sample) were resolved by 2-DE, and silver stained. Individual protein spots with differential intensities were excised out of this particular gel (pH range 3-10) and subjected to MS analysis following in-gel trypsin digestion. This figure represents a typical result from two separate experiments.

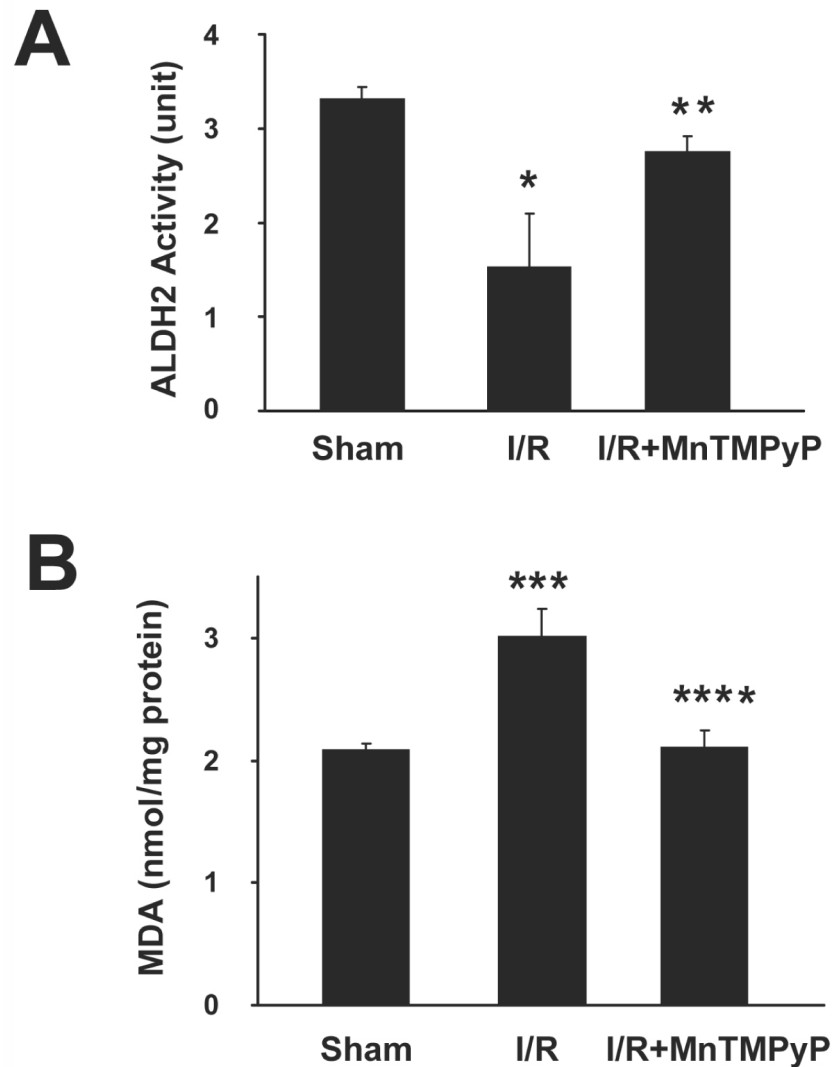


### Oxidatively-modified mitochondrial proteins following hepatic ischemia-reperfusion injury

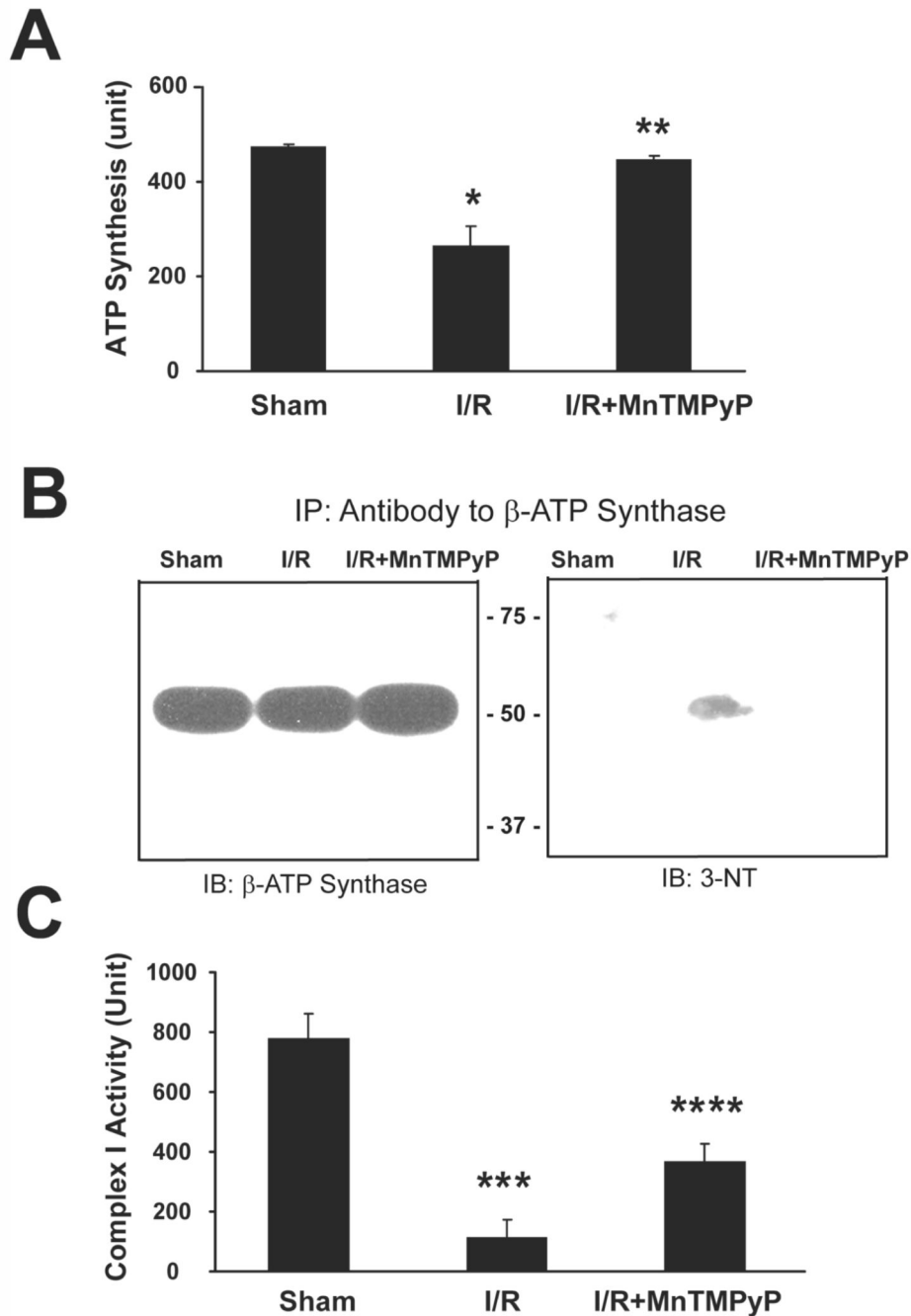


**Figure 5.**

Summary of oxidized and *S*-nitrosylated mitochondrial proteins following hepatic I/R injury in the absence of MnTMPyP. Mitochondrial proteins that are simultaneously oxidized- and *S*-nitrosylated proteins are summarized with respects to the function of each protein.



**Figure 6.** Inactivation of mitochondrial ALDH2 activity and the level of lipid peroxidation in I/R injured mice without or with MnTMPyP. (A) Catalytic activities of mitochondrial ALDH2 from the indicated liver samples (2-h reperfusion) were determined. (B) Hepatic malondialdehyde (MDA) levels in different mice were measured and presented. \*significantly different from the sham controls at  $p < 0.05$ ; \*\*different from the I/R subjected samples at  $p = 0.056$ ; \*\*\*significantly different from the sham controls at  $p < 0.025$ ; \*\*\*\*significantly different from the I/R subjected samples at  $p < 0.01$ .



**Figure 7.** Inhibition of ATP synthase and complex I activities following hepatic I/R injury and nitration of Tyr residue in  $\beta$ -ATP synthase. (A) Mitochondrial ATP synthase activities from sham control and I/R injured mouse livers (2-h reperfusion) without or with MnTMPyP (I/R + MnTMPyP) are presented. (B) Mitochondrial  $\beta$ -ATP synthase (1 mg proteins) in different groups as indicated were immunoprecipitated with the anti- $\beta$ -ATP synthase antibody as described,<sup>23</sup> separated on 12% SDS-PAGE, and subjected to immunoblot analysis using the anti- $\beta$ -ATP synthase antibody (left) or the anti-3-NT antibody (right). (C) NADH-ubiquinone oxidoreductase (Complex I) activities in indicated groups were determined and presented. \*significantly different from the sham controls at  $p < 0.05$ ; \*\*significantly different from the

I/R subjected samples at  $p < 0.05$ ; \*\*\*significantly different from the sham controls at  $p < 0.001$ ;  
\*\*\*\*significantly different from the I/R subjected samples at  $p < 0.01$ .

**Table 1**

Summary of nanoRP LC-MS/MS peptide sequence analyses for oxidized and *S*-nitrosylated proteins in ischemia/reperfusion injury mouse liver mitochondria.

Spot No.	Protein Identified	Accession Number	No. of Peptides Identified
1	78 kDa glucose-regulated protein (GRP 78)	P200029	20
2	75 kDa glucose-regulated protein (GRP 75)	P38647	14
2	Heat shock cognate 71 kDa protein (Hsp 70.8)	P63017	5
2	Heat shock-related 70 kDa protein 2 (Hsp 70.2)	P17156	4
2	Heat shock 70 kDa protein 1A (Hsp 70.3)	Q61696	2
3	60 kDa heat shock protein, mitochondrial (Hsp 60)	P63038	6
4	Not identified		
5	60 kDa heat shock protein, mitochondrial (Hsp 60)	P63038	33
5	ATP synthase beta chain, mitochondrial ( $\beta$ -ATP synthase)	P56480	2
6	Protein disulfide isomerase A3 precursor	Q3TIL2	16
6	ATP synthase alpha chain, mitochondrial ( $\alpha$ -ATP synthase)	P02535	4
6	60 kDa heat shock protein, mitochondrial (Hsp 60)	P63038	2
7	ATP synthase beta chain, mitochondrial ( $\beta$ -ATP synthase)	P56480	6
7	Aldehyde dehydrogenase 2, mitochondrial (ALDH2)	P47738	2
8	ATP synthase beta chain, mitochondrial ( $\beta$ -ATP synthase)	P56480	21
8	Aldehyde dehydrogenase 2, mitochondrial (ALDH2)	P47738	2
9	Aldehyde dehydrogenase 2, mitochondrial (ALDH2)	P47738	16
9	Dihydrolipoamide succinyltransferase	Q9D2G2	6
9	60 kDa heat shock protein, mitochondrial (Hsp 60)	P63038	2
9	Carbamoyl-phosphate synthase	Q8C196	2
9	Dihydrolipoamide branched chain transacylase	P53395	2
9	Pyruvate dehydrogenase protein X component (PDH-X)	Q8BKZ9	2
10	Aldehyde dehydrogenase 2, mitochondrial (ALDH2)	P47738	13
10	4-Trimethylaminobutyraldehyde dehydrogenase (ALDH9)	Q3TG52	3
11	Aldehyde dehydrogenase 2, mitochondrial (ALDH2)	P47738	16
11	4-Trimethylaminobutyraldehyde dehydrogenase (ALDH9)	Q3TG52	2
11	Hydroxymethylglutaryl-CoA synthase, mitochondrial	P54869	2
12	ATP synthase alpha chain, mitochondrial ( $\alpha$ -ATP synthase)	P02535	5
12	Methylmalonate-semialdehyde dehydrogenase (ALDH6)	Q3TDA2	5
13	Methylmalonate-semialdehyde dehydrogenase (ALDH6)	Q3TDA2	6
13	ATP synthase alpha chain, mitochondrial ( $\alpha$ -ATP synthase)	P02535	4
14	Ubiquinol-cytochrome-c reductase complex core protein I	Q9CZ13	9
15	Not identified		
16	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	10
16	Isovaleryl-CoA dehydrogenase, mitochondrial	Q9JH15	2
17	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	12
17	2-Oxoisovalerate dehydrogenase alpha subunit, mitochondrial	P50136	2
18	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	15
18	2-Oxoisovalerate dehydrogenase alpha subunit, mitochondrial	P50136	3
18	Isovaleryl-CoA dehydrogenase, mitochondrial	Q9JH15	2
19	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	12
19	Isovaleryl-CoA dehydrogenase, mitochondrial	Q9JH15	3
20	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	16
20	Carbamoyl-phosphate synthase, mitochondrial	Q8C196	3
20	Isovaleryl-CoA dehydrogenase, mitochondrial	Q9JH15	3
20	Acetyl-CoA acetyltransferase, mitochondrial	Q8QZT1	2
21	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	17
21	Acetyl-CoA acetyltransferase, mitochondrial	Q8QZT1	5
21	3-Ketoacyl-CoA thiolase A, peroxisomal	Q921H8	3
21	Acyl-CoA dehydrogenase, medium-chain, mitochondrial	P45952	3
22	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	24
22	Acetyl-CoA acetyltransferase, mitochondrial	Q8QZT1	7
22	3-Ketoacyl-CoA thiolase A, peroxisomal	Q921H8	4
22	Acyl-CoA dehydrogenase, medium-chain, mitochondrial	P45952	4
22	Carbamoyl-phosphate synthase, mitochondrial	Q8C196	4
23	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	18
23	Acetyl-CoA acetyltransferase, mitochondrial	Q8QZT1	5
23	3-Ketoacyl-CoA thiolase A, peroxisomal	Q921H8	4
24	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	21
24	3-Ketoacyl-CoA thiolase A, peroxisomal	Q921H8	7
24	Acetyl-CoA acetyltransferase, mitochondrial	Q8QZT1	3
25	Electron transfer flavoprotein $\alpha$ -subunit ( $\alpha$ -ETF)	Q99LC5	4
26	Electron transfer flavoprotein $\alpha$ -subunit ( $\alpha$ -ETF)	Q99LC5	4
26	Hydroxymethylglutaryl-CoA lyase, mitochondrial	P38060	2
27	Electron transfer flavoprotein, $\beta$ -subunit ( $\beta$ -ETF)	Q810V3	6
27	ATP synthase alpha chain, mitochondrial ( $\alpha$ -ATP synthase)	P02535	5
28	Electron transfer flavoprotein, $\beta$ -subunit ( $\beta$ -ETF)	Q810V3	6
28	3-Hydroxyacyl-CoA dehydrogenase type II	O08756	3
28	Enoyl-CoA hydratase, mitochondrial	Q8BH95	3

Spot No.	Protein Identified	Accession Number	No. of Peptides Identified
28	Hydroxyacyl-CoA dehydrogenase type II	Q99N15	2
28	ATP synthase alpha chain, mitochondrial ( $\alpha$ -ATP synthase)	P02535	2
29	NADH-ubiquinone oxidoreductase 24 kDa subunit, mitochondrial	Q9D6J6	2
30	ATP synthase D chain, mitochondrial	Q9DCX2	8
31	Glutathione S-transferase P 1	P19157	4
31	Glutathione peroxidase 1 (GPx)	P11352	3
31	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	2
31	Acyl-protein thioesterase 1	P97823	2
32	78 kDa glucose-regulated protein (GRP 78)	P200029	14
33	Stress-70 protein, mitochondrial (GRP 75)	P38647	22
33	Heat shock cognate 71 kDa protein (HSP 70.8)	P63017	10
33	78 kDa glucose-regulated protein (GRP 78)	P200029	6
33	60 kDa heat shock protein, mitochondrial (Hsp 60)	P63038	5
33	Carbamoyl-phosphate synthase, mitochondrial	Q8C196	5
33	Heat shock-related 70 kDa protein 2 (Hsp 70.2)	P17156	5
33	Pyruvate carboxylase	Q3T9S7	2
33	Heat shock 70 kDa protein 1A (Hsp 70.3)	Q61696	2
34	60 kDa heat shock protein, mitochondrial (Hsp 60)	P63038	54
34	ATP synthase beta chain, mitochondrial ( $\beta$ -ATP synthase)	P56480	4
34	Methylmalonate-semialdehyde dehydrogenase (ALDH6)	Q3TDA2	2
35	60 kDa heat shock protein, mitochondria (Hsp 60)	P63038	15
35	Protein disulfide isomerase A3 precursor	Q3TIL2	12
35	Methylmalonate-semialdehyde dehydrogenase (ALDH6)	Q3TDA2	2
35	ATP synthase alpha chain, mitochondrial ( $\alpha$ -ATP synthase)	P02535	2
36	ATP synthase beta chain, mitochondrial ( $\beta$ -ATP synthase)	P56480	40
36	Aldehyde dehydrogenase 2, mitochondrial (ALDH2)	P47738	9
36	60 kDa heat shock protein, mitochondrial (Hsp 60)	P63038	2
36	4-Trimethylaminobutyraldehyde dehydrogenase (ALDH9)	Q3TG52	2
36	Protein disulfide isomerase A3 precursor	Q3TIL2	2
37	ATP synthase alpha chain, mitochondrial ( $\alpha$ -ATP synthase)	P02535	19
37	Methylmalonate-semialdehyde dehydrogenase (ALDH6)	Q3TDA2	10
37	Glutamate dehydrogenase 1, mitochondrial (GDH)	P26443	6
37	Carbamoyl-phosphate synthase, mitochondrial	Q8C196	5
37	ATP synthase beta chain, mitochondrial ( $\beta$ -ATP synthase)	P56480	2
38	ATP synthase alpha chain, mitochondrial ( $\alpha$ -ATP synthase)	P02535	19
38	Methylmalonate-semialdehyde dehydrogenase (ALDH6)	Q3TDA2	9
38	Carbamoyl-phosphate synthase, mitochondrial	Q8C196	2
39	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	5
39	Succinyl-CoA ligase beta-chain, mitochondrial	Q9Z2I9	3
39	ATP synthase beta chain, mitochondrial ( $\beta$ -ATP synthase)	P56480	2
40	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	15
40	Acetyl-CoA acetyltransferase, mitochondrial	Q8QZT1	5
40	Pyruvate dehydrogenase E1, alpha subunit	P35486	4
40	2-Oxoisovalerate dehydrogenase alpha subunit	P50136	2
40	Acyl-CoA dehydrogenase, medium-chain	P45952	2
41	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	17
41	Acetyl-CoA acetyltransferase, mitochondrial	Q8QZT1	6
41	Acyl-CoA dehydrogenase, medium-chain	P45952	4
41	Fumaryl acetoacetase	P35505	4
41	Isovaleryl-CoA dehydrogenase, mitochondrial	Q9JH15	3
41	Acetyl-CoA acyltransferase A	Q921H8	2
41	Carbamoyl-phosphate synthase, mitochondrial	Q8C196	2
42	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	24
42	Acetyl-CoA acetyltransferase, mitochondrial	Q8QZT1	7
42	Acyl-CoA dehydrogenase, medium-chain	P45952	6
42	Carbamoyl-phosphate synthase, mitochondrial	Q8C196	5
42	Acetyl-CoA acyltransferase A	Q921H8	3
42	Hydroxyacyl-CoA dehydrogenase	Q5U5Y5	2
43	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	32
43	Acetyl-CoA acetyltransferase, mitochondrial	Q8QZT1	9
43	Acyl-CoA dehydrogenase, medium-chain	P45952	6
43	Carbamoyl-phosphate synthase, mitochondrial	Q8C196	2
44	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	24
44	Acetyl-CoA acetyltransferase, mitochondrial	Q8QZT1	9
44	Acetyl-CoA acyltransferase A	Q921H8	4
44	Aspartate aminotransferase, mitochondrial	P05202	2
44	Hydroxyacyl-CoA dehydrogenase	Q5U5Y5	2
45	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	6
45	Electron transfer flavoprotein $\alpha$ -subunit ( $\alpha$ -ETF)	Q99LC5	6
45	Voltage-dependent anion-selective channel protein 1 (VDAC1)	Q60932	3
45	ATP synthase alpha chain, mitochondrial ( $\alpha$ -ATP synthase)	P02535	2
45	Hydroxymethylglutaryl-CoA lyase, mitochondrial	P38060	2
46	Electron transfer flavoprotein $\alpha$ -subunit ( $\alpha$ -ETF)	Q99LC5	7

Spot No.	Protein Identified	Accession Number	No. of Peptides Identified
46	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	5
47	Electron transfer flavoprotein, $\beta$ -subunit ( $\beta$ -ETF)	Q810V3	12
47	3-Hydroxyacyl-CoA dehydrogenase type II	O08756	6
47	Short chain L-3-hydroxyacyl-CoA dehydrogenase	Q99N15	4
48	Electron transfer flavoprotein, $\beta$ -subunit ( $\beta$ -ETF)	Q810V3	5
48	ATP synthase alpha chain, mitochondrial ( $\alpha$ -ATP synthase)	P02535	4
48	Short chain L-3-hydroxyacyl-CoA dehydrogenase	Q99N15	3
48	Enoyl-CoA hydratase, mitochondrial	Q8BH95	3
48	Glutathione S-transferase P 1	P19157	3
48	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	2
48	Trifunctional enzyme beta subunit, mitochondrial	Q99JY0	2
49	ATP synthase alpha chain, mitochondrial ( $\alpha$ -ATP synthase)	P02535	16
49	Glutathione peroxidase 1 (GPx)	P11352	7
49	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	4
49	Glutathione S-transferase P 1	P19157	3
50	ATP synthase D chain, mitochondrial	Q9DCX2	11
50	Glutathione peroxidase 1 (GPx)	P11352	3
50	Glutathione S-transferase P 1	P19157	3
50	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	2
50	ATP synthase alpha chain, mitochondrial ( $\alpha$ -ATP synthase)	P02535	2

Biotin-NM labeled oxidized- or *S*-nitrosylated proteins were purified with streptavidinagarose beads, washed twice, resolved by 2-DE, and silver stained. Each protein spot as indicated was picked up with a razor blade and subjected to protein identification by mass spectrometric analysis.<sup>23,29,30</sup> Since our original aim was to identify early signs of oxidative modifications, we only identified the oxidatively-modified proteins after 2-h reperfusion.