GASTROENTEROLOGY 2008;135:1344–1357

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

KWAN–HOON MOON,* BRIAN L. HOOD,‡ PARTHA MUKHOPADHYAY,§ MOHANRAJ RAJESH,§
MOHAMED A. ABDELMEGEED,* YONG–IL KWON,* THOMAS P. CONRADS,‡ TIMOTHY D. VEENSTRA,‡
BYOUNG–JOON SONG,* and PAL PACHER§

*Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, Bethesda; ‡Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, Inc, NCI-Frederick, Frederick; and §Section on Oxidative Stress and Tissue Injury, Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, Bethesda, Maryland

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 2-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 activities of oxidatively modified mitochondrial proteins were measured. Results: Comparative 2-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, histologic damage, increased inducible nitric oxide synthase expression, and *S*-nitrosylation and/or nitration of various key mitochondrial proteins. MnTMPyP pretreatment also restored I/R-induced suppressed activities of mitochondrial aldehyde dehydrogenase, 3-ketoacyl-CoA thiolases, and adenosine triphosphate 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 increased hepatic injury.

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

At earlier stages, increased superoxide and other ROS, derived from the activation of various sources (eg, xathine oxidoreductases[2,3]), play a critical role in tissue damage. The activities of the complex enzymes in the mitochondrial respiratory chain are also inhibited,[4–6] 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 inducible nitric oxide synthase [iNOS] induction) favors the formation of the more potent oxidant peroxynitrite ($ONOO^-$),[7] causing impaired cellular functions.[8]

Abbreviations used in this paper: 2-DE, 2-dimensional polyacrylamide gel electrophoresis; 3-NT, 3-nitrotyrosine; α-ATP synthase, ATP synthase α-subunit; ALDH2, mitochondrial aldehyde dehydrogenase 2; Asc, L-sodium ascorbate; β-ATP synthase, ATP synthase β-subunit; biotin-NM, biotin-N-maleimide; complex I, NADH-ubiquinone oxidoreductase; DTT, dithiothreitol; HSP, heat shock protein; iNOS, inducible nitric oxide synthase; I/R, ischemia-reperfusion; MDA, malondialdehyde; MnTMPyP, metalloporphyrin peroxynitrite scavenger/SOD mimetics; ROS/RNS, reactive oxygen/nitrogen species; SOD, superoxide dismutase.

0016-5085/08/$34.00
doi:10.1053/j.gastro.2008.06.048

After a longer period of reperfusion, increased amounts of proinflammatory cytokines produced from resident macrophage-Kupffer cells and infiltrating leukocytes are critical in inflicting tissue damage.[9] Both elevated ROS/RNS and proinflammatory cytokines such as tumor necrosis factor-α also activate various cell death signaling pathways via c-Jun-N-terminal kinase (JNK)[10–12] and p38 kinase,[12,13] leading to apoptosis and/or necrosis.[10,11,14–18] Consistent with the importance of these pathways, a variety of pharmacologic agents such as specific JNK inhibitors[11] and various antioxidants (eg, ascorbate, glutathione, *S*-adenosylmethionine, vitamine E) attenuate the severity of I/R-mediated liver injury.[3,8,19–21] Accumulation of oxidatively modified proteins, lipid peroxides, and nucleic acids may lead to mitochondrial dysfunction and tissue damage.[8,9,21–25] Despite numerous reports on the role of oxidative stress and mitochondrial dysfunction in hepatic I/R injury,[4–9,26–28] 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.[23,29,30] 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),[20,31,32] 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-β-ATP synthase antibody, dithiothreitol (DTT), MnTMPyP, and L-sodium ascorbate (Asc) were obtained from Sigma Chemical Co (St. Louis, MO). Specific antibodies to prohibitin or horseradish peroxidase-conjugated monoclonal antibody (mAb)-biotin were purchased from Oncogene Science (Cambridge, MA) and Cell Signaling (Beverly, MA), respectively. Specific antibodies to iNOS and 3-nitrotyrosine (3-NT) were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA) and Upstate Biotechnologies (Lake Placid, NY), respectively.

Animals, Hepatic I/R Protocol, and Histologic Analysis

All animal experiments were conducted in accordance with the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee. Young male C57BL/6J mice from Jackson Laboratory (Bar Harbor, ME) were anesthetized with a single dose of pentobarbital (65 mg/kg, intraperitoneally [IP]), and a midline laparotomy incision was performed to expose the liver of anesthesized mice as described.[33,34] 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, IP) 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 hour, after which the microaneurysm clamps were removed. The duration of reperfusion was 2 hours (or 10 hours and 24 hours shown in Supplementary Figures; see Supplementary material online at www.gastrojournal.org). 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 histopathologic evaluation. After embedding formalin-fixed liver tissues and cutting 5-μm slices, all sections were stained with H&E. Histologic evaluation was performed in a blinded manner.[33,34]

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

The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured in serum samples using a clinical chemistry analyzer system (PROCHEM-V; Drew Scientific, Oxford, CT).[23,33,34] 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 nmol/L ATP produced/min/mg protein at ambient temperature. NADH-ubiquinone oxidoreductase (complex I) activity was determined by measuring optical density at 340 nm and subtracting the optical density values in the presence of rotenone, an inhibitor of NADH-ubiquinone oxidoreductase.[35] 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 ≥ 5 per group) treated differently using differential centrifugation followed by 2 separate washing steps, as described.[22,23,29] Relative purities of the mitochondrial fractions were determined by immunoblot analysis using a specific antibody against mitochondrial α-ATP synthase or cytosolic peroxiredoxin-II. Little contamination with cytosolic proteins in our mitochondrial fractions and vice versa was observed (Supplementary Figure 1; see Supplementary material online at www.gastrojournal.org). Labeling of oxidatively modified proteins with biotin-NM was performed concurrently for 3 different groups including the sham-operated mice (Supplementary Figure 2; see Supplementary material online at www.gastrojournal.org).[23,29,30] Purified biotin-NM-labeled oxidized and *S*-nitrosylated proteins bound to the streptavidin-agarose beads were washed twice to remove nonspecifically bound proteins before being resolved using 2-dimensional polyacrylamide gel electrophoresis (2-DE) and were 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.[23,29,30]

Data Processing and Statistical Analysis

All data in this report represent results from at least 3 separate experiments, unless stated otherwise. Statistical analyses were performed using the Student *t* test, with $P < .05$ being considered statistically significant. Other materials and methods not described here were performed as previously described.[23,29,30,33,34]

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 hour of warm ischemia followed by reperfusion for 2 hours, serum ALT and AST activities were increased ~33-fold and 8-fold, respectively, compared with sham-operated controls (Figure 1*A* and *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 hours and 24 hours compared with sham controls, although much higher ALT and AST levels were detected in mice reperfused for 10 hours than 24 hours (Supplementary Figure 3; see Supplementary material online at www.gastrojournal.org). However, MnTMPyP significantly suppressed the elevated ALT and AST levels in I/R-injured mice. Sham-operated mice showed normal hepatic histology (Figure 1*C*, *top*). In contrast, increased signs of blood cell infiltration with sinusoidal congestion and some necrotic hepatocytes were observed in mouse livers reperfused for 2 hours (Figure 1*C*, *middle*). These initial signs of I/R-induced tissue injury were markedly attenuated in mice pretreated with MnTMPyP (Figure 1*C*, *bottom*). Liver histology data also showed marked necrosis after reperfusion for 10 hours (Supplementary Figures 4 and 5; see Supplementary material online at www.gastrojournal.org) or 24 hours (Supplementary Figures 6 and 7; see Supplementary material online at www.gastrojournal.org). MnTMPyP significantly attenuated the histopathological damage under these conditions (Supplementary Figures 4–7).

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

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

Identification of Oxidatively Modified Mitochondrial Proteins in I/R Injury

Increased ROS/RNS, which can be effectively prevented using various antioxidants, plays an important role in hepatic I/R injury.[7,8,18–21] Because of elevated levels of nitrite, iNOS, 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 vs *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) vs *S*-nitrosylated (and/or *S*-glutathionylated) proteins (Supplementary Figure 2 for overview).[23] 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

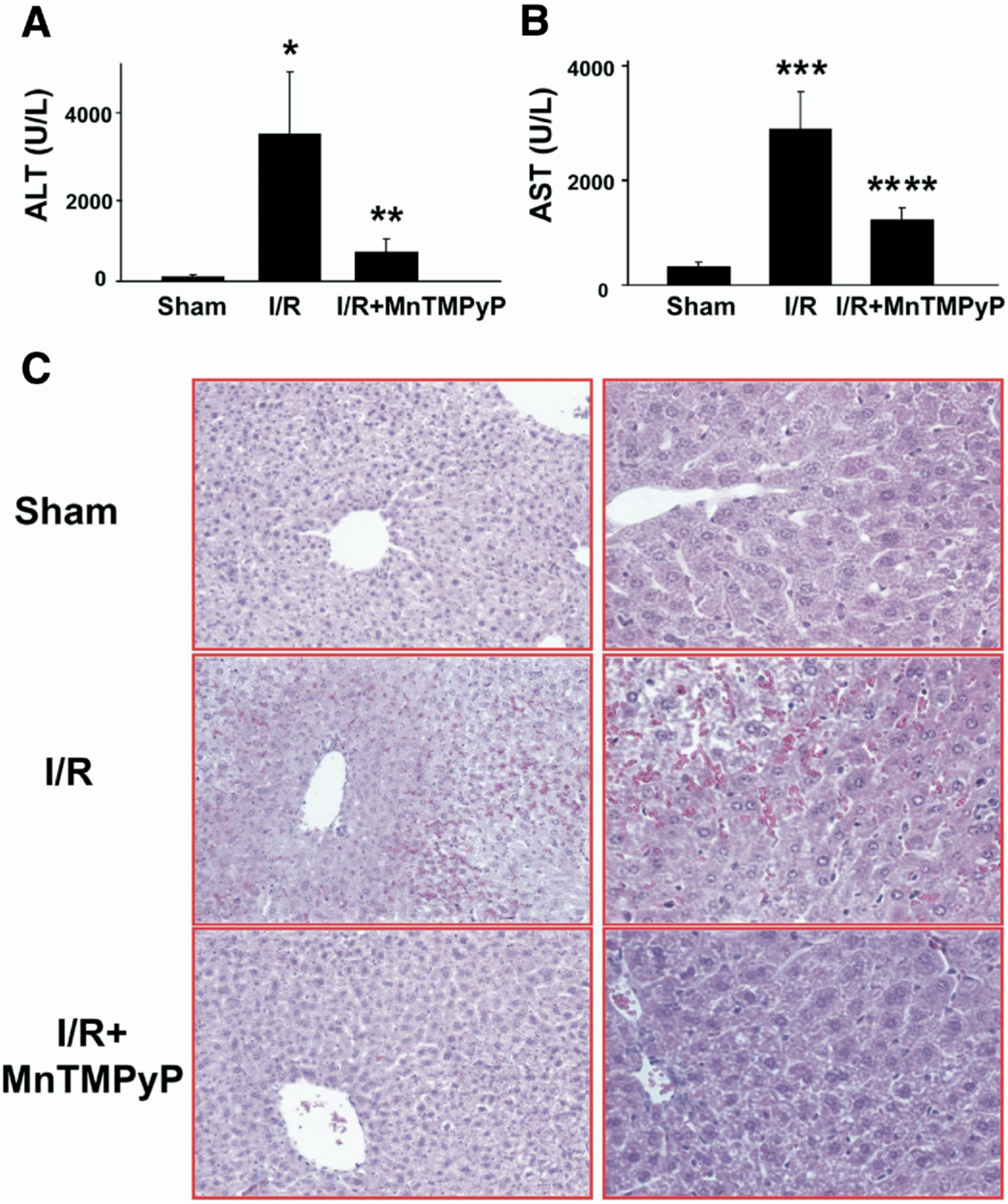

Figure 1. The levels of serum transaminases and histologic evaluation following I/R injury in the absence and presence of MnTMPyP. (*A* and *B*) Serum levels of ALT (*A*) and AST (*B*) following I/R injury (2-hour reperfusion) without or with MnTMPyP are shown. (*C*) Typical H&E-stained liver slides for different groups are also presented. Original magnification: *left panel,* ×100; right panel, ×400. *Significantly different from the sham controls at $P < .001$; **significantly different from the I/R injured mice at $P < .005$; ***significantly different from the sham controls at $P < .0001$; ****significantly different from the I/R subjected samples at $P < .0005$.

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 (Figure 3, *right panel*, *lane 1*). The levels of biotin-NM-labeled oxidized proteins (identified after DTT treatment: Figure 3, *lane 2*) or *S*-nitrosylated proteins (identified after Asc treatment: Figure 3, *lane 4*) were markedly elevated in I/R injured mice (2-hour reperfusion) compared with 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 (Figure 3, *right panel*, *lane 5*). In contrast, MnTMPyP did not decrease the intensity of oxidized (disulfides and/or sulfenic acids) proteins (Figure 3, *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 vs *S*-nitrosylated (and/or *S*-glutathionylated) proteins (Figure 4).[23] 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 (Figure 4*A* and *D*, respectively). The number and abundance of oxidized (Figure 4*B*) and *S*-nitrosylated proteins (Figure 4*E*) found in mouse livers reperfused for 2 hours, however, were markedly increased compared with sham controls. Our

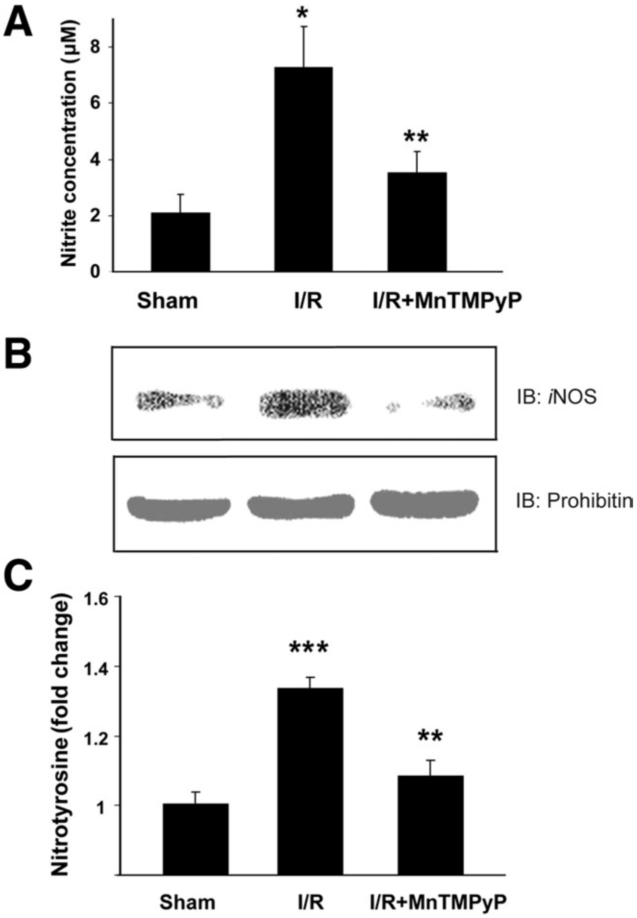

Figure 2. The hepatic levels of nitrite, iNOS, and nitrotyrosine following of I/R injury in the absence and presence of MnTMPyP. The hepatic levels of nitrite (*A*), iNOS (*B*), and nitrotyrosine (*C*) without or with MnTMPyP pretreatment are presented. *Significantly different from the sham control samples at $P < .05$; **significantly different from the I/R subjected samples (2-hour reperfusion) at $P < .05$; ***significantly different from the sham control samples at $P < .005$.

results showed that MnTMPyP did not decrease the levels of oxidized proteins (Figure 4*C*); however, it drastically decreased the levels of *S*-nitrosylated proteins when mouse livers were reperfused for 2 hours (Figure 4*F*). More oxidized and *S*-nitrosylated proteins (Supplementary Figures 8 and 9, respectively; see Supplementary material online at www.gastrojournal.org) were observed after reperfusion for 10 hours and 24 hours 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 Figure 8), although a greater effect of MnTMTyP seems to be observed with the *S*-nitrosylated proteins (ie, Asc-treated samples in Supplementary Figure 9). However, it is unclear why MnTMPyP reversed the oxidized proteins after reperfusion for 10 hours but not for 2 hours.

Thirty-one oxidized and 19 *S*-nitrosylated proteins acquired from mouse liver mitochondria after I/R injury (2-hour reperfusion) that showed an increased staining intensity (Figure 4*B* and *E*) were subjected to mass spectrometry analysis. The protein sequence data acquired for each spot are provided in Table 1 and summarized in Figure 5. Protein sequence analysis revealed that many mitochondrial proteins are identified as being oxidized as well as *S*-nitrosylated, suggesting that these 2 modifications occur concurrently after I/R injury. These proteins include the following: oxidative phosphorylation (NADH-ubiquinone oxidoreductase [complex I], ubiquinol-cytochrom-c-reductase [complex III], and α- and β-ATP synthase [complex V]); antioxidative defense system (aldehyde dehydrogenase [ALDH2], methylmalonate-semialdehyde dehydrogenase [ALDH6], 4-trimethylaminobutyraldehyde dehydrogenase [ALDH9], glutathione transferase, glutathione peroxidase, and others); fatty acid β-oxidation and intermediary metabolism (acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacy-CoA dehydrogenase, 3-ketoacyl-CoA thiolase, acetyl-CoA acetyltransferase, hydroxymethylglutaryl-CoA synthase, hydroxymethylglutaryl-CoA lyase, isovaleryl-CoA dehydrogenase, carbamoyl-phosphate synthase, and others); TCA cycle and energy supply (pyruvate dehydrogenase, dihydrolipoamide succinyltransferase, glutamate dehydrogenase, pyruvate carboxylase, and others); molecular chaperon proteins (heat shock protein [Hsp] 60, Hsp 70.2, Hsp 70.3, Hsp 70.8 and glucose-regulated protein [GRP] 75, GRP78, and others); mitochondrial electron transport and ion channeling proteins (α- or β-subunit of electron transfer flavoprotein and voltage-dependent anion channel protein1).

Many proteins such as ATP synthase (Figure 4; *spots 12, 13, 37, 38*), ALDH2 (Figure 4; *spots 7–11*), ALDH6 (Figure 4; *spots 12, 13, 34–38*), acetyl-CoA acetyltransferase (Figure

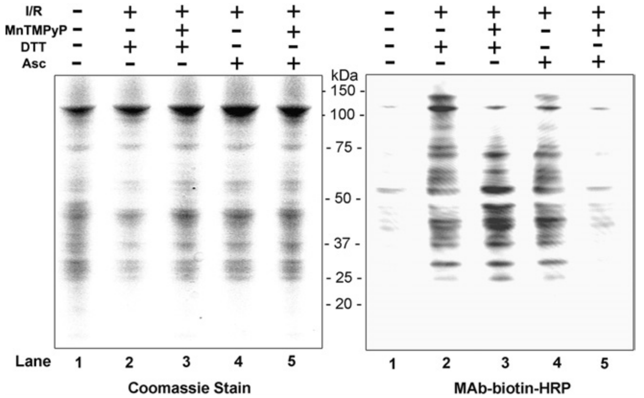

Figure 3. Alteration of the levels of oxidatively modified proteins in mouse livers following I/R injury. (*Left panel*) Equal amounts of mitochondrial proteins (20 μg/well) were isolated from sham-control or I/R injured mouse livers (2-hour reperfusion) with and without MnTMPyP and analyzed by 12% SDS-PAGE subjected to Coomassie blue staining. (*Right panel*) Biotin-NM labeled oxidized- or *S*-nitrosylated-mitochondrial proteins (20 μg/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 2 separate experiments.

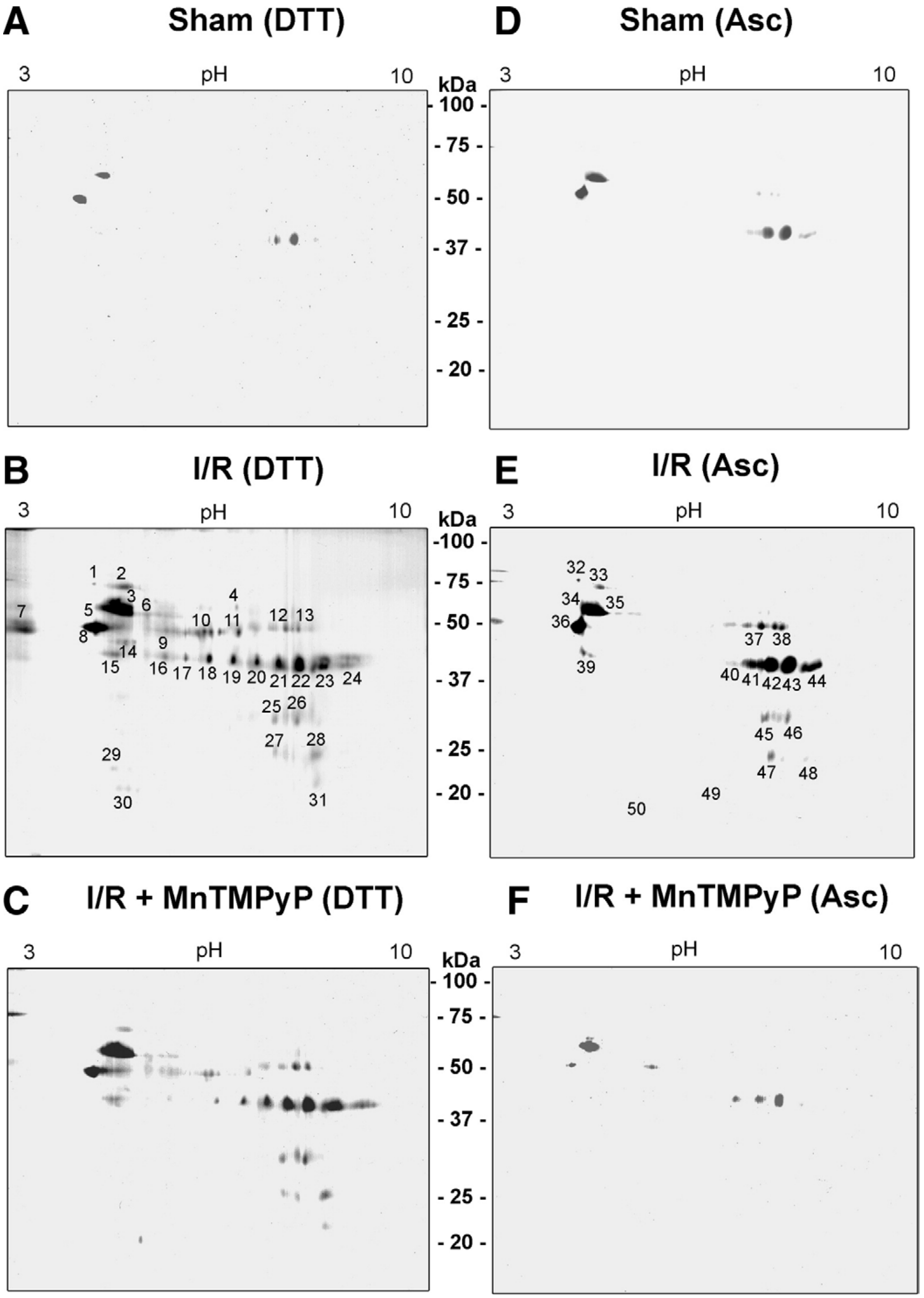

Figure 4. Comparison of oxidized and *S*-nitrosylated mitochondrial proteins by 2-DE in hepatic I/R injury without or with MnTMPyP. Oxidized mitochondrial proteins (10 mg/sample) from sham-control (*A*) and I/R injured mice (2-hour reperfusion) without (*B*) or with MnTMPyP (*C*) (I/R + MnTMPyP) were 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-hour 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 mass spectrometry (MS) analysis following in-gel trypsin digestion. This Figure represents a typical result from 2 separate experiments.

4; *spots 20–24, 40–44*), acyl-CoA dehydrogenase (Figure 4; *spots 21, 22, 40–43),* 3-ketoacyl-CoA thiolase (Figure 4; *spots 16–24, 39–44*), and others were repeatedly detected from spots with different pI values, suggesting they were also covalently modified by either phosphorylation or hyperoxidation to sulfinic and/or sulfonic acids. Several proteins were also identified from spots with apparently lower molecular weights of the parent protein (eg, Figure 4; *spots 27, 28, 45, 48–50* for α-ATP synthase). These spots likely represent fragments of the full-length proteins (eg, 58 kilodaltons for α-ATP synthase). Increased degradation of oxidized proteins was also observed with β-ATP synthase, 3-ketoacyl-CoA thiolase, and other proteins even after 2-hour 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*-nitrosyla-

Table 1. Summary of nanoRPLC Tandem Mass Spectrometry 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-Kilodalton glucose-regulated protein (GRP 78)	P200029	20
2	75-Kilodalton glucose-regulated protein (GRP 75)	P38647	14
2	Heat shock cognate 71 kilodalton protein (Hsp 70.8)	P63017	5
2	Heat shock-related 70 kilodalton protein 2 (Hsp 70.2)	P17156	4
2	Heat shock 70 kilodalton protein 1A (Hsp 70.3)	Q61696	2
3	60-Kilodalton heat shock protein, mitochondrial (Hsp 60)	P63038	6
4	Not identified		
5	60-Kilodalton heat shock protein, mitochondrial (Hsp 60)	P63038	33
5	ATP synthase β chain, mitochondrial (β-ATP synthase)	P56480	2
6	Protein disulfide isomerase A3 precursor	Q3TIL2	16
6	ATP synthase α chain, mitochondrial (α-ATP synthase)	P02535	4
6	60-Kilodalton heat shock protein, mitochondrial (Hsp 60)	P63038	2
7	ATP synthase β chain, mitochondrial (β-ATP synthase)	P56480	6
7	Aldehyde dehydrogenase 2, mitochondrial (ALDH2)	P47738	2
8	ATP synthase β chain, mitochondrial (β-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-Kilodalton 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 α chain, mitochondrial (α-ATP synthase)	P02535	5
12	Methylmalonate-semialdehyde dehydrogenase (ALDH6)	Q3TDA2	5
13	Methylmalonate-semialdehyde dehydrogenase (ALDH6)	Q3TDA2	6
13	ATP synthase α chain, mitochondrial (α-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	Q9JHI5	2
17	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	12
17	2-Oxoisovalerate dehydrogenase α subunit, mitochondrial	P50136	2
18	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	15
18	2-Oxoisovalerate dehydrogenase α subunit, mitochondrial	P50136	3
18	Isovaleryl-CoA dehydrogenase, mitochondrial	Q9JHI5	2
19	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	12
19	Isovaleryl-CoA dehydrogenase, mitochondrial	Q9JHI5	3
20	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	16
20	Carbamoyl-phosphate synthase, mitochondrial	Q8C196	3
20	Isovaleryl-CoA dehydrogenase, mitochondrial	Q9JHI5	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

Table 1. Continued

Spot No.	Protein identified	Accession number	No. of peptides identified
25	Electron transfer flavoprotein α-subunit (α-ETF)	Q99LC5	4
26	Electron transfer flavoprotein α-subunit (α-ETF)	Q99LC5	4
26	Hydroxymethylglutaryl-CoA lyase, mitochondrial	P38060	2
27	Electron transfer flavoprotein, β-subunit (β-ETF)	Q810V3	6
27	ATP synthase α chain, mitochondrial (α-ATP synthase)	P02535	5
28	Electron transfer flavoprotein, β-subunit (β-ETF)	Q810V3	6
28	3-Hydroxyacyl-CoA dehydrogenase type II	O08756	3
28	Enoyl-CoA hydratase, mitochondrial	Q8BH95	3
28	Hydroxyacyl-CoA dehydrogenase type II	Q99N15	2
28	ATP synthase α chain, mitochondrial (α-ATP synthase)	P02535	2
29	NADH-ubiquinone oxidoreductase 24 kilodalton 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-Kilodalton glucose-regulated protein (GRP 78)	P200029	14
33	Stress-70 protein, mitochondrial (GRP 75)	P38647	22
33	Heat shock cognate 71 kilodalton protein (HSP 70.8)	P63017	10
33	78-Kilodalton glucose-regulated protein (GRP 78)	P200029	6
33	60-Kilodalton heat shock protein, mitochondrial (Hsp 60)	P63038	5
33	Carbamoyl-phosphate synthase, mitochondrial	Q8C196	5
33	Heat shock-related 70 kilodalton protein 2 (Hsp 70.2)	P17156	5
33	Pyruvate carboxylase	Q3T9S7	2
33	Heat shock 70 kilodalton protein 1A (Hsp 70.3)	Q61696	2
34	60-Kilodalton heat shock protein, mitochondrial (Hsp 60)	P63038	54
34	ATP synthase β chain, mitochondrial (β-ATP synthase)	P56480	4
34	Methylmalonate-semialdehyde dehydrogenase (ALDH6)	Q3TDA2	2
35	60-Kilodalton 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 α chain, mitochondrial (α-ATP synthase)	P02535	2
36	ATP synthase β chain, mitochondrial (β-ATP synthase)	P56480	40
36	Aldehyde dehydrogenase 2, mitochondrial (ALDH2)	P47738	9
36	60-Kilodalton 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 α chain, mitochondrial (α-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 β chain, mitochondrial (β-ATP synthase)	P56480	2
38	ATP synthase α chain, mitochondrial (α-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 β chain, mitochondrial	Q9Z2I9	3
39	ATP synthase β chain, mitochondrial (β-ATP synthase)	P56480	2
40	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	15
40	Acetyl-CoA acetyltransferase, mitochondrial	Q8QZT1	5
40	Pyruvate dehydrogenase E1, α subunit	P35486	4
40	2-Oxoisovalerate dehydrogenase α 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	Q9JHI5	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

Table 1. Continued

Spot No.	Protein identified	Accession number	No. of peptides identified
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 α-subunit (α-ETF)	Q99LC5	6
45	Voltage-dependent anion-selective channel protein 1 (VDAC1)	Q60932	3
45	ATP synthase α chain, mitochondrial (α-ATP synthase)	P02535	2
45	Hydroxymethylglutaryl-CoA lyase, mitochondrial	P38060	2
46	Electron transfer flavoprotein α-subunit (α-ETF)	Q99LC5	7
46	3-Ketoacyl-CoA thiolase, mitochondrial	Q8BWT1	5
47	Electron transfer flavoprotein, β-subunit (β-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, β-subunit (β-ETF)	Q810V3	5
48	ATP synthase α chain, mitochondrial (α-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 β subunit, mitochondrial	Q99JY0	2
49	ATP synthase α chain, mitochondrial (α-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 α chain, mitochondrial (α-ATP synthase)	P02535	2

NOTE. Biotin-NM labeled oxidized- or *S*-nitrosylated proteins were purified with streptavidin-agarose 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.[23,29,30] Because our original aim was to identify early signs of oxidative modifications, we only identified the oxidatively modified proteins after 2-hour reperfusion.

tion of Cys residue(s) and nitration of Tyr residue(s).[22,23,29] Indeed, several proteins such as ALDH2[40] and 3-ketoacyl-CoA thiolase[41] 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 whether this activity could be restored in the presence of MnTMPyP. Hepatic ALDH2 activity in sham control mice was 3.30 ± 0.13 units compared with 1.52 ± 0.58 and 2.74 ± 0.17 units in 2-hour reperfused 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 (Figure 6*A*). Significant suppression of ALDH2 activity was also observed after reperfusion for 10 hours or 24 hours. However, MnTMPyP treatment fully restored the ALDH2 activity (Supplementary Figure 10*A*; see Supplementary material online at www.gastrojournal.org). Because ALDH2 is known to metabolize toxic lipid aldehydes such as MDA,[42] 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 reperfused for 2 hours (Figure 6*B*). The increased MDA level in I/R-injured mice was abolished by pretreatment with MnTMPyP. MDA levels were also elevated in 10-hour or 24-hour reperfused mice and significantly attenuated by MnTMPyP pretreatment (Supplementary Figure 10*B*).

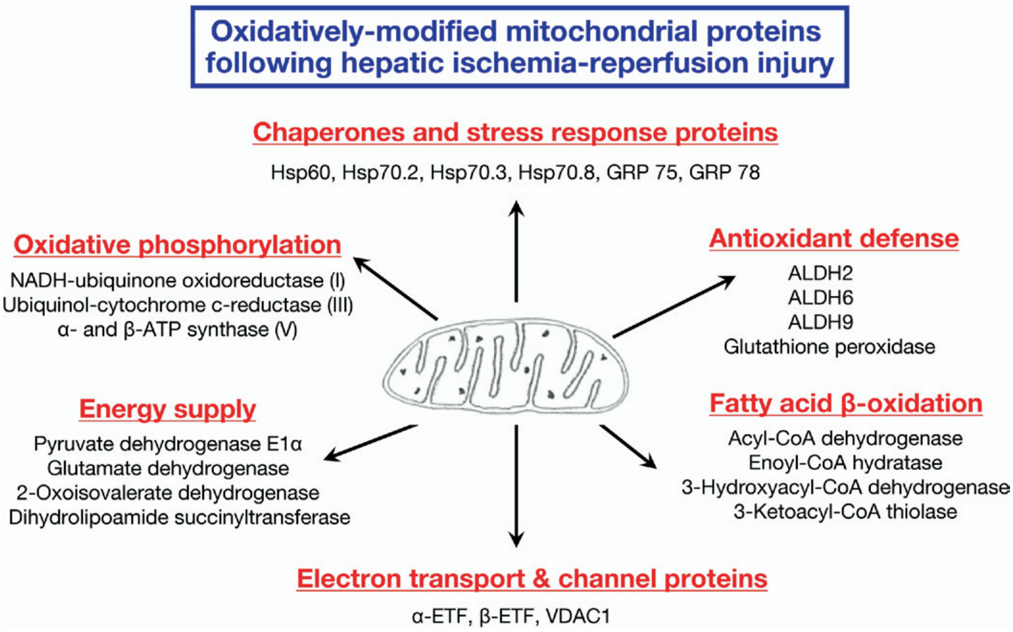

Figure 5. Summary of oxidized and *S*-nitrosylated mitochondrial proteins following hepatic I/R injury in the absence of MnTMPyP. Mitochondrial proteins that are simulataneously oxidized- and *S*-nitrosylated proteins are summarized with respects to the function of each protein.

One His (His[352]) and 2 Cys residues (Cys[92] and Cys[382]) are essential for the activity of 3-ketoacyl-CoA thiolase.[41] 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 with sham control mice (5.82 ± 0.01 units). 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.[4–6,43] 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.[43] Therefore, we measured the activity of ATP synthase (complex V) in mice with or without MnTMPyP pretreatment. The ATP synthase activity was 472.8 ± 7.5 units in sham control mice and 265.1 ± 41.8 and 444.4 ± 10.2 units, respectively, in 2-hour reperfused mice without and with MnTMPyP treatment (Figure 7*A*). Inhibition of ATP synthase activity was also observed after reperfusion for 10 hours or 24 hours; however, this activity was restored following MnTMPyP treatment (Supplementary Figure 11*A*; see Supplementary material online at www.gastrojournal.org). 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 elucidate further the mechanism for I/R-mediated inhibition of ATP synthase activity, we determined the levels of 3-NT in immunoprecipitated β-ATP synthase from mitochondria of sham controls and I/R-injured mice with or without MnTMPyP. Immunoblot analysis showed that similar levels of β-ATP synthase exist in all conditions (Figure 7*B*, *left panel*). However, 3-NT was only detected in mouse livers reperfused for 2 hours and not in the sham controls (Figure 7*B, 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,[23] 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-hour reperfused mice compared with sham controls (Figure 7*C*), an effect that was prevented by MnTMPyP pretreatment ($P < .007$). Inhibition of complex I activity was also observed after reperfusion for 10 hours or 24 hours, whereas MnTMPyP significantly restored its activity (Supplementary Figure 11*B*).

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.[25,44,45] In addition, some

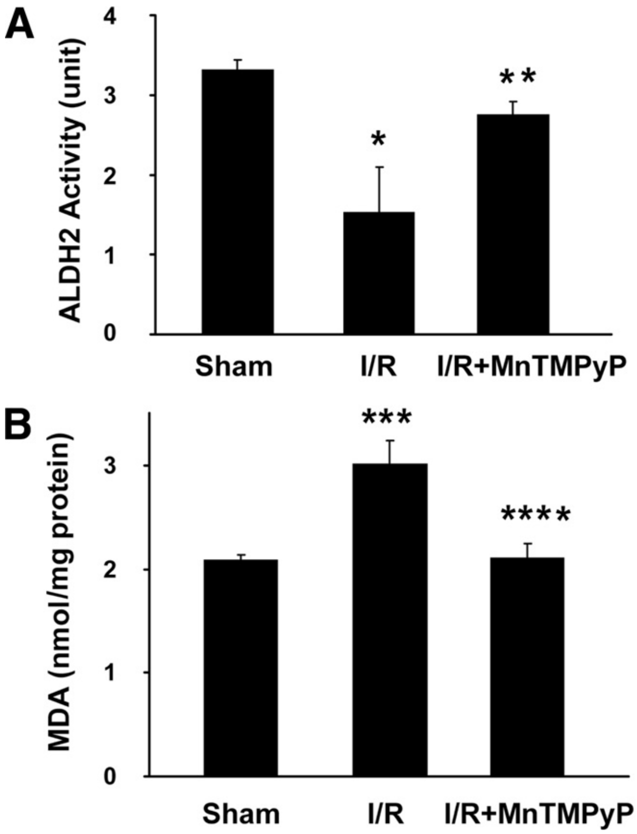

Figure 6. Inactivation of mitochondrial ALDH2 activity and the level of lipid peroxidation in I/R injured mice without or with MnTMPyP. (*A*) Catalytic activities of mitochondrial ALDH2 from the indicated liver samples (2-hour reperfusion) were determined. (*B*) Hepatic malondialdehyde (MDA) levels in different mice were measured and presented. *Significantly different from the sham controls at $P < .05$; **different from the I/R subjected samples at $P = .056$; ***significantly different from the sham controls at $P < .025$; ****significantly different from the I/R subjected samples at $P < .01$.

other proteins were also altered in rabbit hearts following I/R injury.[24,46] However, these studies were conducted using whole cell homogenate without subcellular fractionation and functional characterization of those proteins altered during I/R injury.[25,44,46] 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 hours) 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.[1,9,33,34] Because of the involvement of neutrophils in the longer reperfusion periods, neutrophil-derived oxidative modifications and clorination 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 hours or 24 hours 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 hours vs 10 hours or 24 hours. 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.[10–13]

Numerous mitochondrial proteins involved in energy supply and electron transport system, molecular chaperone activity, antioxidant defense, the urea cycle, degradation of fatty acids, and others have been simultaneously oxidized and *S*-nitrosylated (Figure 4 and Table 1) during

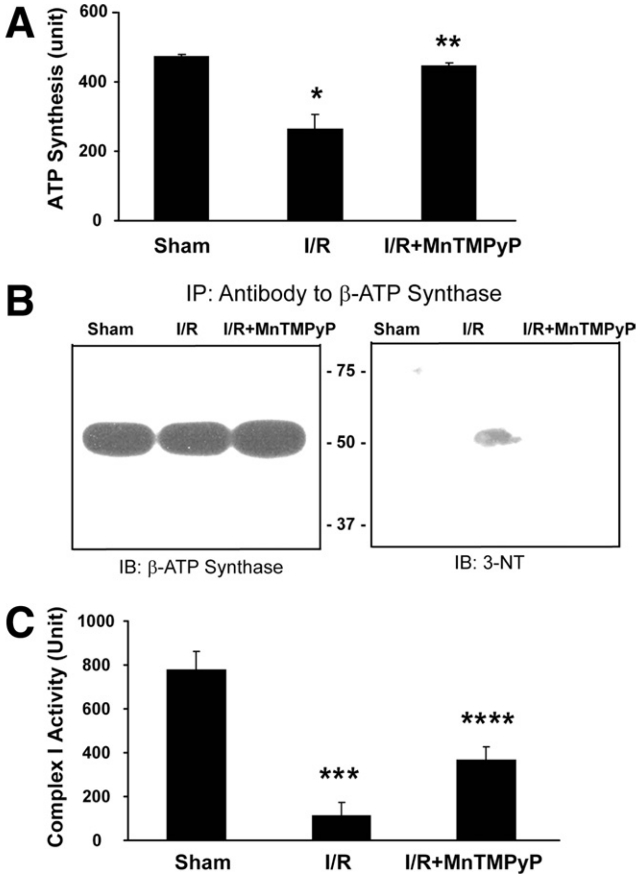

Figure 7. Inhibition of ATP synthase and complex I activities following hepatic I/R injury and nitration of Tyr residue in β-ATP synthase. (*A*) Mitochondrial ATP synthase activities from sham control and I/R injured mouse livers (2-hour reperfusion) without or with MnTMPyP (I/R + MnTMPyP) are presented. (*B*) Mitochondrial β-ATP synthase (1 mg proteins) in different groups as indicated were immunoprecipitated with the anti-β-ATP synthase antibody as described,[23] separated on 12% SDS-PAGE, and subjected to immunoblot analysis using the anti-β-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 $P < .05$; **significantly different from the I/R subjected samples at $P < .05$; ***significantly different from the sham controls at $P < .001$; ****significantly different from the I/R subjected samples at $P < .01$.

I/R injury, as observed in ethanol-exposed animals,[23] 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.[40] 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.[22,23,40] It was also reported that Cys^{323} is critical for the catalytic activity of glutamate dehydrogenase,[47] and its activity is significantly inhibited during hepatic I/R injury.[21] Based on the inactivation of these critical enzymes (eg, 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.[4–6,18,43] 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, and others) and on the beneficial effects of MnTMPyP pretreatment against hepatic damage, increased iNOS-derived NO coupled with superoxide formation and consequent generation of peroxynitrite play a critical role in causing I/R-mediated hepatic injury.[7–9]

Our current results conducted with mouse liver reperfused for 2 hours 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 various protein kinases including JNK and p38 kinase, which are known to be activated during the process of I/R injury.[10–13] Alternatively, multiple pI values of these proteins may represent hyperoxidation of Cys residues to sulfinic and/or sulfonic acids that are not easily reduced by DTT or Asc.[23] 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.[30,48,49] Alternatively, they may simply represent spontaneous fragmentation of oxidized proteins, as observed with cytosolic proteins in alcohol-exposed mouse livers.[23,30] 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.[24,46] Furthermore, we expect that similar covalent modifications (eg, phosphorylation) and/or fragmentations of mitochondrial proteins occur in 10-hour or 24-hour reperfused mice, based on the similar 2-DE patterns of oxidatively modified proteins despite appearance of additional protein spots (Supplementary Figures 8 and 9) compared with those in 2-hour reperfused tissues (Figure 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.[11,50] These results not only represent new information but also imply possible limited energy supply derived from the β-oxidation pathway of fatty acids during I/R. In addition to blocking the fat oxidation pathway, inactivation of ATP synthase (Figure 7) may explain the decreased level of ATP and thus favoring necrosis in I/R injury.[15,17,19] 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 support the pivotal role of ROS/RNS in mitochondrial dysfunction and cell damage during hepatic I/R injury and provide 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 I/R.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2008.06.048.

References

1. Jaeschke H. Molecular mechanisms of hepatic ischemia-reperfusion injury and preconditioning. Am J Physiol Gastrointest Liver Physiol 2003;284:G15–G26.
2. Engerson TD, McKelvey TG, Rhyne DB, et al. Conversion of xanthine dehydrogenase to oxidase in ischemic rat tissues. J Clin Invest 1987;79:1564–1570.
3. Pacher P, Nivorozhkin A, Szabo C. Therapeutic effects of xanthine oxidase inhibitors: renaissance half a century after the discovery of allopurinol. Pharmacol Rev 2006;58:87–114.

4. Jeong C, Lee SM. The beneficial effect of ATP-MgCl(2) on hepatic ischemia/reperfusion-induced mitochondrial dysfunction. Eur J Pharmacol 2000;403:243–250.
5. Kaibori M, Inoue T, Tu W, et al. FK506, but not cyclosporin A, prevents mitochondrial dysfunction during hypoxia in rat hepatocytes. Life Sci 2001;69:17–26.
6. Caraceni P, Bianchi C, Domenicali M, et al. Impairment of mitochondrial oxidative phosphorylation in rat fatty liver exposed to preservation-reperfusion injury. J Hepatol 2004;41:82–88.
7. Ma TT, Ischiropoulos H, Brass CA. Endotoxin-stimulated nitric oxide production increases injury and reduces rat liver chemiluminescence during reperfusion. Gastroenterology 1995;108: 463–469.
8. Pacher P, Beckman JS, Liaudet L. Nitric oxide and peroxynitrite in health and disease. Physiol Rev 2007;87:315–424.
9. Jaeschke H. Mechanisms of liver injury. II. Mechanisms of neutrophil-induced liver cell injury during hepatic ischemia-reperfusion and other acute inflammatory conditions. Am J Physiol Gastrointest Liver Physiol 2006;290:G1083–G1088.
10. Bradham CA, Stachlewitz RF, Gao W, et al. Reperfusion after liver transplantation in rats differentially activates the mitogen-activated protein kinases. Hepatology 1997;25:1128–1135.
11. Uehara T, Bennett B, Sakata ST, et al. JNK mediates hepatic ischemia reperfusion injury. J Hepatol 2005;42:850–859.
12. Massip-Salcedo M, Casillas-Ramirez A, Franco-Gou R, et al. Heat shock proteins and mitogen-activated protein kinases in steatotic livers undergoing ischemia-reperfusion: some answers. Am J Pathol 2006;168:1474–1485.
13. Kobayashi M, Takeyoshi I, Yoshinari D, et al. P38 mitogen-activated protein kinase inhibition attenuates ischemia-reperfusion injury of the rat liver. Surgery 2002;131:344–349.
14. Kim JS, Qian T, Lemasters JJ. Mitochondrial permeability transition in the switch from necrotic to apoptotic cell death in ischemic rat hepatocytes. Gastroenterology 2003;124:494–503.
15. Kim BJ, Ryu SW, Song BJ. JNK- and p38 kinase-mediated phosphorylation of Bax leads to its activation and mitochondrial translocation and to apoptosis of human hepatoma HepG2 cells. J Biol Chem 2006;281:21256–21265.
16. Levrand S, Vannay-Bouchiche C, Pesse B, et al. Peroxynitrite is a major trigger of cardiomyocyte apoptosis in vitro and in vivo. Free Radic Biol Med 2006;41:886–895.
17. Malhi H, Gores GJ, Lemasters JJ. Apoptosis and necrosis in the liver: a tale of two deaths? Hepatology 2006;43:S31–S44.
18. Gero D, Szabo C. Role of the peroxynitrite-poly (ADP-ribose) polymerase pathway in the pathogenesis of liver injury. Curr Pharm Des 2006;12:2903–2910.
19. Jeon BR, Lee SM. S-adenosylmethionine protects post-ischemic mitochondrial injury in rat liver. J Hepatol 2001;34:395–401.
20. Szabo C, Ischiropoulos H, Radi R. Peroxynitrite: biochemistry, pathophysiology and development of therapeutics. Nat Rev Drug Discov 2007;6:662–680.
21. Lee WY, Lee JS, Lee SM. Protective effects of combined ischemic preconditioning and ascorbic acid on mitochondrial injury in hepatic ischemia/reperfusion. J Surg Res 2007;142:45–52.
22. Moon KH, Kim BJ, Song BJ. Inhibition of mitochondrial aldehyde dehydrogenase by nitric oxide-mediated S-nitrosylation. FEBS Lett 2005;579:6115–6120.
23. Moon KH, Hood BL, Kim BJ, et al. Inactivation of oxidized and S-nitrosylated mitochondrial proteins in alcoholic fatty liver of rats. Hepatology 2006;44:1218–1230.
24. Kim N, Lee Y, Kim H, et al. Potential biomarkers for ischemic heart damage identified in mitochondrial proteins by comparative proteomics. Proteomics 2006;6:1237–1249.
25. Vascotto C, Cesaratto L, D'Ambrosio C, et al. Proteomic analysis of liver tissues subjected to early ischemia/reperfusion injury during human orthotopic liver transplantation. Proteomics 2006; 6:3455–3465.
26. Jeon BR, Yeom DH, Lee SM. Protective effect of allopurinol on hepatic energy metabolism in ischemic and reperfused rat liver. Shock 2001;15:112–117.
27. Grattagliano I, Vendemiale G, Lauterburg BH. Reperfusion injury of the liver: role of mitochondria and protection by glutathione ester. J Surg Res 1999;86:2–8.
28. Okatani Y, Wakatsuki A, Enzan H, et al. Edaravone protects against ischemia/reperfusion-induced oxidative damage to mitochondria in rat liver. Eur J Pharmacol 2003;465:163–170.
29. Suh SK, Hood BL, Kim BJ, et al. Identification of oxidized mitochondrial proteins in alcohol-exposed human hepatoma cells and mouse liver. Proteomics 2004;4:3401–3412.
30. Kim BJ, Hood BL, Aragon RA, et al. Increased oxidation and degradation of cytosolic proteins in alcohol-exposed mouse liver and hepatoma cells. Proteomics 2006;6:1250–1260.
31. Pfeiffer S, Schrammel A, Koesling D, et al. Molecular actions of a Mn(III)Porphyrin superoxide dismutase mimetic and peroxynitrite scavenger: reaction with nitric oxide and direct inhibition of NO synthase and soluble guanylyl cyclase. Mol Pharmacol 1998; 53:795–800.
32. Ferrer-Sueta G, Batinic-Haberle I, Spasojevic I, et al. Catalytic scavenging of peroxynitrite by isomeric Mn(III) N-methylpyridylporphyrins in the presence of reductants. Chem Res Toxicol 1999; 12:442–449.
33. Batkai S, Osei-Hyiaman D, Pan H, et al. Cannabinoid-2 receptor mediates protection against hepatic ischemia/reperfusion injury. FASEB J 2007;21:1788–1800.
34. Rajesh M, Pan H, Mukhopadhyay P, et al. Pivotal advance: cannabinoid-2 receptor agonist HU-308 protects against hepatic ischemia/reperfusion injury by attenuating oxidative stress, inflammatory response, and apoptosis. J Leukoc Biol 2007;82: 1382–1389.
35. Cardoso SM, Pereira C, Oliveira R. Mitochondrial function is differentially affected upon oxidative stress. Free Radic Biol Med 1999;26:3–13.
36. Lee VG, Johnson ML, Baust J, et al. The roles of iNOS in liver ischemia-reperfusion injury. Shock 2001;16:355–360.
37. Hines IN, Kawachi S, Harada H, et al. Role of nitric oxide in liver ischemia and reperfusion injury. Mol Cell Biochem 2002;234-235:229–237.
38. Billiar TR. Nitric oxide. Novel biology with clinical relevance. Ann Surg 1995;221:339–349.
39. Harbrecht BG, Billiar TR. The role of nitric oxide in Kupffer cell-hepatocyte interactions. Shock 1995;3:79–87.
40. Moon KH, Abdelmegeed MA, Song BJ. Inactivation of cytosolic aldehyde dehydrogenase via S-nitrosylation in ethanol-exposed rat liver. FEBS Lett 2007;581:3967–3972.
41. Zeng J, Li D. Expression and purification of his-tagged rat mitochondrial 3-ketoacyl-CoA thiolase wild type and His355 mutant proteins. Prot Expr Purif 2004;35:320–326.
42. Hartley DP, Ruth JA, Petersen DR. The hepatocellular metabolism of 4-hydroxynonenal by alcohol dehydrogenase, aldehyde dehydrogenase, and glutathione S-transferase. Arch Biochem Biophys 1995;316:197–205.
43. Tompkins AJ, Burwell LS, Digerness SB, et al. Mitochondrial dysfunction in cardiac ischemia-reperfusion injury: ROS from complex I, without inhibition. Biochim Biophys Acta 2006;1762: 223–231.
44. Avellini C, Baccarani U, Trevisan G, et al. Redox proteomics and immunohistology to study molecular events during ischemia-reperfusion in human liver. Transplant Proc 2007;39:1755–1760.
45. Emadali A, Muscatelli-Groux B, Delom F, et al. Proteomic analysis of ischemia-reperfusion injury upon human liver transplantation reveals the protective role of IQGAP1. Mol Cell Proteomics 2006; 5:1300–1313.
46. White MY, Tchen AS, McCarron HC, et al. Proteomics of ischemia and reperfusion injuries in rabbit myocardium with and without

intervention by an oxygen-free radical scavenger. Proteomics 2006;6:6221–6233.

47. Yang SJ, Cho EH, Choi MM, et al. Critical role of the cysteine 323 residue in the catalytic activity of human glutamate dehydrogenase isozymes. Mol Cells 2005;19:97–103.
48. Widmer R, Kaiser B, Engels M, et al. Hyperammonemia causes protein oxidation and enhanced proteasomal activity in response to mitochondria-mediated oxidative stress in rat primary astrocytes. Arch Biochem Biophys. 2007;464:1–11.
49. Venugopal SK, Chen J, Zhang Y, et al. Role of MAP kinase phosphatase-1 in sustained activation of C-JUN N-terminal kinase (JNK) during ethanol-induced apoptosis in hepatocyte-like VL-17A cells. J Biol Chem. 2007;282:319001–319008.
50. Imber CJ, St. Peter SD, Handa A, et al. Hepatic steatosis and its relationship to transplantation. Liver Transpl 2002;8:415–423.

Received October 18, 2007. Accepted June 19, 2008.

Address reprint requests to: B. J. Song, PhD, Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, 9000 Rockville Pike, Bethesda, Maryland 20892-9410. e-mail: bjs@mail.nih.gov; (301) 594-3113, and Pal Pacher, MD, PhD, Section on Oxidative Stress and Tissue Injury, Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, 9000 Rockville Pike, Bethesda, Maryland 20892-9413. e-mail: pacher@mail.nih.gov.

Supported by the Intramural Research Program of National Institute on Alcohol Abuse and Alcoholism and National Cancer Institute, National Institutes of Health, under contract No. NO1-CO-12400.

The authors thank Dr Norman Salem, Jr, for support.

B.L.H.'s present address is Clinical Proteomics Facility, University of Pittsburgh Cancer Institute University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15213.

T.P.C.'s present address is Department of Pharmacology and Chemical Biology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15213.

Y.-I.K.'s present address is Department of Obstetrics/Gynecology, Kangdong Sacred Heart Hospital, Hallym University Medical College, Seoul, Korea 134-701.

Conflicts of interest: No authors have any conflicts of interest to disclose.