Hepatic Mitochondrial DNA Depletion after an Alcohol Binge in Mice: Probable Role of Peroxynitrite and Modulation by Manganese Superoxide Dismutase

Isabelle Larosche, Philippe Lettérion, Alain Berson, Bernard Fromenty, Ting-Ting Huang, Richard Moreau, Dominique Pessayre, and Abdellah Mansouri


Received August 21, 2009; accepted December 15, 2009

ABSTRACT

Alcohol consumption increases reactive oxygen species (ROS) formation, which can damage mitochondrial DNA (mtDNA) and alter mitochondrial function. To test whether manganese superoxide dismutase (MnSOD) modulates acute alcohol-induced mitochondrial alterations, transgenic MnSOD-overexpressing (MnSOD 

superscript +/−) mice, heterozygous knockout (MnSOD 

superscript −/+ ) mice, and wild-type (WT) littermates were sacrificed 2 or 24 h after intragastric ethanol administration (5 g/kg). Alcohol administration further increased MnSOD activity in MnSOD 

superscript +/− mice, but further decreased it in MnSOD 

superscript −/+ mice. In WT mice, alcohol administration transiently increased mitochondrial ROS formation, decreased mitochondrial glutathione, depleted and damaged mtDNA, and decreased complex I and V activities; alcohol durably increased inducible nitric-oxide synthase (NOS) expression, plasma nitrites/nitrates, and the nitration of tyrosine residues in complex V proteins. These effects were prevented in MnSOD 

superscript ++/++ mice and prolonged in MnSOD 

superscript −/− mice. In alcoholized WT or MnSOD 

superscript −/+ mice, mtDNA depletion and the nitration of tyrosine residues in complex I and V proteins were prevented or attenuated by cotreatment with tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl), a superoxide scavenger; N-nitro-L-arginine methyl ester and N-[3-(aminomethyl)benzyl]acetamidine (1400W), two NOS inhibitors; or uric acid, a peroxynitrite scavenger. In conclusion, MnSOD overexpression prevents, and MnSOD deficiency prolongs, mtDNA depletion after an acute alcohol binge in mice. The protective effects of MnSOD, tempol, NOS inhibitors, and uric acid point out a role of the superoxide anion reacting with NO to form mtDNA-damaging peroxynitrite.

There are two types of alcoholism, either excessive amounts of alcohol ingested daily, or frequent episodes of binge drinking separated by short periods of relative temperance (Mathurin and Deltenre, 2009). Both types of alcohol consumption can damage the liver (Mathurin and Deltenre, 2009).

Alcohol abuse can cause liver disease via oxidative stress, mitochondrial dysfunction, and the up-regulation of proapoptotic, proinflammatory, and profibrogenic cytokines (Dey and Cederbaum, 2006). Ethanol increases the generation of reactive oxygen species (ROS; Kukielka et al., 1994). Increased ROS formation damages hepatic proteins, lipids, and DNA (Wieland and Lauterburg, 1995). Mitochondrial DNA (mtDNA) is more susceptible to oxidative damage than nuclear DNA (nDNA) due to incomplete DNA repair capacity in mitochondria and the proximity of mtDNA to the respiratory chain, a major source of ROS in the cell. Ethanol-induced mtDNA lesions include 8-hydroxydeoxyguanosine (Wieland and Lauterburg, 1995), apurinic/apyrimidinic sites, and mtDNA strand breaks (Mansouri et al., 1999). Alcohol-induced mtDNA lesions may favor the premature occurrence of mtDNA deletions (Mansouri et al., 1997) and may trigger mtDNA depletion (Mansouri et al., 1999). Oxidatively damaged mtDNA mol-
ecules may be degraded by endonucleases, whereas oxidative mtDNA lesions on the remaining mtDNA templates may hamper the progress of polymerase γ, thus impairing mtDNA resynthesis (Demeilliers et al., 2002). After prolonged alcohol administration, decreased mtDNA levels may reduce synthesis of the mtDNA-encoded proteins that are part of mitochondrial complexes (Coleman and Cunningham, 1990). Decreased levels of mtDNA-encoded proteins, and oxidative modifications of both mtDNA- and nDNA-encoded mitochondrial proteins, can impair mitochondrial function after repeated alcohol binges (Demeilliers et al., 2002).

Mitochondrial manganese superoxide dismutase (MnSOD) accelerates the spontaneous dismutation of the superoxide anion produced by the mitochondrial respiratory chain (Wallace, 1999). MnSOD generates hydrogen peroxide, which is detoxified into water by mitochondrial glutathione peroxidase (GPx), mitochondrial peroxiredoxins, and peroxisomal catalase (Wallace, 1999). MnSOD is inducible by ROS, cytokines, and ethanol (Koch et al., 1994) but inactivated by peroxynitrite (MacMillan-Crow et al., 1998).

We have shown that MnSOD-overexpressing (MnSOD++) transgenic mice, but not wild-type (WT) mice, developed increased hepatic iron concentrations and mtDNA depletion after 7 weeks of alcohol treatment (Larosche et al., 2009). The iron chelator deferoxamine prevented both iron accumulation and mtDNA depletion, suggesting that iron-mediated hydroxyl radical formation played a role in the mtDNA damage caused by prolonged alcohol administration in MnSOD+++ mice (Larosche et al., 2009). Unlike prolonged alcohol administration, a single alcohol binge may not increase hepatic iron stores. We therefore hypothesized that the effects of MnSOD overexpression on alcohol-mediated mtDNA depletion could be different after acute or prolonged alcohol administration.

Peroxynitrite is a strong oxidant generated by the spontaneous reaction of nitric oxide with superoxide (Epe et al., 1996; Radi et al., 2002) and might be the DNA-damaging species after an alcohol binge. Indeed, alcohol administration increases inducible nitric-oxide synthase (iNOS) expression (Yuan et al., 2006), which, together with the alcohol-mediated increased in superoxide anion formation, could increase hepatic peroxynitrite formation. Peroxynitrite and/or peroxynitrite-generated reactive intermediates can nitrate proteins and can damage lipids and DNA (Epe et al., 1996; Radi et al., 2002). Peroxynitrite plays an important role in alcohol-induced liver lesions (McKim et al., 2003; Venkatraman et al., 2004; Bailey et al., 2009), and it may be the reactive species responsible for acute mtDNA depletion after administration of paracetamol in mice (Cover et al., 2005).

An important function of MnSOD may be to limit peroxynitrite formation. MnSOD accelerates the dismutation of the superoxide anion, thus decreasing its steady-state levels (Liochev and Fridovich, 2007), which should decrease the chances that NO reacts with the superoxide to form peroxynitrite within mitochondria. In keeping with the role of peroxynitrite in alcohol-induced liver lesions (McKim et al., 2003; Venkatraman et al., 2004; Bailey et al., 2009), and the ability of MnSOD to decrease peroxynitrite formation, increasing MnSOD activity with a recombinant adenovirus protected rats against alcohol-induced apoptosis, necrosis, and inflammation (Wheeler et al., 2001).

In the present study, we compared the effects of an acute alcohol binge on hepatic mtDNA in WT, MnSOD++, and heterozygous MnSOD knockout (MnSOD+/−) mice. We also studied the effects of tempol (4-hydroxy-2,2,6,6-tetramethylpyrroline- N-oxyl), a superoxide scavenger (Wilcox and Pearlman, 2008), Nω-nitro-l-arginine methyl ester (l-NAME), a broad-spectrum NOS inhibitor (McKim et al., 2003); 1400W, a selective iNOS inhibitor (McKim et al., 2003); and uric acid, a peroxynitrite scavenger (Garcia-Ruiz et al., 2006) on alcohol-induced mtDNA depletion. Our results suggest that peroxynitrite is the reactive species involved in the mtDNA-depleting effects of an acute alcohol binge in mice. MnSOD overexpression prevents, whereas partial MnSOD deficiency prolongs, mtDNA depletion and mitochondrial dysfunction after acute alcohol intoxication.

Materials and Methods

Materials and Chemicals. 2′,7′-Dichlorodihydrofluorescein di-acetate (H2DCF-DA), the mouse monoclonal antibody against the α subunit of ATP synthase (complex V) and the mouse monoclonal antibody against the 30-kDa subunit of complex I were purchased from Invitrogen (Carlsbad, CA). The mouse monoclonal antibody against iNOS was obtained from BD Biosciences Transduction Laboratories (Le Pont de Claux, France). The mouse monoclonal anti-3-nitrotyrosine antibody was obtained from Calbiochem (San Diego, CA). The mouse monoclonal antibody against subunit 2 of cytochrome c oxidase (COX2); the goat polyclonal antibody against mitochondriod transcription factor A (Tfam); and the rabbit polyclonal antibodies against MnSOD, copper-zinc superoxide dismutase (Cu/ZnSOD) and peroxisome proliferator-activated receptor γ coactivator 1 (PGC-1), were all purchased from ThermoFisher (Gennevilliers, France). The caspase-3 activity kit was purchased from Biorad Research Laboratories (Plymouth Meeting, PA). Hybrid-N+ membranes, Hybond-C-extra membranes, and [α-32P]dCTP were purchased from GE Healthcare (Orsay, France). The Expand Long Template PCR system was from Roche Applied Science (Mannheim, Germany). All other products were purchased from Sigma-Aldrich (St. Louis, MO).

Animals and Treatments. The MnSOD+++ transgenic mice used in this study were developed in Epstein’s laboratory (Raineri et al., 2001). We used strain 274, a strain in the C57BL/6J genetic background that expresses the Sod2 mouse transgene at levels 2 to 3 times the Sod2 expression in WT mice (Raineri et al., 2001). The heterozygous knockout MnSOD+/− mice used in this study have been described previously (Van Remmen et al., 1999). WT C57BL/6J mice were obtained from Janvier (Le Genest Saint Isle, France) and used for breeding with the MnSOD+++ mice and with the MnSOD+/− mice. Female WT mice and male MnSOD+/− mice or WT and MnSOD+++ mice were backcrossed into an inbred strain for at least nine generations. The mice obtained by breeding were then genotyped. Animals were fed a standard diet ad libitum (A04-10 biscuits; UAR, Villemoisson-sur-Orge, France). Animals received humane care, and all experiments were performed according to national guidelines for the use of animals in biomedical research.

Because we had to breed the animals before the experiments and because genotyping revealed variable numbers of MnSOD++, MnSOD−−, and WT littermates each time, the numbers of available animals of each genotype varied from one experiment to another experiment. At 2 months of age, MnSOD+++ mice, MnSOD−− mice, and their WT littermates were given either water alone or alcohol (5 g/kg b.wt.) diluted in water [30% (v/v)] by
intragastric intubation. Animals were killed by cervical dislocation 2 or 24 h after alcohol or water administration. Thirty minutes before ethanol administration, some WT and MnSOD−/− mice received tempol (200 mg/kg b.wt.), L-NAME (80 mg/kg b.wt.), 1400 W (10 mg/kg b.wt.), or uric acid (500 mg/kg b.wt.) and were killed 2 h after ethanol administration. Other mice received a first dose of these substances 30 min before alcohol administration and a second dose 8 h after the ethanol treatment, and they were killed 24 h after ethanol administration.

**Plasma Ethanol, Plasma Nitrite/Nitrate Levels, and Liver and Plasma Iron Concentrations.** Plasma ethanol was measured with a commercial kit (ADH-NAD Reagent Multiple Test Vial; Sigma-Aldrich) based on the alcohol-mediated conversion of oxidized nicotinamide adenine dinucleotide (NAD+) to reduced nicotinamide adenine dinucleotide (NADH) in the presence of alcohol dehydrogenase. Plasma nitrite/nitrate concentrations were determined using the Griess reaction, as described previously (Fischt et al., 1996). Liver iron was extracted by acid digestion of tissue samples as described previously (Torrance and Bothwell, 1968), followed by determination of iron content on an AU400 automat (Olympus Diagnostic, Rungis, France) using the commercial kit OSR6186 (Olympus Diagnostic). Plasma iron was assessed with the same kit.

**Hepatic MnSOD and Cu,ZnSOD Activities, Mitochondrial GPx Activity, and Mitochondrial Glutathione Levels.** Native gel assays were used to assess MnSOD and Cu,ZnSOD activities (Sutton et al., 2005). Total hepatic proteins (100 µg) were resuspended in 40% glycerol and 0.025% bromphenol blue and loaded on a nondenaturing 15% polyacrylamide gel. Migration was performed at 4°C for 12 h at 120 V, and superoxide dismutase activity was assessed within the gel, as described previously (Sutton et al., 2005). Liver mitochondria were prepared as described previously (Larosche et al., 2009). GPx activity was measured in mitochondrial fractions by following the conversion of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) into oxidized nicotinamide-adenine dinucleotide phosphate (NADP+) at 340 nm for 2 min in the presence of hydrogen peroxide, glutathione reductase, and reduced glutathione (Larosche et al., 2009). The mitochondrial content of reduced glutathione was determined by the method of Tietze (Larosche et al., 2009).

**mtDNA Levels and Long PCR Experiments.** Slot blot hybridization was used to look for mtDNA depletion (Mansouri et al., 1999). Hapatic DNA was isolated by the phenol/chloroform method, and 300 ng was blotted onto Hybond-N+ nylon membranes (GE Healthcare). Membranes were hybridized with a 10.9-kilobase mtDNA probe (nucleotides 4964–15,896) generated by long PCR and labeled by random priming. Membranes were then stripped and hybridized with a mouse Cyt b-1 mtDNA probe (Invitrogen, Cergy Pontoise, France). mtDNA and nDNA were assessed by denaturation analysis of autoradiographs.

We used long PCR experiments to detect mtDNA lesions hampering polymerase progress and altering replication (Demeilliers et al., 2002). This long PCR technique is based on the amplification of a long (8636-bp) and a short (316-bp) mtDNA fragment. Forward primer A (5′-CGACAGCTTGAGGCTCAACTGGG-3′; nucleotides 470–492) and reverse primer B (5′-CCATTGCCTCCATTCTGACC-3′; nucleotides 785–762) amplified a 316-bp mtDNA fragment, whereas forward primer C (5′-TACTAGTCGGCAGGCTTCAAAGGC-3′; nucleotides 4964–4987) and reverse primer D (5′-GGTGATCTTTGGTGGGTT-3′; nucleotides 13,599–13,579) amplified an 8636-bp mtDNA fragment. PCR reactions were performed with the Expand Long PCR system (Roche Applied Science) according to the manufacturer’s instructions using 40 pmol of primers. The thermocycler profile included initial denaturation at 94°C for 2 min, 26 cycles of 95°C for 45 s, 61°C for 10 s, and 68°C for 8 min, and a final extension at 68°C for 7 min. PCR products (20 µl) were separated on 1.6% agarose gels stained with ethidium bromide. Photographs were taken under UV transillumination and scanned to determine the respective intensities of the 316- and 8636-bp PCR products.

**Mitochondrial ROS Production.** Mitochondrial ROS production was determined with H$_2$DCF-DA, which forms the green fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of hydrogen peroxide and other peroxides (Larosche et al., 2009). Each well of a 96-well plate was filled with respiration buffer (130 mM sucrose, 50 mM KCl, 5 mM MgCl$_2$, 5 mM KH$_2$PO$_4$, 0.05 mM EDTA, and 5 mM HEPES, pH 7.4) containing 2 µM H$_2$DCF-DA and mitochondria (5–20 µg protein/ml). The reaction was started by the addition of 5 mM each of glutamate and malate. Fluorescence was recorded at 480/520 nm with a FLUOstar fluorimeter (BMG Labtech GmbH, Offenburg, Germany) for 60 min at 30°C.

**Protein Levels and Activities of Respiratory Chain Complexes I, II, III, IV, and V and Probes for 3-Nitrotyrosine Residues in Proteins.** Alterations in the levels and activity of mitochondrial complexes I and V were examined using blue native polyacrylamide gel electrophoresis (BN-PAGE) as described by Schägger and von Jagow (1991). Mitochondrial extracts were treated with n-dodecyl-$\beta$-d-maltoside. This mild detergent allows the separation of respiratory chain complexes, whereas their subunit assembly and activity remain intact. BN-PAGE was performed using a home-made 5 to 13% polyacrylamide gel gradient with a 4% stacking gel. The anode buffer contained 50 mM Bis-Tris and the cathode buffer contained 50 mM Tricine, 15 mM Bis-Tris, and 0.02% of the Serva Blue G dye. Protein samples (35 µg) in 2 µl of 5% Serva Blue G, 750 mM aminocaproic acid, and 50 mM Bis-Tris/HCl, pH 7.0, were applied to a 10-well gel. Electrophoresis was conducted at 100 V at 4°C until the samples entered the stacking gel (approximately 1 h), when the voltage was increased to 200 V. When the dye front reached the middle of the gel, the initial cathode buffer containing 0.02% Serva Blue G was replaced by a cathode buffer containing 0.002% Serva Blue G. The banding pattern was scanned and quantified by densitometry to measure the level of protein loaded on the gel for each complex. Immediately after electrophoresis, enzymatic colorimetric reactions were performed on the BN-PAGE. The in situ activity of complex I (NADH-oxidoreductase) was determined by incubating gel slices with 2 mM Tris/HCl, pH 7.4, 0.1 mg/ml NADH, and 2.5 mg/ml nitro blue tetrazolium at room temperature for 20 min. The reaction product was preserved by fixing the gel in 50% methanol and 7% acetic acid for 15 min. The ATPase activity of complex V was assessed by incubating the BN-PAGE gel slices overnight at room temperature in 35 mM Tris, 270 mM glycine, 14 mM MgSO$_4$, 0.2% Pb(NO$_3$)$_2$, and 8 mM ATP, pH 7.8. The gel was washed and preserved in distilled water. The violet-colored complex I and the aconitase complex V bands were scanned and quantified by densitometry.

In some experiments, separated complexes on BN-PAGE were transferred electrophoretically to nitrocellulose membranes for Western blot detection of 3-nitrotyrosine. Membranes were blocked with 5% nonfat dry milk for 1 h at room temperature and incubated overnight at 4°C with a mouse monoclonal antibody against 3-nitrotyrosine. Membranes were washed several times and incubated with horseradish peroxidase-conjugated anti-mouse secondary antibody for 1 h. Binding of the secondary antibody was visualized by enhanced chemiluminescence.

**Western Blot Analysis of iNOS, COX2, Tfam, PGC-1, the 30-kDa Subunit of Complex I, and the α Subunit of Complex V (ATP Synthase).** To assess protein expression, equal amounts of total liver proteins (50 µg) were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. Membranes were blocked and incubated overnight at 4°C with an antibody against iNOS, COX2, Tfam, PGC-1, the 30-kDa subunit of complex I or the α subunit of ATP synthase. After three washings with phosphate-buffered saline (PBS), membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) in PBS containing 0.05% Tween 20 for 1 h at room temperature. Blots were then washed with the enhanced chemiluminescence method and exposed to an X-ray film. Blots were stripped and exposed to an anti-β-actin antibody. Protein bands were quantified by densitometry.
Liver Morphology, Caspase-3 Activity, and Terminal Deoxynucleotidyl Transferase-Mediated DTTP-Biotin Nick End-Labeling Assay. A liver fragment for light microscopy was fixed with 10% formalin in PBS, dehydrated in an alcoholic series, placed in toluene baths, and embedded in paraffin. Sections (3 μm in thickness) were prepared for hematoxylin and eosin staining. The in situ detection of apoptosis was performed with the TUNEL assay using the ApoTag peroxidase in situ apoptosis detection kit (Chemicon, Hampshire, UK; Larosche et al., 2009), as described by the manufacturer. For the caspase-3 activity assay, livers were minced and homogenized in 1 mM EDTA, 50 mM HEPES, 0.1% 3-(3-cholamidopropyl)dimethylammonio)-1-propane-sulfonate, 5 mM dithiothreitol, 4 mg/ml leupeptin, and 4 mg/ml pepstatin, pH 7.4. After centrifugation at 14,000g, caspase-3 activity was measured in the supernatant with a fluorescent assay kit (Larosche et al., 2009).

Statistical Analysis. Because the different parameters investigated were similar in mice sacrificed either 2 or 24 h after water administration, these mice were pooled into a single group and served as zero time controls (designed as 0 h) for mice sacrificed 2 or 24 h after alcohol administration. We used two-way ANOVA to assess the effects of the MnSOD genotype, alcohol treatment time, and their interactions. The means of diverse groups were then compared with Fisher’s protected least significant difference (PLSD) post hoc tests. When cotreatments were performed (l-NAME, 1400W, uric acid, or tempol) in WT and MnSOD+/− mice, two-way ANOVA was first used to study the effects of genotype and alcohol treatment time in each cotreatment group. One-way ANOVA tests followed by Fisher’s PLSD tests were then used to compare the effects of the addition or not of a particular cotreatment, first within the group of WT mice and then within the group of MnSOD+/− mice. Values were expressed as means ± S.E.M., and the significance level was set at P < 0.05.

Results

Similar Ethanol and Iron Levels in MnSOD+++, WT, and MnSOD+/− Mice. Two hours after the binge, serum ethanol concentrations were similar in WT, MnSOD+++, and MnSOD+/− mice (Table 1). Plasma and liver iron contents were also similar in naive or intoxicated WT, MnSOD+++, or MnSOD+/− mice (Table 1).

Ethanol Administration Further Increases MnSOD Activity in MnSOD+++ Mice but Further Decreases It in MnSOD+/− Mice, with No Changes in Cu/ZnSOD Level or Activity. Western blots and activity gels were performed to assess hepatic MnSOD protein (Fig. 1A) and activity (Fig. 1B), respectively. As expected, the basal (0-h) MnSOD protein and its basal activity were higher in MnSOD+++ mice than in WT mice and were conversely lower in MnSOD+/− mice than in WT mice (P < 0.05, two-way ANOVA followed by PLSD test; Fig. 1, A and B).

Although alcohol administration did not change MnSOD protein at 2 h, it increased it at 24 h (Fig. 1A). In keeping with the increased protein expression, alcohol administration increased MnSOD activity in WT mice and further augmented the already high MnSOD activity in MnSOD+++ mice (P < 0.05, two-way ANOVA followed by PLSD test; Fig. 1B). In contrast, despite the increased MnSOD protein expression 24 h after the binge, alcohol administration further decreased the already low MnSOD activity in MnSOD+/− mice (P < 0.05, two-way ANOVA followed by PLSD test; Fig. 1B), suggesting enzyme inactivation as discussed further on.

The protein level and the activity of Cu,ZnSOD were similar in the nine groups of mice (Fig. 1, C and D).

Unchanged Mitochondrial GPx Activity in Alcohol-Treated MnSOD+++ Mice but Decreased GPx Activity in Alcohol-Treated WT or MnSOD+/− Mice. We also assessed hepatic mitochondrial GPx activity in the nine groups of mice (Table 2). Basal GPx activity in untreated mice (0 h) was slightly lower in MnSOD+/− mice than in WT mice. Alcohol administration did not change mitochondrial GPx activity in MnSOD+++ mice (Table 2) but decreased it by 26 and 35% at 2 and 24 h in WT mice and by 29 and 24% at 2 h and 24 h in MnSOD+/− mice (P < 0.05, two-way ANOVA followed by PLSD test).

Absent, Transient, or Persistent Mitochondrial GSH Depletion in Alcohol-Treated MnSOD+++ WT, and MnSOD+/− Mice, Respectively. We then assessed hepatic mitochondrial GSH content in the nine groups of mice (Table 3). Although basal GSH concentrations were not significantly different in MnSOD+++ WT, and MnSOD+/− mice, the effects of ethanol differed markedly between the three genotypes. Whereas ethanol administration did not deplete hepatic GSH in MnSOD+++ mice, it transiently decreased mitochondrial GSH by 49% at 2 h and by 19% at 24 h in WT mice and caused severe and prolonged GSH depletion in MnSOD+/− mice, whose hepatic mitochondrial GSH was decreased by 63% at 2 h and 53% at 24 h (P < 0.05, two-way ANOVA followed by PLSD test; Table 3).

Transient Increase in Mitochondrial ROS Formation in Alcohol-Treated WT or MnSOD+++ Mice but Persistent Increase in Mitochondrial ROS Formation in Alcohol-Treated MnSOD+/− Mice. To evaluate mitochondrial ROS production, we isolated hepatic mitochondria from WT and MnSOD+/− mice and incubated these mitochondria with both complex I substrates and H2DCF-DA, which forms the fluorescent DCF in the presence of peroxides (Fig. 2A). Basal mitochondrial ROS production was similar in WT, MnSOD++, and MnSOD+/− mice (Fig. 2A). In WT and MnSOD+++ mice treated with ethanol, DCF fluorescence markedly increased at 2 h but returned to control values by 24 h. In contrast, DCF fluorescence was durably increased.

Table 1

<table>
<thead>
<tr>
<th>Mice</th>
<th>Plasma Ethanol</th>
<th>Plasma Iron</th>
<th>Liver Iron</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 h</td>
<td>0 h</td>
<td>2 h</td>
</tr>
<tr>
<td></td>
<td>g/l</td>
<td>μM</td>
<td>μM</td>
</tr>
<tr>
<td>WT</td>
<td>0.51 ± 0.05</td>
<td>24 ± 6</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>MnSOD+++</td>
<td>0.54 ± 0.05</td>
<td>26 ± 4</td>
<td>19 ± 7</td>
</tr>
<tr>
<td>MnSOD+/−</td>
<td>0.58 ± 0.04</td>
<td>24 ± 7</td>
<td>23 ± 7</td>
</tr>
<tr>
<td>WT</td>
<td>111 ± 16</td>
<td>120 ± 14</td>
<td>123 ± 9</td>
</tr>
<tr>
<td>MnSOD+++</td>
<td>130 ± 12</td>
<td>135 ± 17</td>
<td>118 ± 12</td>
</tr>
<tr>
<td>MnSOD+/−</td>
<td>123 ± 27</td>
<td>118 ± 20</td>
<td>121 ± 16</td>
</tr>
</tbody>
</table>
in alcohol-treated MnSOD+/− mice by 118% at 2 h and by 132% at 24 h (Fig. 2A).

**Both a Superoxide Scavenger and a Peroxynitrite Scavenger Administered in Vivo Attenuate the Alcohol-Mediated Increase in Ex Vivo Mitochondrial ROS Formation.** Thirty minutes before receiving ethanol, some mice received an intraperitoneal injection of tempol (200 mg/kg b.wt.), a superoxide scavenger, or uric acid (500 mg/kg b.wt.), a peroxynitrite scavenger (Fig. 2, B and C). A second dose of the scavenger was administered 8 h after ethanol administration in mice that were sacrificed 24 h after ethanol administration. These experiments assess the in vivo but not the in vitro effects of the scavengers, because the isolation of mitochondria involves several dilutions and washings that effectively remove the scavengers from mitochondria. Whereas tempol or uric acid administration did not modify ROS formation in the mitochondria of control mice (0 h), the presence of these scavengers in vivo attenuated the ethanol administration-mediated increase in ex vivo mitochondrial ROS formation (Fig. 2, B and C).

**Absent, Transient, or Persistent mtDNA Depletion in Alcohol-Treated MnSOD+/+, WT, and MnSOD+/− Mice, Respectively.** Slot blot hybridization was used to assess hepatic DNA levels. Total DNA was first hybridized with an mtDNA-specific probe and then an nDNA-specific probe (Fig. 3A). Because acute ethanol administration did not modify nDNA levels (Fig. 3A), the mtDNA/nDNA hybridization ratio was used to quantify TABLE 2

Hepatic mitochondrial GPx activity in naive or alcohol-treated WT, MnSOD+/+, and MnSOD+/− mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>GPx Activity (nmol/min/mg protein)</th>
<th>0 h</th>
<th>2 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>205 ± 7</td>
<td>152 ± 9*</td>
<td>134 ± 9*</td>
</tr>
<tr>
<td>MnSOD+/+</td>
<td></td>
<td>211 ± 12</td>
<td>182 ± 9*</td>
<td>185 ± 12*</td>
</tr>
<tr>
<td>MnSOD+/−</td>
<td></td>
<td>174 ± 5*</td>
<td>123 ± 14*</td>
<td>132 ± 14*</td>
</tr>
</tbody>
</table>

* Different from untreated mice (0 h) of the same genotype, P < 0.05.

**TABLE 3**

Hepatic mitochondrial GSH in naive or alcohol-treated WT, MnSOD+/+, and MnSOD+/− mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>GSH (nmol/mg protein)</th>
<th>0 h</th>
<th>2 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td></td>
<td>13.2 ± 1.0</td>
<td>6.8 ± 0.9*</td>
<td>10.7 ± 0.9*</td>
</tr>
<tr>
<td>MnSOD+/+</td>
<td></td>
<td>13.8 ± 0.7</td>
<td>11.1 ± 1.1*</td>
<td>11.7 ± 2.6</td>
</tr>
<tr>
<td>MnSOD+/−</td>
<td></td>
<td>10.9 ± 1.2</td>
<td>4.0 ± 0.4*</td>
<td>5.1 ± 1.1*</td>
</tr>
</tbody>
</table>

* Different from untreated mice (0 h) of the same genotype, P < 0.05.

* Different from WT mice studied at the same treatment time (0, 2, or 24 h), P < 0.05.
changes in mtDNA levels (Fig. 3B). Ethanol administration did not deplete mtDNA in MnSOD**+/+** mice. In WT mice, alcohol administration transiently decreased mtDNA levels by 35% at 2 h (P < 0.05; two-way ANOVA followed by PLSD test), whereas mtDNA levels were restored 24 h after the binge. In MnSOD**+/−** mice, ethanol durably decreased mtDNA levels, by 56% at 2 h and by 44% at 24 h (P < 0.05, two-way ANOVA followed by PLSD test; Fig. 3B), suggesting that the compensatory mtDNA resynthesis that occurred in WT mice was impaired in MnSOD**+/−** mice, possibly because of persistent oxidative lesions in mtDNA.

**Absent, Transient, or Persistent mtDNA Lesions Hampering the Progress of Polymerases in Alcohol-Treated MnSOD**+++/+, WT, and MnSOD**+/−** Mice, Respectively.** To assess the presence of mtDNA lesions blocking the progress of polymerases, we performed long PCR experiments to concomitantly amplify a long (8636-bp) and a short (316-bp) mtDNA fragment (Fig. 3C). DNA lesions blocking replication are more likely to be present on a long DNA region than a short fragment (Demeilliers et al., 2002). Indeed, the amplification of the short DNA fragment was similar in all groups of mice (Fig. 3C). We therefore used the long fragment/short fragment intensity ratio as an index of the integrity of mtDNA (Fig. 3D). Two hours after ethanol administration, the 8636-bp product/316-bp product intensity ratio was unchanged in MnSOD-overexpressing mice but was decreased by 51% in WT mice and 59% in MnSOD**+/−** mice (P < 0.05, two-way ANOVA followed by PLSD test). Twenty-four hours after alcohol administration, the amplification of the 8636-bp product had increased in ethanol-treated MnSOD**+++/+** or WT mice but was still decreased by 59% in MnSOD**+/−** mice (P < 0.05, two-way ANOVA followed by PLSD test; Fig. 3D), possibly suggesting that the persistent mtDNA depletion that persisted at that time in MnSOD**+/−** mice was due, in part, to the persistence of polymerase γ-blocking lesions on mtDNA templates, thus hampering mtDNA resynthesis.

**Ethanol Treatment Decreased Tsfam but not PGC-1 Protein Levels in WT and MnSOD**+++/+, Mice.** PGC-1 can increase mtDNA replication (Lin et al., 2003), and Tsfam is necessary for mtDNA replication, transcription, and stability (Kang et al., 2007). We therefore assessed the effects of alcohol administration on the expression of these two proteins (Fig. 4). However, the PGC-1 protein was not affected by alcohol regardless of the genotype (Fig. 4A), and the effects of ethanol on the Tsfam protein were reminiscent of its effects on mtDNA levels. Whereas alcohol administration did not modify Tsfam protein levels in MnSOD**+++/+** mice, it markedly decreased the Tsfam protein 2 h after the binge in WT or MnSOD**+/−** mice, with partial recovery 24 h after the binge (Fig. 4B). In addition to its specific high-affinity interaction with the light and heavy strand promoters on mtDNA, Tsfam also serves as a packaging protein, which wraps the entire mtDNA molecule, so that few Tsfam or mtDNA molecules exist in unbound form (Kang et al., 2007). A corollary is that Tsfam protein depletion can be a consequence of mtDNA depletion (Kang et al., 2007).

**Absent, Transient, or Persistent Respiratory Complex Dysfunction in Alcohol-Treated MnSOD**+++/+, WT, and MnSOD**+/−** Mice, Respectively.** We first used Western blotting to assess the protein expression of selected respiratory proteins but found no changes in the protein levels of the mtDNA-encoded COX2, the nDNA-encoded α subunit of ATP synthase, or the nDNA-encoded 30-kDa subunit of complex I (data not shown). We also looked at respiratory chain complexes separated by BN-PAGE but found no changes in the protein levels of these multisubunit protein complexes (Fig. 5A).

We finally assessed the in-gel activity of complex I and complex V (Fig. 5, B–D). In MnSOD-overexpressing mice, alcohol administration did not modify complex I or complex V activity. In alcohol-treated WT mice, complex I and complex V activities were transiently decreased by 36 and 25%, respectively, at 2 h (P < 0.05, two-way ANOVA followed by PLSD test) but had recovered at 24 h (Fig. 5, C and D). In alcohol-treated MnSOD**+/−** mice, complex I and complex V activities were decreased by 53 and 30%, respectively, at 2 h.
and were still decreased by 66 and 59%, respectively, at 24 h (P < 0.05, two-way ANOVA followed by PLSD test; Fig. 5, C and D).

Exacerbated iNOS Induction in Alcohol-Treated MnSOD−/− Mice Compared with WT or MnSOD+++ Mice. The hepatic expression of the iNOS protein was assessed by Western blotting (Fig. 6A). Baseline iNOS protein expression was similar in untreated WT and MnSOD+++ mice but was significantly higher in untreated MnSOD−/− mice (P < 0.05, two-way ANOVA followed by PLSD test; Fig. 6B). Alcohol significantly increased iNOS protein expression whatever the MnSOD genotype at 24 h but not at 2 h (Fig. 6B). However, because of the already high baseline levels, MnSOD−/− mice reached higher iNOS levels 24 h after alcohol administration than alcohol-treated WT mice (Fig. 6B).

Absent, Late, or Persistent Increase in Plasma Nitrite/Nitrate Levels in Alcohol-Treated MnSOD+++ Mice, Respectively. We also assessed plasma nitrite/nitrate levels (Table 4). In MnSOD+++ mice, ethanol administration did not significantly modify plasma nitrite/nitrate levels. In WT mice, ethanol administration significantly increased nitrite/nitrate concentrations 24 h but not at 2 h. In MnSOD−/− mice, ethanol treatment significantly augmented plasma nitrite/nitrate concentrations at both 2 and 24 h (P < 0.05, two-way ANOVA followed by PLSD test; Table 4).

Exacerbated Nitrination of Tyrosine Residues in the Complex I and Complex V Proteins of Alcohol-Treated MnSOD−/− Mice. Exacerbated iNOS induction and mitochondrial ROS formation in MnSOD−/− mice could increase the formation of peroxynitrite and increase the nitration of protein tyrosine residues. To look for 3-nitrotyrosine adducts, mitochondrial respiratory complexes were separated by BN-PAGE and transferred onto nitrocellulose membranes. The blots were then revealed with an antibody against 3-nitrotyrosine (Fig. 6C). In the proteins of complex I, 3-nitrotyrosine was only detected in MnSOD−/− mice. In the proteins of the more heavily labeled complex V, 3-nitrotyrosine was detected in the three genotypes, although it was much more abundant in MnSOD−/− mice than in WT or MnSOD+++ mice (Fig. 6C). Whereas alcohol administration did not modify 3-nitrotyrosine levels in MnSOD+++ mice, it increased 3-nitrotyrosine 24 h after the binge in both WT and MnSOD+++ mice and also increased complex V tyrosine nitrations 2 h after the binge in MnSOD−/− mice (Fig. 6C).

Tempol, L-NAME, 1400W, or Uric Acid Attenuated Nitrization of Tyrosine Residues in the Complex I and Complex V Proteins of Alcohol-Treated MnSOD−/− Mice. Tempol (a superoxide anion scavenger), L-NAME (a broad-spectrum NOS inhibitor), 1400W (a specific inhibitor of iNOS), or uric acid (a peroxynitrite scavenger) signifi-
alcohol for 24 h. Hematoxylin and eosin staining showed normal liver architecture, no steatosis, no liver cell necrosis and no inflammation in any of the groups of mice at 24 h (data not shown).

To look for apoptosis, we evaluated caspase-3 activity at 2 and 24 h and performed a TUNEL assay at 24 h. However, caspase-3 activity remained unchanged, and the TUNEL assay was negative in ethanol-treated WT, MnSOD\(^{+/+}\), and MnSOD\(^{+-}\) mice (data not shown).

Discussion

This is the first study to show that transgenic mice over-expressing MnSOD are protected against mtDNA damage and mtDNA depletion after an alcohol binge, whereas partially deficient MnSOD\(^{+-}\) mice undergo more sustained mtDNA damage than WT mice (Fig. 3). This is also the first study to suggest that peroxynitrite plays a major role in mtDNA depletion triggered by an alcohol binge in WT or MnSOD\(^{+-}\) mice (Table 5).

Chronic alcohol consumption has been shown to increase the formation of hydrogen peroxide in mitochondria (Wieland and Lauterburg, 1995) and to induce iNOS protein expression within hepatocytes (Wang and Cederbaum, 2008). In the present study, a single alcohol binge increased oxidant production in isolated mouse liver mitochondria (Fig. 2A). The binge also increased plasma nitrite/nitrate levels and the hepatic expression of the iNOS protein 24 h after the binge (Table 4; Fig. 6B). Although the presence of NOS within mitochondria is debated, the NO formed elsewhere can cross mitochondrial membranes to react with superoxide and form peroxynitrite within the mitochondria (Radi et al., 2002).

Peroxynitrite and/or peroxynitrite-generated reactive intermediates can nitrate the tyrosine residues of proteins (Radi et al., 2002) and can damage DNA (Epe et al., 1996). In the present study, 3-nitrotyrosine residues were detected in complex V proteins 24 h after the alcohol binge in WT mice (Fig. 6). Two hours after the binge, mtDNA was depleted in WT mice (Fig. 3B), probably because damaged mtDNA molecules had been degraded by mitochondrial endonucleases (Mansouri et al., 1999; Demeilliers et al., 2002). The remaining mtDNA molecules also presented mtDNA lesions, as shown by the selective impairment of amplification of a long mtDNA fragment contrasting with the normal amplification of a short mtDNA fragment (Fig. 3D). Lesions hampering the progress of polymerases are much more likely to be present on a long stretch of DNA than a short DNA region (Mansouri et al., 1999; Demeilliers et al., 2002).

To further assess the role of peroxynitrite in mtDNA depletion and in protein nitration, we tested the effects of radical scavengers or NOS inhibitors on alcohol-induced mtDNA depletion (Table 5) and on the nitration of tyrosine residues in complex I and complex V proteins in WT and MnSOD\(^{+-}\) mice (Table 5). Tempol, a superoxide scavenger (Wilcox and Pearlman, 2008), l-NNAME, a broad-spectrum NOS inhibitor (McKim et al., 2003), 1400W, a selective iNOS inhibitor (McKim et al., 2003), and uric acid, a peroxynitrite scavenger (García-Ruiz et al., 2006), all prevented or attenuated alcohol-induced mtDNA depletion (Table 5) and complex I and V protein nitration (Fig. 6). These results suggest that the reaction of superoxide with
Fig. 5. Effects of alcohol administration on the activities of respiratory chain complexes I and V in WT, MnSOD\textsuperscript{+++} and MnSOD\textsuperscript{+-} mice. Mice killed 2 or 24 h after the intragastric administration of alcohol (5 g/kg) served as pooled zero-time controls (0 h) for the alcohol treatment. Other mice were killed 2 or 24 h after the intragastric administration of alcohol. A, liver mitochondrial lysates (35 μg of proteins) underwent BN-PAGE to separate intact oxidative phosphorylation complexes. B, bands corresponding to complex I or complex V were excised and incubated with specific reagents to determine the in situ activity of complex I and complex V, as shown on representative gels. C and D, values on the histograms represent the ratio of the densitometric reading for the activity-associated band intensity over the densitometric reading of the protein band intensity. Two-way ANOVA indicated an effect of genotype and alcohol treatment time as well as an interaction between genotype and treatment time.

# different from untreated mice (0 h) of the same genotype, \( P < 0.05 \).

\*, different from WT mice studied at the same treatment time (0, 2, or 24 h), \( P < 0.05 \).

Fig. 6. Effects of alcohol administration on iNOS protein expression in WT, MnSOD\textsuperscript{+++}, and MnSOD\textsuperscript{+-} mice and effects of alcohol administration with or without ROS scavengers or NOS inhibitors on the nitration of tyrosine residues in mitochondrial complex I and V proteins. Mice killed 2 or 24 h after the intragastric administration of water served as pooled zero-time controls (0 h) for the alcohol treatment. Other mice were killed 2 or 24 h after the intragastric administration of alcohol (5 g/kg). Thirty minutes before ethanol, some mice received an intraperitoneal injection of tempol (200 mg/kg b.wt.), L-NAME (80 mg/kg b.wt.), 1400W (10 mg/kg b.wt.), or uric acid (500 mg/kg b.wt.). In mice that were sacrificed 24 h after ethanol administration, a second injection of the cotreatment was administered 8 h after ethanol administration. A, hepatic proteins underwent SDS-PAGE and transfer to nitrocellulose membranes. Western blots were revealed with anti-iNOS and anti-β-actin antibodies. B, the iNOS/β-actin intensity ratio was quantified (means ± S.E.M. for six to eight mice). Two-way ANOVA revealed an effect of genotype and an effect of alcohol treatment time.

# different from untreated mice of the same genotype, \( P < 0.05 \).

\*, different from WT mice studied at the same treatment time (0, 2, or 24 h), \( P < 0.05 \).

C, equal amounts of liver mitochondrial proteins (35 μg) underwent BN-PAGE to separate intact oxidative phosphorylation complexes as shown in Fig. 5A. Separated complexes were transferred to nitrocellulose membranes for Western blot detection of 3-nitrotyrosine residues in the proteins of complex I and complex V.
Effects of alcohol administration on plasma nitrite/nitrate concentrations in WT, MnSOD+/+, and MnSOD−/− mice

Mice killed 2 or 24 h after the intragastric administration of water served as pooled zero-time controls (0 h) for the alcohol treatment. Other mice were killed 2 or 24 h after the intragastric administration of alcohol (5 g/kg). Two-way ANOVA indicated an effect of genotype and alcohol treatment time. Results are means ± S.E.M. for 5 to 12 mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Plasma Nitrite/Nitrate Conc.</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
<td>2 h</td>
</tr>
<tr>
<td>WT</td>
<td>50 ± 5</td>
<td>70 ± 14</td>
</tr>
<tr>
<td>MnSOD−/+</td>
<td>38 ± 12</td>
<td>44 ± 12</td>
</tr>
<tr>
<td>MnSOD−/−</td>
<td>63 ± 10</td>
<td>85 ± 15</td>
</tr>
</tbody>
</table>

* Different from untreated (0 h) of the same genotype, P < 0.05.

TABLE 5 Effects of a superoxide anion scavenger (tempol), a nonselective NOS inhibitor (l-NNAME), a specific iNOS inhibitor (1400W), and a peroxynitrite scavenger (uric acid) on ethanol-induced mtDNA depletion in WT, MnSOD+/+, and MnSOD−/− mice

Mice killed 2 or 24 h after the intragastric administration of water served as pooled zero-time controls (0 h) for the alcohol treatment. Other mice were killed 2 or 24 h after the intragastric administration of alcohol (5 g/kg). Thirty minutes before ethanol, some mice received an intraperitoneal injection of tempol (200 mg/kg b.wt.), L-NAME (80 mg/kg b.wt.), 1400 W (10 mg/kg b.wt.), or uric acid (500 mg/kg b.wt.). In mice that were sacrificed 24 h after ethanol administration, a second injection of the cotreatment was administered 8 h after ethanol administration. Values represent means ± S.E.M. for 5 to 18 mice. Two-way ANOVA indicated a global effect of genotype or treatment time or a significant interaction between genotype and treatment time (P < 0.05, two-way ANOVA).

<table>
<thead>
<tr>
<th>Cotreatment</th>
<th>mtDNA/nDNA Hybridization Ratio in WT Mice</th>
<th>0 h</th>
<th>2 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100 ± 4</td>
<td>64 ± 4</td>
<td>103 ± 5</td>
<td></td>
</tr>
<tr>
<td>Tempol</td>
<td>102 ± 7</td>
<td>97 ± 10</td>
<td>94 ± 7</td>
<td></td>
</tr>
<tr>
<td>l-NNAME</td>
<td>99 ± 3</td>
<td>85 ± 5</td>
<td>111 ± 4</td>
<td></td>
</tr>
<tr>
<td>1400W</td>
<td>105 ± 11</td>
<td>99 ± 10</td>
<td>94 ± 9</td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td>99 ± 3</td>
<td>96 ± 3</td>
<td>97 ± 4</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cotreatment</th>
<th>mtDNA/nDNA Hybridization Ratio in MnSOD−/− Mice</th>
<th>0 h</th>
<th>2 h</th>
<th>24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>101 ± 2</td>
<td>44 ± 4</td>
<td>67 ± 6</td>
<td></td>
</tr>
<tr>
<td>Tempol</td>
<td>98 ± 7</td>
<td>102 ± 11</td>
<td>72 ± 9</td>
<td></td>
</tr>
<tr>
<td>l-NNAME</td>
<td>99 ± 3</td>
<td>78 ± 4</td>
<td>93 ± 2</td>
<td></td>
</tr>
<tr>
<td>1400W</td>
<td>106 ± 9</td>
<td>88 ± 8</td>
<td>103 ± 10</td>
<td></td>
</tr>
<tr>
<td>Uric acid</td>
<td>91 ± 4</td>
<td>76 ± 3</td>
<td>96 ± 3</td>
<td></td>
</tr>
</tbody>
</table>

* Different from untreated (0 h) of the same genotype receiving the same cotreatment, if any (P < 0.05, two-way ANOVA).

* Different from WT mice studied at the same treatment time (0, 2, or 24 h) and receiving the same cotreatment, if any (P < 0.05, two-way ANOVA).

* Different from mice of the same genotype studied at the same treatment time but without any cotreatment (P < 0.05, one-way ANOVA within the WT group or within the MnSOD−/− group).

Whereas MnSOD overexpression prevented all the mitochondrial effects of the alcohol binge, partial MnSOD deficiency prolonged the mitochondrial effects of alcohol that were only transient in WT mice. Unlike WT mice, MnSOD−/− mice still exhibited increased mitochondrial ROS formation (Fig. 2A), decreased mtDNA levels (Fig. 3B), decreased mtDNA integrity (Fig. 3D), decreased activities of mitochondrial complexes I and V (Fig. 5), and low mitochondrial GSH levels (Table 3) 24 h after the binge. Several intertwined effects may lead to protracted formation of reactive oxygen/nitrogen species in alcohol-treated MnSOD−/− mice. First, as already mentioned, the higher intramitochondrial superoxide concentrations of MnSOD−/− mice may increase peroxynitrite formation within mitochondria. Second, exacerbated oxidative stress may increase iNOS expression in MnSOD−/− mice. In keeping with results showing that superoxide dismutase prevented iNOS induction in two previous studies (Mendes et al., 2003; San Martin et al., 2007), iNOS expression was higher in alcohol-treated MnSOD−/− mice than in alcohol-treated WT mice (Fig. 6). Therefore, both decreased superoxide inactivation by the deficient MnSOD and increased induction of iNOS by the alcohol-induced oxidative stress may cause higher and more sustained peroxynitrite formation in alcohol-treated MnSOD−/− mice than in alcohol-treated WT mice.

Third, decreased respiratory complex activities may prolong mitochondrial ROS formation after alcohol administration to MnSOD−/− mice. Although complex I was only transiently inactivated in alcohol-treated WT mice, it was durably inactivated (at both 2 and 24 h) in alcohol-treated MnSOD−/− mice (Fig. 5). Impaired electron flow along the respiratory chain can cause electrons to accumulate within the chain, and some of the accumulated electrons to react with oxygen to form the superoxide anion, thus increasing mitochondrial ROS formation (Esposito et al., 1999). Indeed, whereas mitochondrial ROS formation was only transiently increased in alcohol-treated WT mice, it was still markedly increased 24 h after the binge in MnSOD−/− mice (Fig. 2A). Increased mitochondrial formation of superoxide at 24 h together with the marked induction of iNOS at that time (Fig. 6B) could increase peroxynitrite formation and explain the major nitration of complex I and complex V proteins in alcohol-treated MnSOD−/− mice 24 h after the binge (Fig. 6C). A last mech-

...
anism contributing to prolonged mitochondrial dysfunction in alcohol-treated MnSOD-/- mice may involve the paradoxical effect of alcohol on MnSOD activity in these mice. MnSOD has been shown to be inducible by ROS, cytokines, and ethanol (Koch et al., 1994), and its activity indeed increased 24 h after the alcohol binge in WT and MnSOD+/-+ mice (Fig. 1). In sharp contrast, however, alcohol administration further decreased the already low MnSOD activity in MnSOD-/- mice (Fig. 1). Although MnSOD is inducible by ethanol, it is inactivated by peroxynitrite (MacMillan-Crow et al., 1998). Sustained increases in peroxynitrite formation and protein nitration are probably the reason for this further decrease in MnSOD activity in alcohol-treated MnSOD-/- mice. A similar mechanism has been suggested for the decreased MnSOD activity in acute cardiac allograft rejection (Nilakantan et al., 2005). This secondary MnSOD inactivation may further hamper the intramitochondrial inactivation of superoxide and further enhance its reaction with NO to form peroxynitrite in alcohol-treated MnSOD+/- mice.

The activity of respiratory complexes has to be severely decreased (in excess of a 70–80% inhibition threshold) to decrease the overall respiratory rate (Rossignol et al., 1999), and the respiratory rate has to be severely decreased to cause necrosis or steatosis (Fromenty and Pessayre, 1995). Presumably, these thresholds were not reached and/or the impairment was not sustained enough in the liver, as neither necrosis nor steatosis was observed, even in MnSOD-/- mice.

The effects of MnSOD overexpression on alcohol-mediated mtDNA depletion could be different after acute or prolonged alcohol administration. Unlike the protective effect of MnSOD observed in the present study, MnSOD overexpression worsened the effects of chronic ethanol treatment on mtDNA levels and integrity in mice (Larosche et al., 2009). Recent studies have shown previously unsuspected connections between MnSOD activity and the tendency to develop hepatic iron accumulation in diverse circumstances favoring iron deposition (Sutton et al., 2006; Nahon et al., 2008; Larosche et al., 2009). In particular, MnSOD-overexpressing transgenic mice, but not WT mice, developed increased hepatic iron concentrations and mtDNA depletion after 7-weeks of alcohol treatment (Larosche et al., 2009). The iron chelator deferoxamine prevented both iron accumulation and mtDNA depletion, suggesting that iron-mediated hydroxyl radical formation played a role in the mtDNA damage caused by prolonged alcohol administration in MnSOD+/-+ mice (Larosche et al., 2009). One mechanism contributing to iron accumulation during prolonged alcohol administration is decreased expression of hepatic hepcidin, thus increasing intestinal iron absorption and slowly increasing hepatic iron stores (Ohtake et al., 2007).

The binge drinking fad in the young has become an important health issue in some countries (Mathurin and Deltenre, 2009). Although a single alcohol binge was not sufficient to cause liver lesions in this study or a previous study (Mansouri et al., 1999), repeated whisky binges promoted liver injury in rats fed a choline-deficient diet (Nieto and Rojkind, 2007), and human drinkers binging at least once a week can develop cirrhosis (Mathurin and Deltenre, 2009). Further animal studies are needed to better determine the long-term pathogenic consequences of frequent alcohol binges.

In conclusion, MnSOD overexpression prevents, whereas partial MnSOD deficiency prolongs, mtDNA depletion and mitochondrial dysfunction after an acute alcohol binge in mice. The protective effects of MnSOD, tempol, NOS inhibitors, and uric acid suggest that peroxynitrite plays a major role in the mtDNA damage caused by an alcohol binge in mice.

Acknowledgments

We thank Nathalie Vadrot, Dr. A. Abbey-Toby, and Prof. D. Henin (Laboratoire d’Anatomato-Pathologie, Hôpital Bichat, Paris, France) for assessing liver lesions and Drs. H. Van Remmen, A. Richardson (University of Texas Health Science Center, San Antonio, TX), and C. J. Epstein (University of California, San Francisco, CA) for providing heterozygous MnSOD knockout progenitor mice.

References


Address correspondence to: Dr. Abdelhaj Mansour, Institut National de la Santé et de la Recherche Médicale U773, Faculté de Médecine Xavier Bichat, 16 rue Henri Huchard, BP 416, 75018 Paris, France. E-mail: abdel.mansour@inserm.fr