Toxicology and Applied Pharmacology 234 (2009) 326–338

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Prolonged ethanol administration depletes mitochondrial DNA in MnSOD-overexpressing transgenic mice, but not in their wild type littermates

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ARTICLE INFO

Article history:
Received 2 May 2008
Revised 15 October 2008
Accepted 4 November 2008
Available online 20 November 2008

Keywords:
Mitochondria
Liver
Alcohol
Oxidative stress
Manganese superoxide dismutase

ABSTRACT

Alcohol consumption increases reactive oxygen species formation and lipid peroxidation, whose products can damage mitochondrial DNA (mtDNA) and alter mitochondrial function. A possible role of manganese superoxide dismutase (MnSOD) on these effects has not been investigated. To test whether MnSOD overexpression modulates alcohol-induced mitochondrial alterations, we added ethanol to the drinking water of transgenic MnSOD-overexpressing (TgMnSOD) mice and their wild type (WT) littermates for 7 weeks. In TgMnSOD mice, alcohol administration further increased the activity of MnSOD, but decreased cytosolic glutathione as well as cytosolic glutathione peroxidase activity and peroxisomal catalase activity. Whereas ethanol increased cytochrome P-450 2E1 and mitochondrial ROS generation in both WT and TgMnSOD mice, hepatic iron, lipid peroxidation products and respiratory complex I protein carbonyls were only increased in ethanol-treated TgMnSOD mice but not in WT mice. In ethanol-fed TgMnSOD mice, but not ethanol-fed WT mice, mtDNA was depleted, and mtDNA lesions blocked the progress of polymerases. The iron chelator, DFO prevented hepatic iron accumulation, lipid peroxidation, protein carbonyl formation and mtDNA depletion in alcohol-treated TgMnSOD mice. Alcohol markedly decreased the activities of complexes I, IV and V of the respiratory chain in TgMnSOD, with absent or lesser effects in WT mice. There was no inflammation, apoptosis or necrosis, and steatosis was similar in ethanol-treated WT and TgMnSOD mice. In conclusion, prolonged alcohol administration selectively triggers iron accumulation, lipid peroxidation, respiratory complex I protein carbonylation, mtDNA lesions blocking the progress of polymerases, mtDNA depletion and respiratory complex dysfunction in TgMnSOD mice but not in WT mice.

Introduction

Ethanol administration increases the generation of free radicals and reactive oxygen species (ROS) by mitochondria, cytochrome P-450 2E1 (CYP2E1), NADPH oxidases and ferrous iron (Dey and Cerderbaum, 2006; Albano, 2008). Ethanol ingestion can therefore trigger hepatic oxidative stress and mitochondrial dysfunction in humans and experimental animals (Dey and Cerderbaum, 2006; Albano, 2008).

In the liver, ethanol administration oxidatively damages mitochondrial proteins, phospholipids and mitochondrial DNA (mtDNA) (Wieland and Lauterburg, 1995). mtDNA encodes 13 of the oxidative phosphorylation proteins, and is more susceptible to oxidative damage than nuclear DNA (nDNA) due to the absence of protective histones, incomplete DNA repair capacity in mitochondria, and the proximity of mtDNA to the mitochondrial respiratory chain, which is the main site of ROS formation in the cell (Yakes and Van Houten,

Abbreviations: ATP, adenosine triphosphate; BN-PAGE, blue native polyacrylamide gel electrophoresis; COX2, subunit 2 of cytochrome *c* oxidase; Cu,ZnSOD, copper-zinc superoxide dismutase; DCF, 2′,7′-dichlorofluorescein; DNP, 2,4-dinitrophenyl; ECL, enhanced chemiluminescence; H_2DCF-DA, 2′,7′-dichlorodihydrofluorescein diacetate; HRP, horseradish peroxidase; iNOS, inducible nitric oxide synthase; PLSD, protected least significance difference; MnSOD, manganese superoxide dismutase; mtDNA, mitochondrial DNA; NAD^+, oxidized nicotinamide-adenine dinucleotide; NADH, reduced nicotinamide-adenine dinucleotide; $NADP^+$, oxidized nicotinamide-adenine dinucleotide phosphate; NADPH, reduced nicotinamide-adenine dinucleotide phosphate; NBT, Nitro Blue Tetrazolium; nDNA, nuclear DNA; TBARs, thiobarbituric acid reactants; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling; ROS, reactive oxygen species; PBS, phosphate-buffered saline; PGC-1, peroxisome proliferator activated receptor gamma coactivator 1; Tfam, mitochondrial transcription factor A.

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doi:10.1016/j.taap.2008.11.004

1997). The ethanol-induced oxidative stress causes the formation of 8-hydroxydeoxyguanosine in mtDNA (Wieland and Lauterburg, 1995), as well as apurinic/apyrimidinic sites and mtDNA strand breaks. These mtDNA lesions can lead to mtDNA depletion in animals (Mansouri et al., 1999; Mansouri et al., 2001; Demeilliers et al., 2002) and to premature mtDNA deletions in humans (Fromenty et al., 1995; Mansouri et al., 1997). Ethanol consumption can therefore decrease the synthesis of the 13 mtDNA-encoded proteins that are part of respiratory chain complexes I, III, IV and V (Coleman and Cunningham, 1990; Demeilliers et al., 2002).

Whereas most ROS-induced mtDNA damages are quickly repaired in mitochondria, lipid peroxidation-induced bulky DNA adducts and mtDNA cross-links may not be removed from mtDNA (Croteau et al., 1999). After four days of daily intragastric ethanol administration to mice, the accumulation of unrepaired lesions in mtDNA prevented the re-synthesis of new mtDNA molecules, thus contributing to the prolonged persistence of mtDNA depletion after cessation of alcohol administration (Demeillers et al., 2002).

The superoxide anion radical produced by the respiratory chain is detoxified by the successive action of manganese superoxide dismutase (MnSOD) and then mitochondrial glutathione peroxidase-1 (GPx-1) (Wallace, 1999). MnSOD dismutates the superoxide anion into hydrogen peroxide, which is detoxified into water by GPx-1. MnSOD is inducible by ROS, cytokines, and ethanol (Koch et al., 1994). An increase in MnSOD activity should decrease the steady state cellular levels of the superoxide anion (Liochev and Fridovich, 2007). This might decrease its reaction with NO to form peroxynitrite, which seems to play an important role in alcohol-induced liver lesions (McKim et al., 2003). Indeed, increasing MnSOD activity with a recombinant adenovirus protected rats against alcohol-induced apoptosis, necrosis and inflammation in a previous study (Wheeler et al., 2001). This study, however, did not assess mtDNA damage or mitochondrial function (Wheeler et al., 2001).

Although an increased MnSOD activity may have beneficial effects on some targets of ethanol, it could also have deleterious consequences on some other targets. The goal of the present study was to evaluate the effects, if any, of MnSOD overexpression on mtDNA levels after chronic alcohol treatment. We exposed transgenic MnSOD-overexpressing (TgMnSOD) mice and their wild type (WT) littermates to alcohol for seven weeks. We found that prolonged ethanol administration selectively damages and depletes mtDNA in TgMnSOD mice but not in WT mice. The iron chelator, DFO prevents mtDNA depletion in ethanol-treated TgMnSOD mice, suggesting a contributory role of iron.

Methods

Materials and chemicals. The ApoTag peroxidase *in situ* apoptosis detection kit and the OxyBlot protein carbonylation kit were purchased from Chemicon (Hampshire, UK). Dichlorodihydrofluorescein diacetate (H_2DCF-DA) and the mouse monoclonal antibody against the α subunit of ATPase were from Molecular Probes (Eugene, OR). The mouse monoclonal antibody against subunit 2 of cytochrome *c* oxidase (COX2), the goat polyclonal antibodies against mitochondrial transcription factor A (Tfam) and the rabbit polyclonal antibodies against MnSOD, copper-zinc superoxide dismutase (Cu, ZnSOD) and peroxisome proliferator activated receptor gamma coactivator 1 (PGC-1) were all purchased from Tebu-Bio (Le Perray en Yvelines, France). The mouse monoclonal antibody against inducible nitric oxide synthase (iNOS) was from BD Transduction Laboratories (Le Pont de Claix, France), and the rabbit polyclonal antibody against CYP2E1 from Euromedex (Souffelweyersheim, France). The caspase-3 activity kit was purchased from Biomol (Plymouth Meeting, PA). Hybond-N^+ membranes, Hybond-C-extra membranes and α-dCTP[^{32}P] were purchased from GE Healthcare (Orsay, France). The Expand Long Template PCR System was from Roche Applied Science (Mannheim, Germany). Other products were from Sigma (St. Louis, MO).

Animals and treatments. The TgMnSOD mice used in this study were developed in Epstein's laboratory (Raineri et al., 2001). We used strain 274, which is in the C57BL/6J genetic background and expresses the Sod2 mouse transgene at levels 2 to 3 times the Sod2 expression in WT mice (Raineri et al., 2001). WT C57BL/6J mice were obtained from Janvier (Le Genest Saint Isle, France) and used for breeding with the TgMnSOD mice. WT and TgMnSOD mice were backcrossed into an inbred strain for at least 9 generations.

Animals were fed *ad libitum* a standard diet (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. At 2 to 3 months of age, the adult male mice obtained by breeding were genotyped. TgMnSOD mice and their WT littermates were then given either water alone to drink for 7 weeks, or progressively increasing concentrations of alcohol added to the drinking water: 5% (v/v) alcohol for the first week, 10% for the second week, 15% for the third, and then 20% for the fourth week and until the end of the seventh week. Some WT and TgMnSOD mice received either deferoxamine (DFO) mesylate alone s.c. (50 mg/kg/day, 5 days a week) or both DFO mesylate s.c. and alcohol in the drinking water for 7 weeks. Animals were killed by cervical dislocation either immediately at the end of the ethanol treatment or 48 h after the removal of ethanol from the drinking water.

Serum ethanol concentration. Serum ethanol was measured with a commercial kit (ADH-NAD Reagent Multiple Test Vial, Sigma) based on the alcohol-mediated conversion of oxidized nicotinamide adenine dinucleotide (NAD^+) to reduced nicotinamide adenine dinucleotide (NADH) in the presence of alcohol dehydrogenase.

Protein levels and activities of MnSOD and Cu,ZnSOD. For Western blot analysis, proteins (50 μg) underwent SDS-14% polyacrylamide gel electrophoresis. The MnSOD and Cu,ZnSOD proteins were detected with rabbit polyclonal anti-MnSOD and anti-Cu,ZnSOD antibodies. After washings, membranes were incubated with an anti-rabbit HRP-conjugated secondary antibody, and revealed with enhanced chemiluminescence (ECL) reagents. Blots were stripped and exposed to an anti-β-actin antibody. We also used native gel assays to assess MnSOD and Cu,ZnSOD activities (Sutton et al., 2005). Total hepatic proteins (100 μg) were resuspended in 40% glycerol and 0.025% bromophenol blue, and loaded on a non-denaturing 15% polyacrylamide gel. Migration was performed at 4 °C for 12 h at 120 V, and superoxide dismutase activity was assessed within the gel. The assay is based on a competition reaction between superoxide dismutase and the superoxide indicator molecule, nitro blue tetrazolium (NBT). The gel was first soaked for 15 min in 100 mM potassium phosphate buffer (pH 7.8) containing 2.45 mM NBT, and then soaked for another 15 min in the dark in the same buffer containing 28 mM N,N,N′,N′-tetramethylethylenediamine and 28 μM riboflavin. The gel was illuminated for 15 min to initiate the photochemical reaction, and was immediately scanned to assess the MnSOD and CuZnSOD bands by densitometry analysis.

Glutathione, GPx activity and catalase activity assays. The mitochondrial and cytosolic contents of reduced glutathione were determined as described by Tietze (1969). GPx activity was measured in mitochondrial and cytosolic fractions by following the conversion of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) to $NADP^+$ at 340 nm for 2 min in the presence of hydrogen peroxide, glutathione reductase, and reduced glutathione. To measure catalase activity in liver homogenates, 10 to 20 μg of liver proteins were incubated in 50 μL of phosphate buffer and the reaction was initiated by adding 3 mL of 12.5 mM H_2O_2 to the medium. The change in