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Original Contribution

Decreased expression of peroxiredoxin 6 in a mouse model of ethanol consumption

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ABSTRACT

Alcoholic liver disease is multifactorial and oxidative stress is believed to play an intimate role in the initiation and progression of this pathology. The goals of this study were to investigate the effect of chronic ethanol treatment on inducing hepatic oxidative stress and peroxiredoxin 6 expression. After 9 weeks of treatment with an ethanol-containing diet, significant increases in serum ALT activity, liver to body weight ratio, liver triglycerides, CYP2E1 protein expression, and CYP2E1 activity were observed. Chronic ethanol feeding resulted in oxidative stress as evidenced by decreases in hepatic glutathione content and increased deposition of 4-hydroxynonenal and 4-oxononenal protein adducts. In addition, novel findings of decreased PRX6 protein and mRNA and increased levels of carbonylated PRX6 protein were observed in the ethanol-treated animals compared to the pair-fed controls. Lastly, NF-κB activity was found to be significantly increased in the ethanol-treated animals. Concurrent with the increase in NF-κB activity, decreases in both MEK1/2 and ERK1/2 phosphorylation were also observed in the ethanol-treated animals compared to the pair-fed controls. Together, these data demonstrate that chronic ethanol treatment results in oxidative stress, implicating NF-κB activation as an integral mechanism in the negative regulation of PRX6 gene expression in the mouse liver.

Introduction

The term oxidative stress refers to a serious imbalance between the production of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) and the detoxification of these molecules by cellular antioxidant defenses. A state of oxidative stress results from increased production of ROS/RNS or diminished antioxidant defenses, or a combination of the two. ROS/RNS can attack cellular macromolecules; for example, increased ROS production can lead to lipid peroxidation and the generation of secondary aldehydic end products, including 4-hydroxynonenal, which have the capacity to modify proteins and alter their normal function [1]. ROS and RNS are involved in many biological phenomena such as mutations, carcinogenesis, aging, atherosclerosis, inflammation, diabetes mellitus, neurodegenerative diseases, and tissue injury associated with chronic alcohol abuse [2].

Alcoholic liver disease (ALD) is a pathology characterized by early steatosis, which can progress to an inflammatory stage, often referred to as steatohepatitis. In some individuals persistent inflammation may progress to a stage of fibrosis and cirrhosis [3]. ALD is a multifactorial disease process where oxidative stress is believed to play an intimate role in disease initiation and progression. Increased ROS production in response to chronic ethanol consumption can be attributed to numerous factors, which include CYP2E1 induction, mitochondrial dysfunction, Kupffer cell activation, and/or decreased antioxidant defenses [4]. Alcohol-induced decreased antioxidant defenses include depleted glutathione stores and/or decreased antioxidant expression/activity of superoxide dismutase, catalase, glutathione peroxidase, and peroxiredoxins.

Peroxiredoxins (PRX) are a class of thiol-specific antioxidant proteins and are classified into two distinct groups, 2-Cys and 1-Cys, based on the number of conserved cysteine residues directly involved in catalysis. Members of the 2-Cys class include PRX 1–5, while PRX6 is the lone member of the 1-Cys class. These proteins are primarily located in the cytosol, but are also found in the mitochondria. PRX exert their antioxidant role by exhibiting peroxidase activity [5]. PRX6 has been shown to be unique compared to its family members due to its ability to reduce phospholipid hydroperoxides [6]. PRX6 has also been shown to be essential in protecting tissue from various oxidative insults [7–9].

Due to the fact that chronic ethanol consumption results in liver injury and oxidative stress and that PRX6 is believed to be an important antioxidant in many tissues, our aim was to investigate how the expression of PRX6 was affected by chronic ethanol. Using a mouse model of chronic ethanol feeding, we observed the typical hallmarks of early ALD including steatosis, CYP2E1 induction, and increased oxidative stress. Lastly, we made the novel observations of decreased PRX6 protein and mRNA and increased levels of carbonylated PRX6 protein in conjunction with an increase in NF-κB activity and a decrease in MAP kinase phosphorylation in the ethanol-fed animals.

Abbreviations: ALD, alcoholic liver disease; EDC, ethanol-derived calories; 4HNE, 4-hydroxynonenal; IHC, immunohistochemical; 4ONE, 4-oxononenal; PCR, polymerase chain reaction; PRX, peroxiredoxins; ROS, reactive oxygen species; RNS, reactive nitrogen species; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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Materials and methods

Animals

All procedures involving animals were approved by the Institutional Animal Care and Use Committee of the University of Colorado and were performed in accordance with published National Institutes of Health Guidelines. Male C57/Bl6 mice (8 per group) were fed a modified Lieber-DeCarli diet (Bio-Serv, Frenchtown, NJ) for 9 weeks that consisted of 45% fat-derived calories and 16% protein-derived calories and the balance of the calories comprised varying concentrations of carbohydrate-derived and ethanol-derived calories (EDC). Animals began the study on a diet containing 2% ethanol (v/v) (10.8% EDC) and the amount of ethanol was increased each week until the diet contained 5% ethanol (v/v) (26.9% EDC). The animals continued with this treatment for the remainder of the study. Each ethanol-fed animal was pair-fed with a control animal that was provided a similar diet where the ethanol content was substituted by carbohydrates. Food consumption was measured and recorded daily and body weights were measured at weekly cage changes. On completion of the feeding regimen the animals were anesthetized via intraperitoneal injection of sodium pentobarbital and euthanized by exsanguination. Blood samples were collected from the inferior vena cava for determination of plasma ALT activity using an assay kit from Diagnostic Chemicals Limited (Oxford, CT). Livers were removed, weighed, and homogenized and subcellular fractions were prepared as described elsewhere [10].

Biochemical analysis

Liver triglycerides were measured from 2:1 chloroform:methanol extracts of liver homogenates using a kit from Diagnostic Chemicals Limited. Total hepatic glutathione levels were measured using a kit from Cayman Chemical (Ann Arbor, MI). A TransAM NF-κB p65 activation assay from Active Motif (Carlsbad, CA) was used to assess NF-κB activation in control and ethanol-treated mice. Whole cell extracts were used in the assessment of NF-κB activation and these samples were prepared using a nuclear extract kit from Active Motif. CYP2E1 activity was assessed by measuring the rate of oxidation of *p*-nitrophenol to *p*-nitrocatechol as previously described [11]. Protein concentrations were measured using either a BCA Protein Assay from Pierce (Rockford, IL) or a Bio-Rad Protein Assay (Hercules, CA).

Biotin hydrazide derivatization and NeutrAvidin affinity selection

To isolate the carbonylated proteins from control and ethanol-fed samples, 300 μg of whole liver extracts was treated with 0.5 mM EZ-link biotin hydrazide (Pierce) for 2 h at room temperature. The samples were then desalted three separate times using protein desalting columns from Pierce to remove any excess, unreacted biotin hydrazide. Fifty microliters of 50% NeutrAvidin agarose resin slurry (Pierce) was added to each sample and incubated for 1.5 h on a rotating shaker at room temperature. The beads were washed 3–5 times with PBS and then boiled for 5–10 min in 50 μL of SDS-PAGE loading buffer in order to remove proteins captured on the beads.

Western blotting

Whole liver extracts, subcellular fractions, or NeutrAvidin captured proteins were separated on a polyacrylamide gel via standard SDS-PAGE procedures, and then transferred to Hybond-P transfer membrane (GE Health Sciences, Buckinghamshire, UK). The membrane was then blocked in a solution of 5% nonfat milk in TBST. The membrane was probed with primary antibodies against CYP2E1 (Calbiochem, San Diego, CA), PRX6 (Abcam, Cambridge, MA), phosphorylated ERK1/2, phosphorylated MEK1/2, ERK1/2, and MEK1/2 (Cell Signaling, Danvers, MA). A horseradish peroxidase-conjugated secondary antibody was then applied. The membrane was developed using ECL-Plus reagent from GE Health Sciences (Buckinghamshire, UK) and the chemiluminescence was visualized using a STORM 860 scanner from Molecular Dynamics (Sunnyvale, CA). The membrane was then stripped and reprobed with a primary antibody against β-actin (Sigma, St. Louis, MO) or cytochrome p450 reductase (CPR) (Abcam, Cambridge MA) to ensure equal protein loading.

Histology and immunohistochemistry

After the livers were excised from the study animals a small portion of the tissue was placed into a vial containing 10% neutral buffered formalin. The tissue samples were then processed, paraffin-imbedded, and mounted on slides by Colorado Histoprep (Ft. Collins, CO). One set of slides was stained with hematoxylin and eosin for histological examination of pathology and the remainder of the slides were deparaffinized and processed for immunohistochemical analysis. The sections were stained with primary antibodies against CYP2E1 (Calbiochem), PRX6 (Abcam), and 4HNE-KLH and 4ONE-KLH (Synpep, Dublin, CA) and were developed using Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA) and counterstained with hematoxylin.

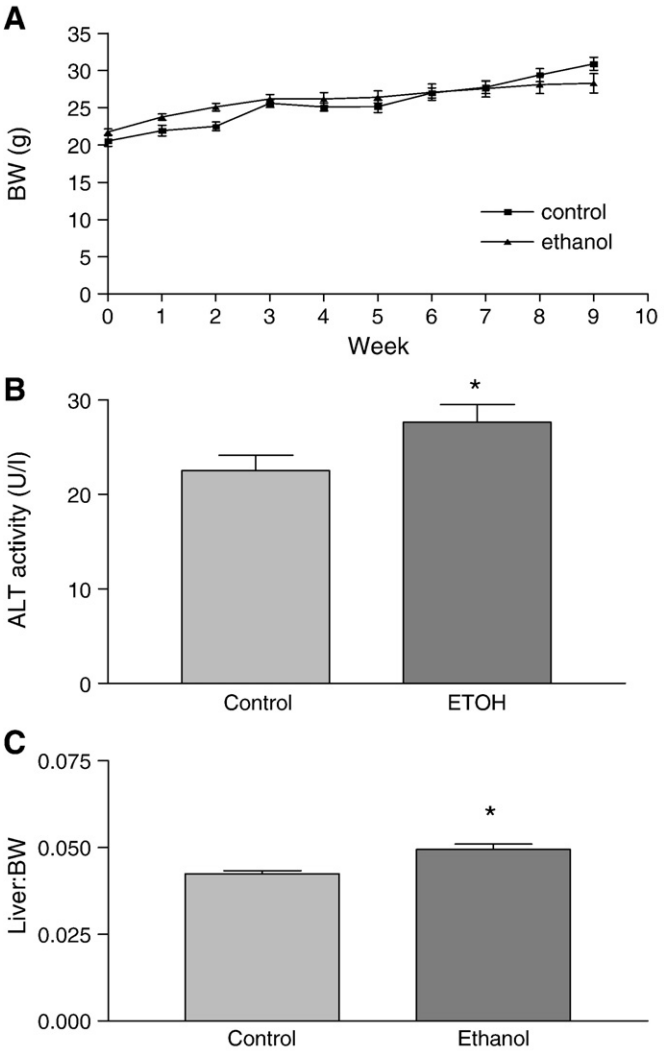

Fig. 1. Chronic ethanol administration results in liver injury: (A) Bodyweights, (B) plasma ALT activity, (C) liver to bodyweight ratios, $n=8$, ±SEM (*$P<0.05$).