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Original Contribution

Effects of *N*-acetylcysteine on ethanol-induced hepatotoxicity in rats fed via total enteral nutrition

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Abstract

The effects of the dietary antioxidant *N*-acetylcysteine (NAC) on alcoholic liver damage were examined in a total enteral nutrition (TEN) model of ethanol toxicity in which liver pathology occurs in the absence of endotoxemia. Ethanol treatment resulted in steatosis, inflammatory infiltrates, occasional foci of necrosis, and elevated ALT in the absence of increased expression of the endotoxin receptor CD14, a marker of Kupffer cell activation by LPS. In addition, ethanol treatment induced CYP2E1 and increased TNFα and TGFβ mRNA expression accompanied by suppressed hepatic IL-4 mRNA expression. Ethanol treatment also resulted in the hepatic accumulation of malondialdehyde (MDA) and hydroxynonenal (HNE) protein adducts, decreased antioxidant capacity, and increased antibody titers toward serum hydroxyethyl radical (HER), MDA, and HNE adducts. NAC treatment increased cytosolic antioxidant capacity, abolished ethanol-induced lipid peroxidation, and inhibited the formation of antibodies toward HNE and HER adducts without interfering with CYP2E1 induction. NAC also decreased ethanol-induced ALT release and inflammation and prevented significant loss of hepatic GSH content. However, the improvement in necrosis score and reduction of TNFα mRNA elevation did not reach statistical significance. Although a direct correlation was observed among hepatic MDA and HNE adduct content and TNFα mRNA expression, inflammation, and necrosis scores, no correlation was observed between oxidative stress markers or TNFα and steatosis score. These data suggest that ethanol-induced oxidative stress can contribute to inflammation and liver injury even in the absence of Kupffer cell activation by endotoxemia.

Keywords: *N*-Acetylcysteine; Adducts; Cytokines; Alcohol; Hepatotoxicity; Lipid peroxidation

Introduction

In the recent years a growing body of experimental evidence indicates that inflammatory reactions and oxidative stress play a major role in alcohol hepatotoxicity [1–4]. Nonetheless the relative contributions of these factors in the processes leading to alcohol-induced liver damage (ALD) remain in dispute. One reason is that the various rodent models of ALD differ significantly with regard to animal

Abbreviations: ALD, alcohol-induced liver damage; TEN, total enteral nutrition; PUFA, polyunsaturated dietary fatty acids; NAC, *N*-acetylcysteine; UEC, urine ethanol concentrations; BEC, blood ethanol concentration; ORAC, oxygen radical absorbance capacity; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; FL, fluorescein; MDA, malondialdehyde; HNE, 4-hydroxynonenal; IL, interleukin; TNF, tumor necrosis factor; TGF, tumor growth factor; HER, hydroxyethyl; SOD, superoxide dismutase; NFκB, nuclear factor κB.

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age, nutritional status, growth rate, and the development of endotoxemia preceding pathological alterations [1,4–7]. Increased lipid peroxidation, impaired antioxidant status, the appearance of free radical adducts derived from fatty acid breakdown and CYP2E1-dependent ethanol metabolism to the 1-hydroxyethyl radical have all been shown to correlate with the development of pathology [3,8–10]. Antioxidant treatment in vitro has been demonstrated to protect hepatocytes overexpressing CYP2E1 from the synergistic toxicity of polyunsaturated fats such as arachidonic acid and iron [11]. Similarly, in vivo, feeding with the glutathione precursor L-oxothioazolidine-4-carboxylic acid and with dietary antioxidant extracts from cocoa and green tea, as well as gene therapy resulting in hepatic overexpression of either Cu/Zn or Mn superoxide dismutase has been shown to be protective against ALD [4,12–16]. It is noteworthy that these later observations were performed in rat intragastric ethanol-infusion models such as that originally developed by Tsukamoto et al. [5] where pathology is accompanied by endotoxemia and Kupffer cell activation as measured by increased production of oxidants via NADPH oxidase and increased expression of the endotoxin receptor CD14 [4,12–18]. We have developed a total enteral nutrition (TEN) model in which ethanol is also infused intragastrically, but in which ethanol substitutes for carbohydrate as part of an isocaloric diet. In this model, development of ALD (steatohepatitis accompanied by focal necrosis) above and beyond simple steatosis is dependent upon low dietary carbohydrate content ($\leq$5% total calories) and high polyunsaturated dietary fatty acids (PUFA) (high PUFA/carbohydrate ratio) [19,20]. Moreover, in this later model, ALD develops without significant elevations in endotoxin [21,22]. Since Kupffer cell activation by endotoxins might represent a major source of free radical species, in the current study, we have used the dietary antioxidant *N*-acetylcysteine (NAC) to determine whether oxidative stress might also be responsible for ethanol toxicity in the absence of the proinflammatory stimulation by endotoxins. NAC is a dietary antioxidant and glutathione precursor widely utilized in the treatment of acute oxidative liver injury by acetaminophen overdose [23,24] and which has been suggested to be a possible dietary therapy for ALD clinically [25].

Materials and methods

Animals and experimental design

All the animal studies described below were approved by the Institutional Animal Care and Use Committee at the University of Arkansas for Medical Sciences. All animals received humane care according to the criteria outlined in the *Guide for the Care and Use of Laboratory Animals* at an AAALAC approved animal facility at ACHRI. Groups of $N = 6–13$ male Sprague-Dawley rats, 300 g, were fitted with an intragastric cannula and infused with liquid diets using TEN as described previously [21,22]. Controls were infused at the NRC recommended caloric intake of 187 kcal/kg$^{.75}$/day allowing growth at comparable rates to ad libitum chow-fed animals [21,22,26]. Control diets contained 16% protein (whey peptides), 41% carbohydrate (dextrose and maltodextrin), and 43% fat (corn oil) together with NRC recommended levels of vitamins and minerals [26,27]. In the ethanol-treated (EtOH) groups, ethanol was used to substitute isocalorically for carbohydrate calories at a dose of 10 g/kg/day. Thereafter, fat calories were reduced as the ethanol infusion was increased to a final level of 13 g/kg/day to produce a final diet composition of 16% protein, 5% carbohydrate, 43% fat, and 36% ethanol calories and to maintain a constant level of 5% carbohydrates throughout the study as described previously [21,22]. In addition, control and ethanol groups were treated with NAC at 1.2 g/kg/day added to the diets. Urine ethanol concentrations (UEC) were measured daily using an Analox Instuments GL5 analyzer. All rats were sacrificed after 45 days of infusion.

Biochemical analysis

Blood ethanol concentration (BEC) at sacrifice was measured by Analox and serum ALT levels were assessed as a measure of liver damage using the Infinity ALT liquid stable reagent (Thermo Electron Corp., Waltham, MA) according to manufacturer's protocols. Liver microsomes were prepared by differential ultracentrifugation and *p*-nitrophenol hydroxylation was measured as described previously [19]. Carbon tetrachloride-dependent lipid peroxidation was measured as described by Johansson and Ingelman-Sundberg [28]. CYP2E1 apoprotein expression was measured by Western blot as described previously [19] using a rabbit polyclonal antibody raised against purified rat CYP2E1. Intracellular soluble antioxidant capacity was measured in protein-precipitated liver cytosol using the oxygen radical absorbance capacity (ORAC) assay [29,30]. Briefly, the assays were carried out on a FLUOstar Galaxy plate reader, which was equipped with an incubator and two injection pumps. The temperature of the incubator was set to 37°C. The procedures were based on the modified $ORAC_{FL}$ method [30]. 2,2′-Azobis(2-amidinopropane) dihydrochloride (AAPH) was used as a peroxyl radical generator and Trolox as a standard. Forty microliters of sample or blank (phosphate buffer), and Trolox calibration solutions (in phosphate buffer), was transferred to 48-well microplates in duplicate based upon a set layout. The plate reader was programmed to record the fluorescence of fluorescein (FL) every cycle. In cycle 1, the instrument reads the fluorescence in each of the wells. In cycle 2, the computer was programmed to pipette 400 μL FL from pump 1 into the respective wells to give a final FL concentration of 14 μM followed by reading the fluorescence. During cycle 3, the instrument read the fluorescence of all wells in the plate.