This student paper was written as an assignment in the graduate course

Free Radicals in Biology and Medicine

(77:222, Spring 2001)

offered by the

Free Radical and Radiation Biology Program
B-180 Med Labs
The University of Iowa
Iowa City, IA 52242-1181
Spring 2001 Term

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Alcohol and Liver Diseases

By

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For 77:222, Spring 2001
May 03, 2001

Abbreviations

ADH, Alcohol dehydrogenase
ALD, Alcoholic liver disease
CYP2E1, Cytochrome P450 2E1
ESR, Electron spin resonance
MDA, Malondialdehyde
MEOS, Microsomal ethanol oxidizing system
NOS, Nitric oxide synthases
POBN, (4-pyridyl-1-oxide)-N-t- butylnitrone

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Abstract

Excessive ethanol consumption activates a number of systems that generate oxygen free radicals and reactive aldehydic species. It was demonstrated that hydroxyethyl radicals are generated during ethanol metabolism by the microsomal monoxygenase system, involving the alcohol-inducible cytochrome P450 2E1 (CYP2E1). The formation of hydroxyethyl radicals is associated with stimulation of lipid peroxidation and development of liver damage. The presence of protein adducts in the liver of alcohol abusers with an early phase of histological liver damage indicates that adduct formation is one of the key events in the pathogenesis of alcoholic liver disease. Moreover, hydroxyethyl free radicals are also capable of inducing the production of specific antibodies, fibrosis, and interfere with signal transduction, which can be observed in ethanol-fed animals as well as in patients abusing alcohol. This report explores the possible role of free radicals produced by excessive ethanol consumption in the pathogenesis of liver injury, and proposes a future study to explore other mechanisms.

Introduction

Alcoholic liver disease (ALD) is a major health problem worldwide. It is the ninth leading cause of death in the United States and accounts for nearly half of all cirrhosis-related deaths [1]. One aspect of alcohol toxicity that has received increasing attention in recent years concerns the contribution of free radical intermediates in the pathogenesis of liver injury [2]. Although hepatic toxicity of ethanol is likely multifactorial, direct and indirect evidence for the role of ROS in the development of chronic liver disease and hepatic fibrosis has repeatedly been obtained in patients with alcoholic liver disease and in animal models of acute and chronic ethanol injury [2]. A number of experimental studies have demonstrated that either acute or chronic alcohol administration to experimental animals increases the formation of lipid

peroxidation products, such as lipoperoxides, conjugated dienes and malondialdehyde (MDA) and decreases tissue levels of antioxidants [2]. The impairment of cellular antioxidant defenses along with the formation of oxygen-derived radicals has been proposed to play a role in causing oxidative damage associated with alcoholic liver disease. However, other free radical intermediates might also contribute to trigger alcohol-dependent oxidative injury [3].

Hepatocyte ethanol metabolism and ROS production

The hepatotoxic effects of ethanol and ROS production are closely tied to ethanol metabolism. There are three well-recognized pathways for hepatic ethanol metabolism: 1) cytosolic alcohol dehydrogenase (ADH), 2) hepatic catalase, located in the peroxisomes and 3) the microsomal ethanol oxidizing system (MEOS) located in the endoplasmic reticulum [4].

ADH converts ethanol to acetylaldehyde and the oxidation is tightly coupled to reduction of NAD^+:

$$CH_3\text{-}CH_2\text{-}OH + NAD^+ \xrightarrow[ADH]{} CH_3\text{-}CH{=}O + NADH$$

Although various extrahepatic isozymes of ADH exist, cytosolic hepatic ADH accounts for the majority of ethanol conversion to acetaldehyde. In the oxidation of ethanol to acetaldehyde and again in the conversion of acetaldehyde to acetate, NAD^+ is reduced to NADH. When large amounts of ethanol are consumed, an excess of reduced NADH is generated. This can cause a variety of metabolic disorders of hepatic protein, glucose, and lipid metabolism. The latter of these results in early central perivenular steatosis, the hallmark of ALD [5].

Catalase can metabolize ethanol in vitro in the presence of H_2O_2. However, this is probably not important *in vivo* since H_2O_2 concentrations do not normally achieve high enough levels to allow this reaction to proceed [6].

Finally, and probably most important in chronic ALD, ethanol can be oxidized to acetaldehyde in the cellular microsomes or microsomal enzyme oxidizing system (MEOS) by a cytochrome p450-dependent enzyme known as CYP2E1. CYP2E1, which is inducible by ethanol and other alcohols, can oxidize ethanol and a variety of other organic compounds. Because CYP2E1 has a lower Km than cytosolic ADH for ethanol (8 to 10 mM compared to 0.2 to 2 mM, respectively), hepatic ADH is probably responsible for most ethanol metabolism under normal conditions. However, with chronic ethanol consumption, CYP2E1 levels increase and ethanol is metabolized to acetyladehyde preferentially by CYP2E1 [7].

$$CH_3\text{-}CH_2\text{-}OH + NADP+ \xrightarrow[CYP2E1]{} CH_3\text{-}CH{=}O + NADPH$$

CYP2E1 activity is loosely coupled to NADPH oxidase, which results in the generation of ROS and other species such as α-hydroxyethyl radical. Ethanol-mediated induction of CYP2E1 has been linked to oxidative stress, lipid peroxidation, and the fibrosis of chronic ALD [8].

Generation of ethanol-derived free radicals

Different studies using Electron Spin Resonance (ESR) spectroscopy in combination with spin trapping technique have reported that a carbon-centered free radical intermediate, identified as 1-hydroxyethyl radical, is produced by rat liver microsomes incubated in the presence of ethanol and NADPH [9]. Several other reports also have shown that hydroxyethyl radical generation occurs during ethanol oxidation by microsomes from alcohol dehydrogenase (ADH)-deficient (ADH^-) deer-mice or human livers [10]. It is important to mention here that free

radical intermediates are similarly produced during microsomal oxidation of various aliphatic alcohols including propanol, butanol and pentanol, indicating a common metabolic pathway for radical production from alcohols [11]. The possible role of ethanol-derived radicals in relation to alcohol toxicity has been shown by the observation that hydroxyethyl radicals are also produced during ethanol metabolism *in vivo*. By applying the EPR spectroscopy technique of spin trapping to the study of ethanol- treated alcohol dehydrogenase-deficient deer-mice (*Peromyscus maniculatus*), Knecht *et al.* (1990) have detected the alpha- (4-pyridyl-1-oxide)-N-t- butylnitrone (POBN)/alpha-hydroxyethyl radical adduct in bile from animals administered [1-^{13}C] ethanol and the spin trap POBN (figure1) [27].

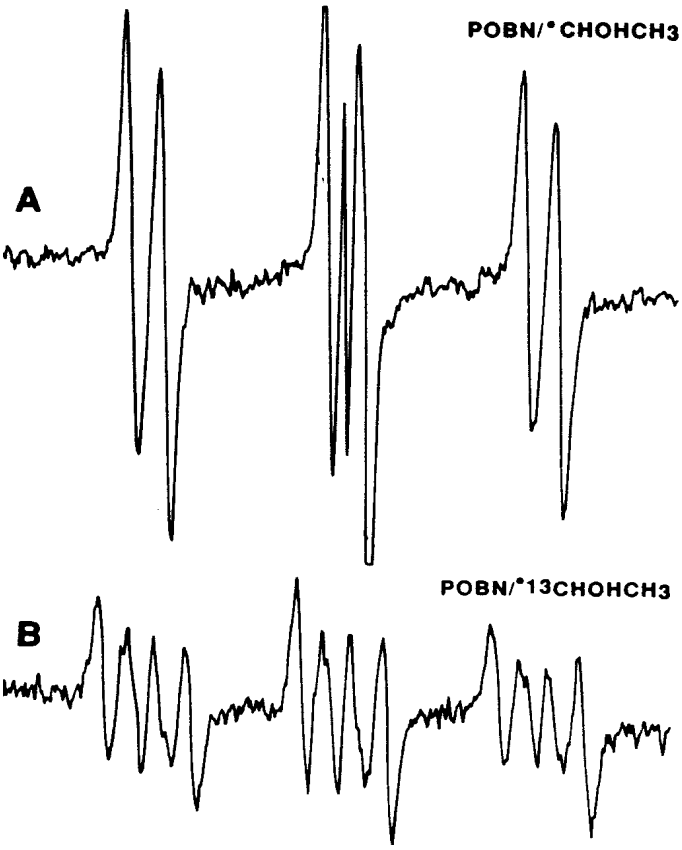

Figure 1 EPR spectra of radical adduct signal of bile from ADH$^-$ deer-mice that were chronically treated with ethanol-containing high fat diet and given an acute dose of ethanol (3.7 g/kg) and POBN (100mg/kg) , A. B, as in A, except acute [1-^{13}C] ethanol [27].

Free radicals and oxidative injury associated with alcohol abuse

a) Formation of protein –aldehyde adducts

Adducts of proteins with acetaldehyde, the first metabolite of ethanol, have been described in a number of studies. Acetaldehyde forms adducts primarily via binding to reactive lysine residues of preferred target proteins [12]. There seems to be several preferred target proteins for aldehyde attack *in vivo.* The continuously growing list of the primary targets includes erythrocyte membrane proteins, hemoglobin, albumin, tubulin, lipoproteins, and collagens. Not surprisingly, adduct formation with acetaldehyde, hydroxyethyl radicals and ethanol-metabolizing cytochrome P4502EI enzyme, seem to occur *in vivo* [13].

Formation of protein adducts with reactive aldehydic products has provided a basis for new hypotheses to explain the pathogenesis of ALD (figure 2). Covalent binding to proteins is known to interfere with protein function particularly when there is a lysine residue in a functionally critical location, such as in tubulin and in lysine-dependent enzymes [14]. Altered microtubule function may subsequently lead to an impairment in protein secretion and plasma membrane.

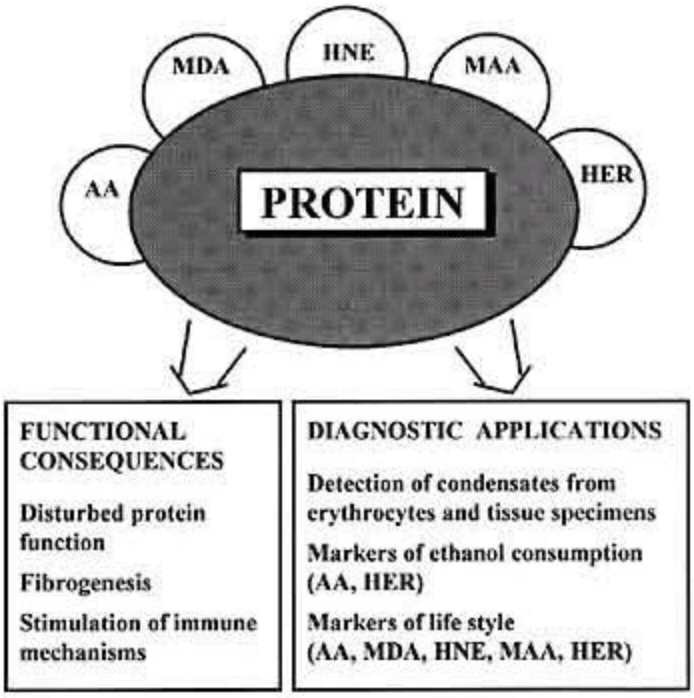

Figure 2 Schematic representation of various reactive components capable of attacking proteins to form adducts during ethanol metabolism. Protein binding with such compounds provides a basis to explain the pathogenesis of alcoholic liver disease and to diagnostic applications. Abbreviations, AA, acetaldehyde, MDA, malondialdehyde, HNE, 4-hydroxynonenal, MAA, malondialdehyde-acetaldehyde hybrid adduct, HER, hydroxyethyl radical assembly. Generation of reactive aldehydes may also contribute to ethanol-induced impairment in receptor-mediated endocytosis [14].

b) Stimulation of fibrogenesis

Hepatic fibrosis is a common, if not a universal, response to chronic liver injury including that resulting from ethanol. Fibrosis is the result of excessive deposition of extracellular matrix proteins by hepatic stellate cells acting in response to factors produced by hepatocytes, Kupffer cells, and infiltrating inflammatory blood cells [15]. These factors include oxidants and their reaction products, cytokines, and other paracrine factors [15]. While the molecular events responsible for increased fibrogenesis and development of cirrhosis in patients with ALD remain unknown, there is increasing evidence that in both processes the production of reactive oxygen species (ROS) and perhaps reactive nitrogen species (RNS) is involved [16]. Studies have further demonstrated that hepatic stellate cells, which are the primary source of extracellular matrix, become readily activated under conditions involving enhanced oxidative stress and lipid peroxidation [16].

c) Stimulation of immune reactions

Alcoholic liver disease is associated with an antigen-driven immune response that targets liver cells [17]. Such immune reactions involve lymphocyte-mediated response to liver autologous human hepatocytes as well as the development of circulating antibodies against epitopes present on the surface of hepatocytes that trigger antibody-dependent immunotoxicity [18].

Moncada *et al*. [19] have shown that immunization of rabbits with rat liver microsomes incubated in vitro with ethanol or with hydroxyethyl radical-albumin adducts leads to the formation of antibodies that specifically recognize the hydroxyethyl epitopes independently from the carrier proteins. Anti-hydroxyethyl radical antibodies have also been observed in the serum of rats chronically fed with alcohol, suggesting their possible use as markers for the formation of ethanol-derived radicals in subjects abusing of alcohol [19]. They have observed that sera of patients with alcoholic liver disease (ALD) contain both IgG and IgA which recognize proteins modified by hydroxyethyl radicals, while practically no reaction is present with the sera of healthy controls or patients with non-alcoholic liver diseases (figure 3) [20]. The antibodies detected in the sera of patients with ALD are specific for hydroxyethyl radical-derived epitopes and do not cross react with acetaldehyde-modified albumin [20].

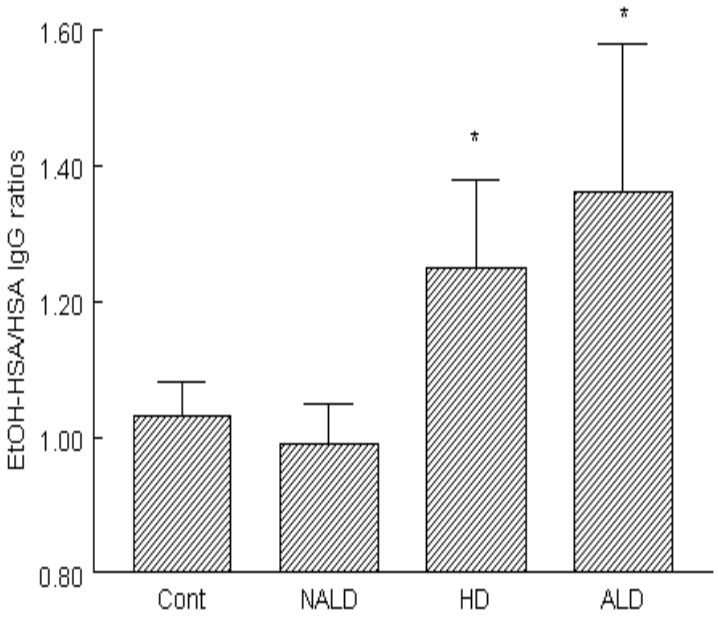

Figure 3 Presence of circulating antibodies reacting with hydroxyethyl radical-modified human serum albumin (EtOH-HSA) in the sera of patients with alcoholic (ALD) or non-alcoholic (NALD) liver diseases, in heavy drinkers (HD) without liver damage and healthy controls. IgG levels were measured by reacting polystyrene ELISA microwell plates covered with 0.05 mg/ml of either EtOH-HSA or unmodified HSA with the different sera (1:20 dilution). * $p<0.05$ [20].

The role played by CYP2E1 in the generation of hydroxyethyl radicals in humans has been recently investigated in a group of alcoholic patients in which CYP2E1 activity has also been estimated by assessing chlorzoxazone oxidation [21]. Dupont *et al*. (1998), observed an increased chlorzoxazone oxidation in 40 out of 51 (78%) alcoholics, while in the remaining 22% of the patients, chlorzoxazone oxidation was within the control range in spite of a similar alcohol intake, indicating a lack of CYP2E1 inducibility (Figure 4). IgG reacting with hydroxyethyl free radical-protein adducts are absent in subjects without CYP2E1 induction, while they are significantly increased in alcoholics with induced CYP2E1 activity. These observations indicate that CYP2E1 activity greatly influences the human formation of hydroxyethyl radicals stimulating specific immune reactions against radical species derived from ethanol [21].

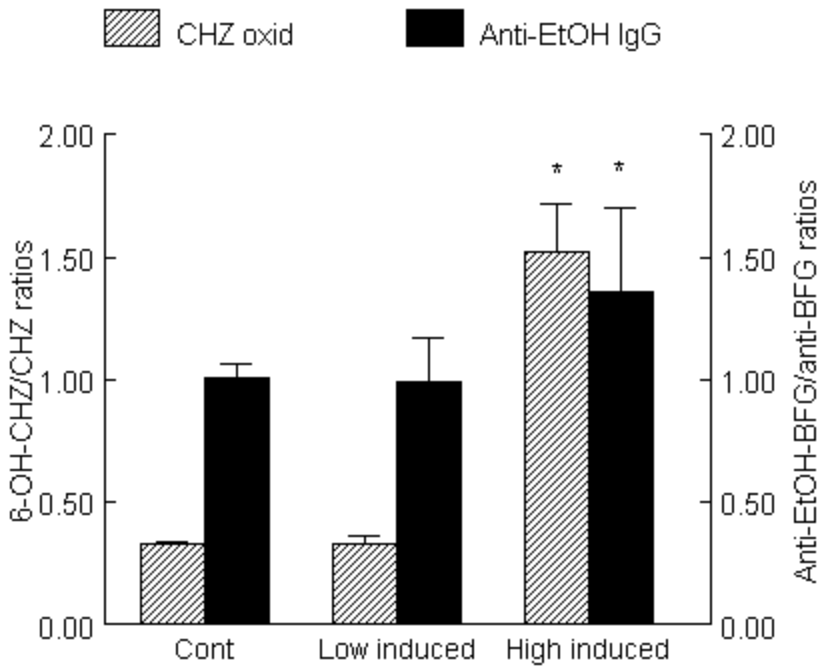

Figure 4 Chlorzoxazone oxidation and IgG reacting with bovine fibrinogen-adducts of hydroxyethyl free radicals (EtOH-BFG) in the sera of healthy controls (Cont) or alcoholics with low-induced or high-induced CYP2E1 activity. The ratios between plasma 6-hydroxychlorzoxazone and chlorzoxazone levels (6-hydroxy-CHZ /CHZ) were used as index of CYP2E1 activity [21].

d) Ethanol effects on hepatocyte apoptosis

Ethanol toxicity is known to promote apoptosis in hepatocytes and the process is likely connected with the formation of ROS, lipid peroxidation, and depletion of glutathione [22]. It is unclear whether the depletion of cytosolic and mitochondrial glutathione is a cause or an affect of the oxidative stress. However, glutathione depletion can trigger apoptosis in some cells [23]. In animal models and in humans with ALD, the sites of maximal hepatocyte apoptosis are around the central veins. This corresponds to areas of maximal ethanol injury, CYP2E1 expression, and ROS generation [24]. Apoptosis can also be triggered by specific cell surface receptor/ligand interactions. In ALD, the best studied are the "death domain" receptors and their ligands which include TNF, Fas ligand (CD 95) and TGF-β1. Patients with alcoholic hepatitis exhibit increased TNF activity, primarily from mononuclear cells. mRNA for TNF-α is increased in the livers of rats fed ethanol intragastrically [25]. Hepatocyte surface expression of

TNF-α receptors is up-regulated during chronic ethanol administration, suggesting a possible sensitizing role for ethanol in the TNF induced apoptosis of ALD [25].

e) Ethanol effects on signal transduction

Recent data suggests an additional link between ethanol and cellular signaling events. Acetylaldehyde, the product of ethanol oxidation by ADH and CYP2E1, has recently been shown to activate both the NF-κB and AP-1 transcription factors in HepG2 cells [26]. Both of these factors play important roles in the regulation of apoptosis and the production of a variety of inflammatory mediators.

Future Study

Many studies have shown that cellular production of nitric oxide ($^{\bullet}NO$) is involved in many physiologic and pathologic processes. $^{\bullet}NO$ is produced by one of several enzymes termed nitric oxide synthases (NOS). Each of these enzymes oxidizes L-arginine to citrulline and $^{\bullet}NO$ (28). There are three major NOS isoforms. Two (NOS-1 and NOS-3) are expressed constituitively (cNOS) and NOS-2 is inducible (iNOS). In contrast to cNOS activity, which is regulated by intracellular [Ca^{2+}], iNOS activity is regulated at the transcriptional level (28). A variety of cytokines, oxidant species, and other exogenous stimuli increase expression of iNOS. Among the transcription factors known to modulate iNOS expression in hepatocytes is NF-κB (29). Some of the biological effects of $^{\bullet}NO$ are mediated through another oxidant product, peroxynitrite ($ONOO^-$) which results from the rapid reaction ($k = 6.7 \times 10^9\ M^{-1}s^{-1}$) between $^{\bullet}NO$ and $O_2^{-\bullet}$ (30). $^{\bullet}NO$ has been reported to both enhance and inhibit lipid peroxidation.

Increased production of $^{\bullet}NO$ by hepatocytes has also been observed as a consequence of chronic alcohol exposure (31). $^{\bullet}NO$ production may be enhanced by TNF and other cytokines.

Hypothesis

The presence of ethanol will enhance the magnitude of oxidant stress in the hepatocyte through the increased formation of $^{\bullet}NO$ and/or $OONO^-$. Consequently, the ethanol exposure will lead to greater degree of RNS production, which might be involved in the pathogenesis of liver disease induced by ethanol. To test this hypothesis various aspects of $^{\bullet}NO$ production and their consequences in liver cells in the presence or absence of ethanol should be measured.

Proposed experimental procedures

Cell lines as an *in vitro* model of liver cells

- HepG2 cells: Human hepatocellular carcinoma cell line.
- E47 cells: HepG2 cell line overexpressing the ethanol-inducible cytochrome P450 2E1 (CYP2E1).
- C34 cells: HepG2 cell line expressing the empty vector as a control cells.

Different cell lines will be cultured with and without different concentrations of ethanol and for different periods of time and the following tests can be done to achieve the following goals.

Goal 1: Detection of increased nitric oxide production

To find out the effect of ethanol on the level on $^{\bullet}NO$ production, $^{\bullet}NO$ production will be quantified by transferring the cell supernatant to a chemical $^{\bullet}NO$ analyzer, which relies on the fact that $^{\bullet}NO$ decomposes to nitrate and nitrite.

INOS protein level

In addition cell pellets will be harvested and subjected to immunoblot analysis for iNOS protein using commercially available antisera to iNOS. If differences in either iNOS expression or $^{\bullet}NO$ production are detected we can proceed with iNOS mRNA analysis by RT-PCR to confirm that this is occurring at the transcriptional level.

L-arginine analogue

In another approach we can employ the L-arginine analogue and inhibitor of NOS, L-NAME (N^{G}-nitro-L-arginine methyl ester), to assess the potential involvement of $^{\bullet}NO$ (32).

Spin Trapping

Reszka et *al*. (1994) have shown that in the presence of strong alkali (0.5 M NaOH) $^{\bullet}NO_2$ reacts with the *aci*-form of nitromethane (NM), $CH_2{=}NO_2^-$, to generate a characteristic, relatively stable spin adduct, $^-O_2N{-}^{\bullet}CH{-}NO_2^-$. Moreover, they have demonstrated that *aci*-NM can be useful analytical probe for the EPR detection of nitric oxide radicals in aqueous solutions (33).

Goal 2: Detection of Peroxynitrite formation

A characteristic reaction of $ONOO^-$ with proteins is nitration of tyrosine residues leading to the formation of 3-nitrotyrosine (34). We can initially examine for evidence of $ONOO^-$ production by immunohistochemistry and immunoblot approaches using commercially available (Cayman Chemical) monoclonal and polyclonal antibody to 3-nitrotyrosine. Quantification of 3-nitrotyrosine using HPLC methods can also be used.

Goal 3: Exploring the role of NADPH and NADH produced by ALH and CYP2E1

As shown above, the oxidation of ethanol to acetaldehyde by ADH and again in the conversion of acetaldehyde to acetate, NAD^+ is reduced to NADH. Moreover, with chronic

ethanol consumption, CYP2E1 levels are increased and ethanol is metabolized to acetaldehyde preferentially by CYP2E1 and more NADPH is produced [7].

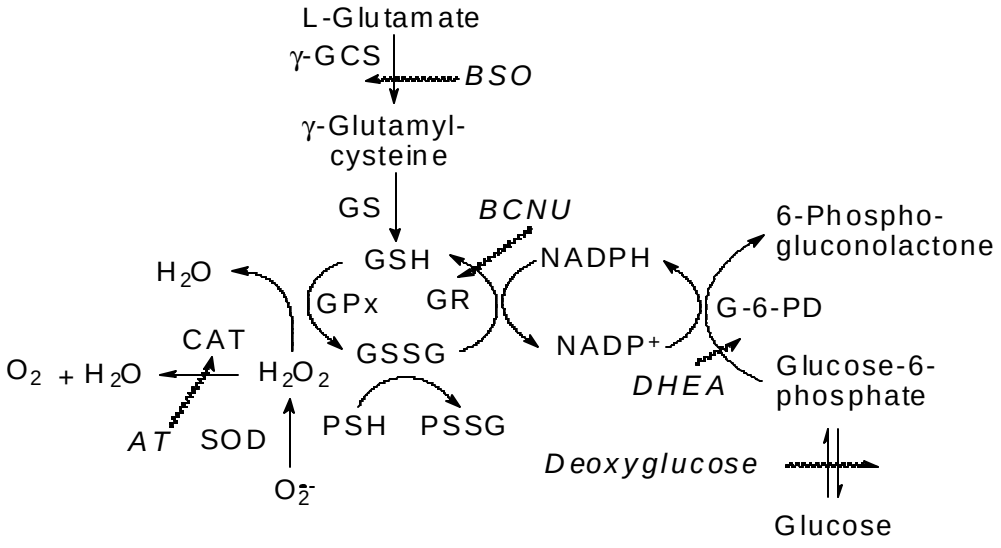

Figure 5 This schematic shows the relationship between different components involved in GSH synthesis and reactions. Note the central role of NADPH in the cycle [Adapted from 35]

As shown in figure 5, NADPH is an essential cofactor for proper GSH function, so it is important to find out the possible impact of the excess NADPH on the GSH level in both cells that produce NADPH (E47) and cells that produce NADH (C34) as control. The following experiments can be performed:

Measurement of NADPH level.

The level of NADPH can be measured by following the absorption at 340 nm. Although both NADH and NADPH are measured at 340 nm, we still can predict the increase in NADPH using the proper control cells.

Measurement of GSH/GSSG level in the test cells.

An increased levels of GSH in the CYP2E1 transfected and alcohol treated cells indicate increased levels of NADPH production as can be seen in figure 5. Untransfected and alcohol treated control cells must be used to role out NADH.

Inhibition of in vivo production of NADPH.

By inhibiting glucose-6-phosphate dehydrogenase enzyme (G6PD) using dehydroepiandrosterone (DHEA), we can block the *in vivo* production of NADPH (figure 5). Upon treatment with ethanol, any increased levels of NADPH or GSH could be attributed to the NADPH produced by CYP2E1 transfected cells.

Expected results and possible problems

An increased levels of $^{\bullet}NO$ and $ONOO^-$ production is expected. The inhibition of $^{\bullet}NO$ production using the analogues is expected to decrease the degree of pathology induced by ethanol treatment. If the inhibition of $^{\bullet}NO$ productions was not critical in reducing the pathology, another possibility could be in the involvement of increased NADH production in the pathology associated with metabolic, protein and lipid synthesis and metabolism.

Summary

In summary, it was demonstrated that alcoholic patients and experimental animals exposed to ethanol display biochemical signs of oxidative damage, suggesting a possible role of free radicals in causing some of the toxic effects of alcohol. The formation of these free radical metabolites might represent a novel mechanism in alcoholic liver injury since hydroxyethyl radicals can promote oxidative damage as well as trigger immunoallergic reactions targeting hepatocytes.

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