

HYDROXYETHYL RADICALS IN ETHANOL HEPATOTOXICITY

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1. ABSTRACT

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 use of electron spin resonance (ESR) spectroscopy associated with spin trapping technique has demonstrated that hydroxyethyl radicals are generated during ethanol metabolism by the microsomal monooxygenase system, involving the alcohol-inducible cytochrome P450 2E1 (CYP2E1). Recent observations in rats fed intragastrically with a high fat diet containing ethanol indicate that the formation of hydroxyethyl radicals is associated with stimulation of lipid peroxidation and development of liver damage. Moreover, by alkylating liver proteins, and particularly CYP2E1, hydroxyethyl free radicals are also capable of inducing the production of specific antibodies which can be observed in ethanol-fed animals as well as in patients abusing alcohol. Recent studies have demonstrated that hydroxyethyl radical-derived antigens are exposed on the plasma membranes of hepatocytes exposed to ethanol where are able to target antibody-dependent cell-mediated immunotoxic reactions towards liver cells. Thus, beside to contribute to alcohol-mediated oxidative damage, hydroxyethyl free radicals can contribute by immunological mechanisms to cause hepatocellular lesions associated with alcohol abuse.

2. INTRODUCTION

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 (1). 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 (1).

Clinical studies support these observations showing that markers of oxidative stress, including protein carbonyls, breath exhalation of pentane and serum titres of antibodies reacting with MDA-protein adducts are higher in patients with alcoholic liver disease (1-5), while the levels of antioxidants such as glutathione and vitamin E are appreciably reduced (1,2,4-6). 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. GENERATION OF ETHANOL-DERIVED FREE RADICALS

In 1987 two independent 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 (7,8). These observations have been confirmed by several other reports (9,10) also showing that hydroxyethyl radical generation occurs during ethanol oxidation by microsomes from alcohol dehydrogenase (ADH)-deficient deer-mice (11) or human livers (12). It is noteworthy 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 (7,13,14). The interest for a possible role of ethanol-derived radicals in relation to alcohol toxicity has been strengthened by the observation that hydroxyethyl radicals are also produced during ethanol metabolism *in vivo* and are detectable in the bile of ADH-deficient deer-mice (15) or of alcohol-fed rats (16-18) receiving an acute dose of ethanol along with the spin trapping agent 4-pyridyl-1-oxide-t-butyl nitron (4-POBN).

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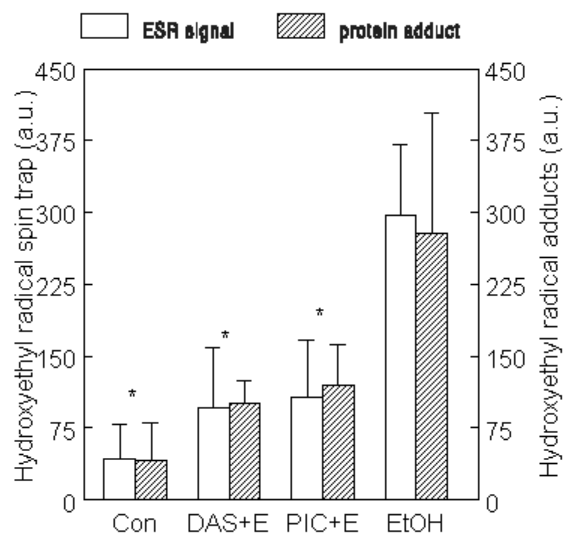


Figure 1. Formation of hydroxyethyl free radicals in intragastric ethanol fed rats and effect of interference with CYP2E1 induction by diallylsulfide (DAS) (200 mg/kg b.wt.day) or phenylethylisothiocyanate (PIC) (1 mmol/kg b.wt.day). Open bars represent the intensity of ESR signals due to the spin trapping of hydroxyethyl radicals produced in liver microsomes from intragastrically alcohol-fed rats incubated *in vitro* 30 min at 37° C with 50 mM ethanol and 25 mM 4-POBN. Stripped bars represent the detection by an ELISA assay of hydroxyethyl radicals-derived antigens in liver microsomes from the same rats using rabbit serum against hydroxyethyl radical-modified-KHL (anti-Et-KLH). The columns represent: ethanol-fed rats (EtOH); rats fed isocaloric diet containing dextrose (Con); rats fed ethanol diet plus DAS (DAS+E); rat fed ethanol diet plus PIC (PIC+E). The results are expressed as arbitrary units and are means of 4-6 different animals in each group. Statistically significant versus EtOH: * $p < 0.002$.

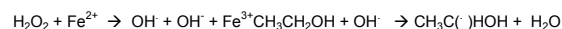
3.1. Role of CYP2E1 in the formation of hydroxyethyl free radicals

In liver microsomes the formation of hydroxyethyl radicals strictly requires the presence of NADPH and functionally active monooxygenase enzymes. The requirement for cytochrome P450 appears essential since hydroxyethyl radicals can be generated by reconstituted monooxygenase systems incubated in the presence of NADPH only if cytochrome P450 reductase and cytochrome P450 are both present (13). Interestingly, in human liver microsomes, also, NADH can support hydroxyethyl radicals production (12). The possible involvement of ethanol-inducible cytochrome P4502E1 isozyme (CYP2E1) in the generation of free radical metabolites from ethanol, as well as from other aliphatic alcohols, is suggested by the observation that liver microsomes prepared from ethanol fed or acetone-pretreated rats are 2-3 fold more active in forming radical intermediates from ethanol, 1-propanol and 1-butanol than microsomes from phenobarbital or methylcholanthrene pretreated animals (13). Consistently, antibodies against CYP2E1 or CYP2E1 inhibitors, such as p-nitrophenol and diethyldithiocarbamate, reduce by about 60% the spin trapping of hydroxyethyl free radicals (13,14). Furthermore, experiments performed in reconstituted

monooxygenase systems containing various purified cytochrome P450 isozymes and cytochrome P450 reductase have shown that CYP2E1 is twice more active than CYP2B1 in generating alcohol derived radicals (13). More recently, using rats receiving ethanol by intragastric feeding according to the Tsukamoto-French model (19) we have observed that hydroxyethyl radical formation, as measured *in vitro* by spin trapping technique or *in vivo* by immunological detection of hydroxyethyl radicals covalently bound to microsomal proteins, increases by about 7 fold following chronic alcohol administration (figure 1) (20). In these animals the treatment with diallylsulfide (DAS) or phenylethylisothiocyanate (PIC), two compounds that interfere with the induction of CYP2E1 by ethanol (21,22), greatly reduces the generation of hydroxyethyl radicals (figure 1). A linear correlation ($r = 0.732$; $p < 0.002$) can be observed between the individual content of CYP2E1 in liver microsomes of these animals and their capacity to form hydroxyethyl radicals (20). A similar correlation between CYP2E1 content and hydroxyethyl radical spin trapping is also appreciable in human microsomal preparations obtained from livers of kidney donors. It has been recently reported that destruction of Kupffer cells by treating *in vivo* intragastric ethanol-fed rats with gadolinium chloride strongly decreases the trapping of hydroxyethyl radicals (18). However, the actual role of Kupffer cells in hydroxyethyl radicals generation requires further investigations, since little is known on possible interference of gadolinium with hepatocyte functions.

3.2. Mechanisms responsible for hydroxyethyl free radicals production by CYP2E1

It is well known that hydroxyethyl free radicals can be produced non-enzymatically by the interaction of ethanol with hydroxyl radicals (OH) originating by iron-catalyzed degradation of hydrogen peroxide through, the so called, Fenton reaction (23,24).



Furthermore, recent studies have suggested that ethanol interaction with ferric iron complexed with phosphate can give rise to hydroxyethyl free radicals (25). Although in microsomal preparations the enzymatic activity of the complete cytochrome P450-dependent monooxygenase system is necessary for hydroxyethyl radical formation (13), several evidences indicate that the process might involve the interaction of iron and reactive oxygen species (O_2^- and H_2O_2). Indeed, addition of azide, to prevent H_2O_2 degradation by catalase, or microsome supplementation with μmolar concentrations of FeCl_3 both increase by several fold the trapping of hydroxyethyl radicals (9-12), whereas iron removal from the incubation buffer by treatment with Chelex-100 resin strongly affect the radical formation (9). The efficiency of iron in catalyzing this reaction appears largely influenced by the nature of the molecules to which it is complexed. For instance, EDTA is about twice more active than citrate, while ADP has little effect (9). In agreement with a role for reactive oxygen species, superoxide dismutase, catalase and hydroxy radicals scavengers benzoate and mannitol are effective in preventing hydroxyethyl radical generation by either rodent and human liver microsomes (9-12). So far, little is known

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about the chemical nature of the oxidizing species involved in ethanol oxidation, but hydroxyl radicals (OH)(26) and perferryl iron complexes (FeO_2^{2+})(11), the latter originating by the direct combination of superoxide anion with iron, has been proposed as possibly responsible for hydroxyethyl radical formation.

The involvement of reactive oxygen species in the production of hydroxyethyl radical is not in contrast with the role played by CYP2E1, since this isozyme has an especially high NADPH oxidase activity which leads to an extensive production of O_2^- and H_2O_2 (13,27,28) Microsomes obtained from rats chronically exposed to alcohol are more active than microsomes from untreated animals in producing O_2^- and H_2O_2 (24,29) and antibodies against CYP2E1 inhibit NADPH oxidase activity (30). Consistently, the formation of alcohol-derived radicals by microsomes from alcohol-fed rats or reconstituted monooxygenase systems containing CYP2E1 is significantly reduced by tryptamine, isoniazid and octylamine (13), compounds that interfere with the NADPH oxidase activity of CYP2E1(24). Thus, according to the current view the interaction of iron with O_2 and H_2O_2 released by CYP2E1 might give rise to oxidizing species able to form hydroxyethyl radical by abstracting one electron from the ethanol molecule.

However, this mechanism does not explain the following observations: i) free radical trapping during the metabolism of equimolar concentrations of ethanol, 1-propanol and 1-butanol increases with the length of the carbon chain (13) in agreement with the increase in the rates of CYP2E1-mediated oxidation of the different alcohols (31); ii) in the absence of iron, CYP2E1 exhibits a high specificity for ethanol oxidation, but not for the production of OH (30); iii) hydroxyethyl radicals can specifically alkylate CYP2E1 (32). Altogether, these observations suggest the possibility that at least part of the hydroxyethyl radicals produced by liver microsomes may originate by interaction of ethanol with of an oxidizing species bound to CYP2E1. We have observed that, favoring the transfer of a second electron to oxycytochrome P-450, either by adding cytochrome b_5 to reconstituted vesicles containing CYP2E1 or by incubating ethanol-induced microsomes in the presence of both NADPH and NADH, decreases the formation of alcohol radicals by about 50%. Thus, we postulate that hydroxyethyl radicals could also originate as a side products during CYP2E1-catalyzed oxidation of ethanol to acetaldehyde and that the ferric cytochrome P-450-oxygen complex ($\text{CYP2E1-Fe}^{3+}\text{O}_2$) might be responsible for one electron oxidation of ethanol according to the following equation:



Recent studies by Stoyanovsky and Cederbaum (33) using spin trapping coupled with HPLC- electrochemical detection analysis have shown that the rate of hydroxyethyl radical formation by pyrazole-treated rat liver microsomes is about 1-1.5 nmol/minute/mg of protein. This rate is 10 times lower as compared to the rate of acetaldehyde formation by two electron ethanol oxidation. Thus, one electron transfer producing hydroxyethyl free radicals

represents a minor pathway in the process of ethanol oxidation by microsomal enzymes.

4. HYDROXYETHYL FREE RADICALS AND OXIDATIVE INJURY ASSOCIATED TO ALCOHOL ABUSE

So far there is not direct evidence that hydroxyethyl radicals might contribute to the stimulation of lipid peroxidation by ethanol. Chemically generated hydroxyethyl free radicals readily react with glutathione, ascorbic acid and α -tocopherol (34), suggesting that ethanol-derived radicals might contribute to the lowering of liver antioxidants observed after chronic alcohol exposure (1). We have observed that in intragastric ethanol-fed rats the individual values of hydroxyethyl radicals covalently bound to microsomal proteins are positively correlated with liver MDA content ($r = 0.709$; $p < 0.005$) (20). Similarly, a positive correlation is evident in patients with alcoholic liver disease between the titres of IgG against hydroxyethyl radicals and those of antibodies against MDA-protein adducts ($r = 0.828$; $p < 0.0001$) (Rolla *et al.* unpublished results). It is, therefore, possible that hydroxyethyl radicals might contribute to cause alcohol-induced oxidative damage.

5. HYDROXYETHYL FREE RADICALS AND IMMUNE REACTIONS ASSOCIATED TO ALCOHOLIC LIVER DISEASE

Alcoholic liver disease is associated with an antigen-driven immune response that targets liver cells (35-37). Such immune reactions involve lymphocyte-mediated response to alcoholic hyalin (38) or liver autologous human hepatocytes (39) as well as the development of circulating antibodies against epitopes present on the surface of hepatocytes from ethanol-fed rabbits (40-41), that trigger antibody-dependent immunotoxicity (42,43). These immunological reactions have been ascribed to the binding of acetaldehyde to proteins (44,45), since, either experimental animals exposed to alcohol (46) or alcoholic patients have high titres of immunoglobulins which react with acetaldehyde-protein adducts (47-51). Although the immunization with acetaldehyde-modified hemoglobin of ethanol-fed guinea pigs has been reported to reproduce experimentally some features of human alcoholic hepatitis (52), immune response toward acetaldehyde adducts can not completely explain the immunoallergic reactions associated to alcoholic liver disease, since anti-acetaldehyde antibodies can also be found in patients with liver diseases unrelated to alcohol (53). Moreover, flow cytometry analysis has failed to demonstrate acetaldehyde adducts on the surface of hepatocytes isolated from rats fed ethanol, unless acetaldehyde metabolism is inhibited by cyanamide (54).

5.1. Evidence for immune reaction toward hydroxyethyl free radicals following alcohol abuse in humans

Moncada *et al.* (55) 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

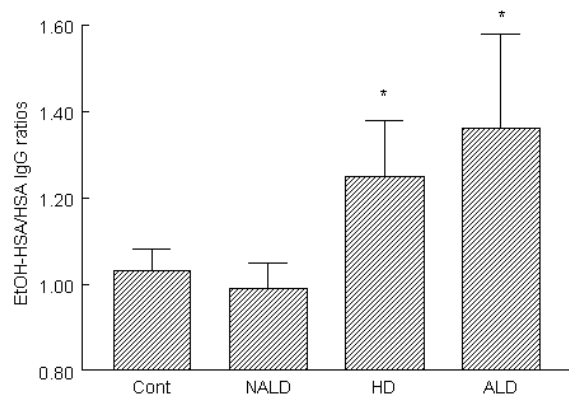


Figure 2. Presence of circulating antibodies reacting with hydroxyethyl radical-modified human serum albumin (EtOH-HSA) in the sera of patients with alcoholic (ALD) or non-alcoholic (NALD) liver diseases, in heavy drinkers without liver damage and healthy controls. IgG levels were measured by reacting polystyrene ELISA microwell plates covered with 0.05 mg/ml of either EtOH-HSA or unmodified HSA with the different sera (1:20 dilution). The secondary antibody consisting of 1:2000 solution of peroxidase-linked goat anti-human IgG was then added, followed by a color developing mixture containing 1-phenyldiamine and hydrogen peroxide. The results are expressed as means of the ratios calculated for the spectrophotometric readings in the plates containing EtOH-HSA or unmodified HSA. Statistically significant versus control: * $p < 0.05$.

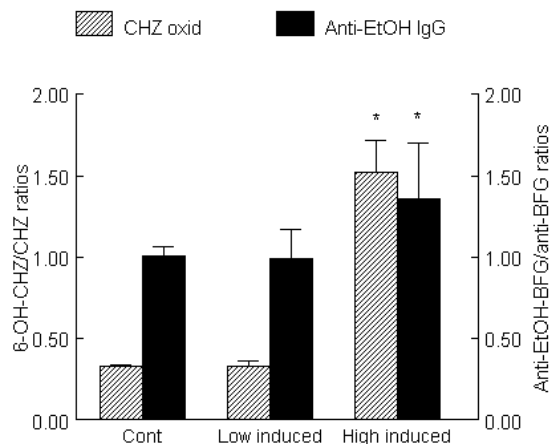


Figure 3. Chlorzoxazone oxidation and IgG reacting with bovine fibrinogen-adducts of hydroxyethyl free radicals (EtOH-BFG) in the sera of healthy controls (Cont) or alcoholics with low-induced or high-induced CYP2E1 activity. The ratios between plasma 6-hydroxychlorzoxazone and chlorzoxazone levels (6-hydroxy-CHZ /CHZ) were used as index of CYP2E1 activity. Anti-EtOH-BFG/anti-oxi-BFG ratios were calculated by dividing the spectrophotometric readings for each of the sera tested in the wells containing as antigen EtOH-BFG and those containing oxy-BFG. Values around 1 indicated the absence of antibodies directed versus the modified proteins. The values are expressed as means \pm SEM. Statistical significance: * $p < 0.001$ versus low-induced alcoholics and controls.

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 (20,55), suggesting their possible use as markers for the formation of ethanol-derived radicals in subjects abusing of alcohol. By mean of a microplate enzyme linked immunoabsorbent assay (ELISA) using as antigen human serum albumin reacted with chemically produced hydroxyethyl radicals, we 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 2) (56). 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 (56). IgG reacting with hydroxyethyl radical epitopes have been also observed in heavy drinkers without clinical signs of hepatic damage. In most of these patients anti-hydroxyethyl radical IgG titres slowly decline upon abstinence and rapidly rise again, when drinking is resumed (Clot *et al.* unpublished observations). 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 (57). We have 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. 3). 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. Moreover, chlorzoxazone oxidation was significantly lower in alcoholics without clinical and biochemical signs of liver disease as compared to patients with alcoholic liver disease (57). These observations not only confirm that hydroxyethyl radicals are actually produced in humans as consequence of excessive alcohol intake, but also indicate that CYP2E1 activity greatly influences the human formation of hydroxyethyl radicals stimulating specific immune reactions against radical species derived from ethanol.

5.2. Characterization of the antigens responsible for immune reactions involving hydroxyethyl radicals

Several studies have demonstrated that alkylation of hepatic proteins by reactive intermediates produced during the metabolism of several drugs, including halothane, dihydralazine, tienilic acid diclofenac and clometacin is associated with the development of circulating antibodies reacting with drug-alkylated hepatic proteins (58-61). According to Pessayre (61) the formation of antibodies against drug-derived metabolites is a consequence of the alkylation of hepatic proteins by reactive metabolites. The presentation of peptides derived from the degradation of these proteins on the macrophage membranes in association with major histocompatibility complex (MHC) class II molecules to CD4⁺ helper T lymphocytes can stimulate the clonal expansion of

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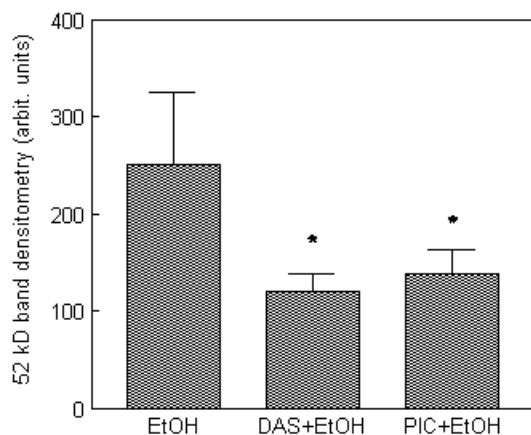


Figure 4. Videodensitometric evaluation of 52 kD protein band, corresponding to CYP2E1-hydroxyethyl radical adduct, in immunoblots of liver microsomes obtained from intragastrically ethanol-fed rats receiving or not diallylsulfide (DAS) (200 mg/kg b.wt.day) or phenylethylisothiocyanate (PIC) (1 mmol/kg b.wt.day). Microsomal proteins (30 μ g) were submitted to SDS-PAGE electrophoresis and transferred to nitrocellulose filters that were further reacted 2 hour with rabbit antiserum anti-hydroxyethyl radical bound albumin (dilution 1:500). Immunoblots were visualized by peroxidase staining and the films scanned using Molecular Dynamic Personal Densitometer and Image-Quant software. The bars refers to the relative volume of the 52 kD bands in microsomes from ethanol fed rats (EtOH); microsomes from rats fed ethanol and DAS (DAS+E); microsomes from rats fed ethanol and PIC (PIC+E). The results are means \pm S.D. * statistically different versus microsomes from ethanol-treated rats $p < 0.05$.

immature B lymphocytes and their differentiation into plasmocytes secreting specific antibodies (61).

Hydroxyethyl free radicals are quite reactive species and can interact with proteins and DNA [62,63]. Upon incubation of rat liver microsomes with NADPH and radioactive ethanol, hydroxyethyl radical residues can be recovered covalently bound to microsomal proteins (64). We have observed that anti-hydroxyethyl radical IgG from alcoholics have high affinity for hydroxyethyl radical adducts produced in liver microsomes during ethanol metabolism (32). Immunoblots of proteins from human liver microsomes incubated *in vitro* with ethanol demonstrate that anti-hydroxyethyl radical antibodies recognize at least four microsomal proteins with apparent molecular weights of 78 kD, 60 kD, 52 kD and 40 kD, respectively (32). The 52 kD protein has been identified by combined immunoblotting and immunoprecipitation techniques as CYP2E1 (32). The identity of 78 kD, 60 kD, and 40 kD proteins recognized by alcoholic sera is presently unknown, but it is possible that they are intimately associated with CYP2E1 in the microsomal membranes. Experiments in reconstituted systems suggest, however, that the 78 kD protein is not NADPH-cytochrome P450 reductase. It is noteworthy, that a 60 kD protein from the liver endoplasmic reticulum is also involved in the development of immune reactions triggered

by the exposure to halothane and diclofenac that are similarly activated by cytochrome P450 (58,60). The formation of hydroxyethyl-CYP2E1 adducts takes place also *in vivo* and can be detected in immunoblots of microsomal proteins obtained from rats acutely treated with a large dose of ethanol as well as in microsomes from intragastric ethanol-fed rats. In the latter, the treatment with CYP2E1 inhibitors DAS and PIC greatly decreases the hydroxyethyl radical binding to CYP2E1 (Figure. 4) and this effect is associated with the presence of low titres of anti-hydroxyethyl radicals IgG in the same animals (20). Thus, alkylation of CYP2E1 by hydroxyethyl radicals might be important in the process leading to the production of anti-hydroxyethyl radical antibodies during chronic alcohol exposure.

5.3. Possible role of hydroxyethyl free radicals in causing immuno-mediated liver injury

The role played by immune reactions directed against liver proteins modified by hydroxyethyl radicals in the pathogenesis of alcoholic liver lesions is still unknown. It has been reported that antibodies present in the sera of patients with hepatitis caused by halothane, α -methyl dopa, diclofenac and clometacin recognize specific epitopes present in the plasma membranes of hepatocytes incubated with these drugs and are able to induce cytotoxicity upon addition of peripheral blood mononuclear cells (65,66). Western blot analysis of plasma membrane proteins from ethanol-treated hepatocytes demonstrates that the sera of patients with alcoholic liver disease recognize 3 main plasma membrane protein bands, one of which corresponds to CYP2E1-hydroxyethyl radical adducts (67). The presence of CYP2E1 adducts on hepatocyte plasma membranes has been confirmed by the use of laser confocal microscopy that has also shown the co-localization on the cell surface of immunofluorescence due to anti-hydroxyethyl radical antibodies and anti-CYP2E1 IgG (67). The plasma membrane localization of CYP2E1-hydroxyethyl radical adducts is not surprising, since functionally active cytochrome P450 isoenzymes, including CYP2E1, have been demonstrated on the extracellular side of either rat and human hepatocyte plasma membranes (68-70). Moreover, Eliasson and Kenna and Robin and coworkers have recently reported that trifluoroacetyl-CYP2E1 and tienilic acid-CYP2C11 adducts on hepatocyte plasma membranes are recognized by the sera of patients suffering from, respectively, halothane- or tienilic acid-induced hepatitis recognize (71,72). The possibility that the development of immuno-toxic reaction towards hydroxyethyl radical-derived antigens might contribute to alcohol-mediated liver damage is suggested by the positive correlation ($r = 0.626$; $p < 0.02$) present between the extent of hepatic damage and the individual levels of hydroxyethyl radical-derived epitopes in liver microsomes of intragastric alcohol-fed rats (20). Furthermore, we have observed that isolated rat hepatocytes exposed *in vitro* to ethanol can be killed by antibody-dependent cell-mediated cytotoxic (ADCC) reactions upon the addition of sera from patients with ALD and normal human blood mononuclear cells (66). These latter findings suggest that hydroxyethyl radical-derived antigens on hepatocyte surface can be regarded as the target for alcohol-altered liver cells antibodies previously detected in patients with alcoholic

liver disease (37)] and associated with an increased risk of developing liver cirrhosis (73).

6. PERSPECTIVE

The results of combined experimental and clinical studies demonstrate that hydroxyethyl free radical intermediates are produced during ethanol metabolism by microsomal CYP2E1-dependent monooxygenase system. 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.

7. ACKNOWLEDGMENTS

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