

Alcohol, oxidative stress and free radical damage

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The involvement of free radical mechanisms in the pathogenesis of alcoholic liver disease (ALD) is demonstrated by the detection of lipid peroxidation markers in the liver and the serum of patients with alcoholism, as well as by experiments in alcohol-fed rodents that show a relationship between alcohol-induced oxidative stress and the development of liver pathology. Ethanol-induced oxidative stress is the result of the combined impairment of antioxidant defences and the production of reactive oxygen species by the mitochondrial electron transport chain, the alcohol-inducible cytochrome P450 (CYP) 2E1 and activated phagocytes. Furthermore, hydroxyethyl free radicals (HER) are also generated during ethanol metabolism by CYP2E1. The mechanisms by which oxidative stress contributes to alcohol toxicity are still not completely understood. The available evidence indicates that, by favouring mitochondrial permeability transition, oxidative stress promotes hepatocyte necrosis and/or apoptosis and is implicated in the alcohol-induced sensitization of hepatocytes to the pro-apoptotic action of TNF- α . Moreover, oxidative mechanisms can contribute to liver fibrosis, by triggering the release of pro-fibrotic cytokines and collagen gene expression in hepatic stellate cells. Finally, the reactions of HER and lipid peroxidation products with hepatic proteins stimulate both humoral and cellular immune reactions and favour the breaking of self-tolerance during ALD. Thus, immune responses might represent the mechanism by which alcohol-induced oxidative stress contributes to the perpetuation of chronic hepatic inflammation. Together these observations provide a rationale for the possible clinical application of antioxidants in the therapy for ALD.

Ethanol: Lipid peroxidation: Free radicals: Immune response: Antioxidants

It is now well accepted that the progression of liver injury consequent to chronic alcohol abuse is a multifactorial event that involves a number of genetic and environmental factors. Among these factors there is a growing interest in the role of free radical-mediated oxidative stress. The involvement of oxidative injury in ethanol toxicity was first proposed in the early 1960s by Di Luzio (1963) and subsequently supported by a number of experimental studies showing that ethanol promotes the formation of a variety of free radical intermediates (oxygen-derived radicals, 1-hydroxyethyl radicals ($\text{CH}_3\text{C}^\bullet\text{HOH}$; HER), NO, lipid-derived radicals) by several cell types, including hepatocytes, Kupffer cells, endothelial cells and infiltrating inflammatory leucocytes (for reviews, see Nordmann *et al.* 1992; Albano, 2002). However, the most convincing evidence to clarify the role of oxidative damage in the

pathogenesis of alcohol-induced liver injury has been obtained using a procedure in which alcohol is fed enterally to rodents (Tsukamoto *et al.* 1985). Using this experimental model Nanji and coworkers (Nanji & French, 1989; Nanji *et al.* 1994a) have shown that rats fed ethanol in combination with diets rich in unsaturated fatty acids from maize or fish oils develop liver damage. This outcome is associated with an increase in the hepatic content of lipid peroxidation products (Nanji *et al.* 1994a,b; Polavarapu *et al.* 1998), suggesting that the generation of aldehydic end products of the peroxidative breakdown of PUFA promotes ethanol toxicity. Interestingly, after 6 weeks of ethanol administration the replacement of fish oil with poorly-oxidizable palm oil or with medium-chain triacylglycerols lowers lipid peroxidation and ameliorates already established liver damage (Nanji *et al.* 1995b). In

Abbreviations: ALD, alcoholic liver disease; CYP, cytochrome P450; GSH, glutathione; HSC, hepatic stellate cells; HER, 1-hydroxyethyl radicals; iNOS, inducible NO synthetase; MDA, malondialdehyde; MPT, mitochondrial permeability transition; mtGSH, mitochondrial GSH; ROS, reactive oxygen species; SOD, superoxide dismutase.

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accordance with these findings, several other studies have demonstrated that supplementation with different antioxidants and free radical scavengers reduces hepatic injury in alcohol-fed rodents (Nanji *et al.* 1994b; Albano *et al.* 1996; Sadrzadeh & Nanji, 1998; Arteel, 2003; Ronis *et al.* 2005). The relevance of these observations to human alcoholic liver disease (ALD) is demonstrated by several clinical studies showing that serum markers of lipid peroxidation, such as conjugated dienes, malondialdehyde (MDA), 4-hydroxynonenal and F2-isoprostanes, are increased in patients with ALD (Clot *et al.* 1994; Aleynik *et al.* 1998; Meager *et al.* 1999). Moreover, immunohistochemical analysis of liver biopsies from patients with alcoholism reveals that proteins adducted by lipid peroxidation products are localized in the areas of liver fatty infiltration, focal necrosis and fibrosis (Niemelä *et al.* 1994).

The present paper will review the most recent insights into the mechanisms responsible for ethanol-induced free radical formation and the contribution of oxidative stress to liver injury by alcohol.

Ethanol and free radical formation

Formation of reactive oxygen species

The formation of reactive oxygen species (ROS) such as the superoxide anion (O_2^-) and H_2O_2 represents an important cause of oxidative injury in many diseases associated with free radical formation. In the presence of trace amounts of transition metals (most frequently Fe) O_2^- and H_2O_2 generate highly-reactive hydroxyl radicals, which are then responsible for the oxidation of biological constituents. Several enzymic systems, including the cytochrome P450 (CYP) 2E1-dependent microsomal monooxygenase system, the mitochondrial respiratory chain and the cytosolic enzymes xanthine oxidase and the aldehyde oxidases have been implicated as sources of O_2^- and H_2O_2 in parenchymal cells during ethanol intoxication (Albano, 2002).

CYP2E1-dependent monooxygenase activity increases by 10–20-fold in alcohol-treated rodents (Ronis *et al.* 1996). In human subjects the determination of CYP2E1 activity by measuring the oxidation of the myorelaxant drug chlorzoxazone reveals an appreciable CYP2E1 induction not only in alcohol abusers (Girre *et al.* 1994), but also in moderate alcohol consumers (Liangpunsakul *et al.* 2005). CYP2E1 has an especially high rate of NADPH oxidase activity, which leads to the production of large quantities of O_2^- and H_2O_2 . In liver microsomes from both human subjects and alcohol-fed rodents the CYP2E1 content is positively correlated with NADPH oxidase activity and lipid peroxidation (for review, see Ronis *et al.* 1996). Increased ROS production is also evident in HepG2 hepatoma cells stably transfected with the *CYP2E1* gene (Caro & Cederbaum, 2004). Thus, the high efficiency of CYP2E1 in reducing O_2 to O_2^- and H_2O_2 could be regarded as one of the key factors contributing to oxidative stress during chronic exposure to alcohol. Consistently, experiments performed using rats enterally fed alcohol (Albano *et al.* 1996; Fang *et al.* 1998; Gouillon *et al.* 2000) have

demonstrated that the induction of CYP2E1 by ethanol is associated with the stimulation of lipid peroxidation, while compounds interfering with CYP2E1 expression reduce oxidative stress and hepatic damage. These findings are in partial contrast with the observation that CYP2E1-knock-out mice are not protected from alcohol toxicity (Kono *et al.* 1999). This discrepancy can be explained, since in ethanol-fed mice CYP2E1 represents <5% of the total hepatic CYP content (Bardag-Gorce *et al.* 2000). Thus, the contribution of CYP2E1 to alcohol toxicity might be blunted by other factors. Nevertheless, more recent studies (Bardag-Gorce *et al.* 2000; Bradford *et al.* 2005) have shown that after ethanol treatment both carbonyl groups and oxidized DNA products are actually lower in CYP2E1-null mice as compared with CYP2E1-expressing mice. Furthermore, chronic enteral administration of ethanol to CYP2E1 transgenic mice, which overexpress CYP2E1 by about 2-fold, causes more liver pathology and transaminase release than in naïve mice (Morgan *et al.* 2002).

The oxidation of mitochondrial proteins and DNA is a common feature of both acute and chronic ethanol exposure (Hoek *et al.* 2002). An enhanced electron leakage from complexes I and III of the mitochondrial respiratory chain along with a stimulation of NADH shuttling to the mitochondria have been proposed to account for the increase in O_2^- production observed in isolated rat hepatocytes incubated acutely with ethanol (Bailey & Cunningham, 2002). Conversely, an impaired synthesis of mitochondria-encoded constituents of the respiratory chain, particularly cytochrome b, consequent to oxidative mitochondrial DNA damage is probably responsible for the enhanced production of ROS observed in liver mitochondria from rats treated chronically with ethanol (Bailey & Cunningham, 2002). An additional mechanism contributing to the mitochondrial formation of ROS might be represented by the interaction between complexes II and III and N-acetyl-D-sphingosine (C2-ceramide) released by hepatocytes in response to TNF- α (Garcia-Ruiz *et al.* 2000). Furthermore, Neve & Ingelman-Sundberg (2001) have described a form of CYP2E1 that lacks the hydrophobic part of the NH_2 terminus, is localized in the matrix of liver mitochondria and might contribute to causing alcohol-induced mitochondrial oxidative damage.

Experiments using the enteral nutrition model (Hines & Wheeler, 2004) have shown that the activation of Kupffer cells in response to circulating bacterial endotoxins stimulates inflammatory reactions within the liver. Moreover, in alcohol abusers as well as in alcohol-fed rodents chronic ethanol intake increases the production of CXC chemokines (chemokines with paired cysteine residues separated by a different amino acid; IL-8 in human subjects and cytokine-induced neutrophil chemoattractant in rats) and macrophage chemotactic protein-1, which promote hepatic leucocyte infiltration by up regulating the expression of CD18 adhesion molecule in neutrophils and its counter receptor intracellular adhesion molecule 1 in endothelial cells (Bautista, 2002). These events account for the increased hepatic sequestration of granulocytes associated with advanced ALD and for the priming of neutrophils to O_2^- production (Bautista, 2002). Accordingly, liver depletion of Kupffer cells with $GdCl_3$ lowers the hepatic

production of O_2^- in the recovery period after 12 h of continuous ethanol infusion (Bautista & Spitzer, 1996) and reduces both liver injury and oxidative stress markers in rats subjected to chronic enteral feeding of alcohol (Arteel, 2003). Similar protection is also evident in mice deficient in intracellular adhesion molecule 1 (Kono *et al.* 2001b) or the phagocyte NADPH oxidase activity (p47phox-knock-out mice; Kono *et al.* 2000). However, more recent investigations that have measured oxidative DNA damage as an index of intracellular oxidative stress (Bradford *et al.* 2005) have shown that the extent of DNA oxidation induced by alcohol administration is comparable in both p47phox-knock-out and naïve mice, whereas CYP2E1-null mice are instead protected from such damage. Together these results indicate the contribution of both intracellular and extracellular sources of ROS to ethanol-induced oxidative stress. However, ROS produced by CYP2E1 appear to be more important in causing intracellular oxidative injury than ROS originating from activated phagocytes (Bradford *et al.* 2005).

Role of iron in ethanol-induced oxidative stress

Alcohol abuse in human subjects is often associated with impaired utilization and/or increased deposition of Fe in the liver (Irving *et al.* 1988). Moreover, a recent survey (Ioannou *et al.* 2004) has demonstrated that even moderate alcohol consumption increases the risk of hepatic Fe overload. Although the presence of Fe, particularly of low-molecular-weight non-protein Fe complexes, exacerbates oxidative damage by ethanol (Caro & Cederbaum, 2004) and primes hepatic macrophages to produce ROS and proinflammatory cytokines (Tsukamoto *et al.* 1999), it is not yet clear how ethanol favours Fe accumulation in the liver and the actual role of Fe in the progression of ALD. It has been proposed that oxidative modifications of cytosolic Fe regulatory protein 1 might cause the inappropriate repression of ferritin synthesis and the concomitant stimulation of transferrin receptor synthesis. This outcome would lead to an increased Fe uptake combined with an impaired capability of hepatocytes to store Fe (Rouault, 2003). In support of this hypothesis, preliminary data from Suzuki *et al.* (2004) show that the incubation of isolated rat hepatocytes with ethanol (25 mmol/l) increases the expression of transferrin receptor and enhances transferrin uptake. The actual contribution of Fe to alcohol toxicity also awaits further investigation. Dietary Fe supplementation worsens liver pathology and lipid peroxidation in the enteral-alcohol feeding model (Tsukamoto *et al.* 1995). However, in the same experimental model the liver content of low-molecular-weight Fe is decreased (Rouach *et al.* 1997) or unchanged (Kamimura *et al.* 1992). Conflicting results have also been obtained using Fe chelators, as the administration of the oral Fe chelator 1,2-dimethyl-3-hydroxypyrid-4-one in combination with chronic enteral feeding of ethanol reduces non-haem-Fe levels, lipid peroxidation and fat accumulation, whereas the long-acting parenteral Fe chelator hydroxyethylstarch-desferoxamine has no effect (Sadrzadeh *et al.* 1994b, 1997).

Nitric oxide and alcohol-induced oxidative injury

NO is a N-centred free radical produced from L-arginine by constitutive NO synthetase and inducible NO synthetase (iNOS) enzymes. In hepatocytes and endothelial and Kupffer cells membrane-bound constitutive NO synthetase produces pulses of NO for short periods in response to specific stimuli, and the NO so formed mediates intra- and intercellular signalling (Li & Billiar, 1999). Once stimulated by cytokines or endotoxins the same cells respond with the activation of cytosolic iNOS, which leads to the continuous formation of large amounts of NO (Li & Billiar, 1999). The reaction of NO with O_2^- leads to the formation of highly-reactive peroxynitrite ($ONOO^-$), which can inactivate several enzymes and impair mitochondrial functions (Beckman & Koppenol, 1996). Chamulitrat & Spitzer (1996) have shown that in rats chronically exposed to ethanol the generation of NO triggered by the infusion of endotoxins is 3-fold higher when compared with that of naïve animals. Furthermore, iNOS-knock-out mice are protected against the oxidative stress, tyrosine nitration and hepatic injury induced by chronic enteral feeding of alcohol (Arteel, 2003). A comparable protection is also given in wild-type mice by treatment with N-(3-amino-methyl)benzyl-acetaminidine (1400 W), a highly-selective iNOS inhibitor (McKim *et al.* 2003), suggesting the possible contribution of NO generated by iNOS to alcohol-related oxidative stress. On the other hand, the inhibition of NO formation enhances O_2^- release by Kupffer cells (Bautista & Spitzer, 1994). Moreover, the stimulation of NO production in hepatocytes increases their resistance to ethanol-induced oxidative stress by lowering the intracellular low-molecular-weight Fe content (Sergeant *et al.* 1997). This protective action of NO is consistent with the observation that treatment with the non-selective NO inhibitor N-nitro-L-arginine methyl ester worsens alcohol-related liver damage (Nanji *et al.* 1995a). These contrasting results might reflect the different effects exerted by NO associated with the timing, the location and the rate of its production. Nonetheless, the actual role of NO in the pathogenesis of oxidative stress induced by alcohol awaits further investigation.

Ethanol-derived free radicals

The use of electron-spin-resonance spectroscopy in combination with a spin-trapping technique has revealed that ethanol oxidation by CYP2E1 results in the formation of a free radical metabolite identified as HER (Albano *et al.* 1994). In addition, it has been reported that the *in vivo* trapping of HER is decreased by Kupffer cell destruction with $GdCl_3$ (Knecht *et al.* 1995) as well as in both NADPH oxidase- and iNOS-knock-out mice (Kono *et al.* 2000; McKim *et al.* 2003). These findings suggest the possibility that the interaction of ethanol with $ONOO^-$ might also contribute to the formation of HER. However, the actual importance of these observations requires further investigation.

Although HER are produced by rat liver microsomes at a rate ten times lower than the rate of ethanol conversion to acetaldehyde (Stoyanovsky & Cederbaum, 1998), their

high reactivity towards ascorbic acid, glutathione (GSH), α -tocopherol and DNA (Nakao & Augusto, 1998; Stoyanovsky *et al.* 1998) might be relevant in relation to alcohol toxicity. When rat liver microsomes are incubated with NADPH and [14 C]ethanol the HER residues account for the majority of the radioactivity that can be recovered covalently bound to microsomal proteins (Albano *et al.* 1993). Immunoblots using antibodies raised against HER–protein adducts have demonstrated the binding of HER to at least four microsomal proteins, including CYP2E1 (Clot *et al.* 1996). The formation of HER–CYP2E1 adducts also occurs *in vivo* following both acute and chronic ethanol intoxication (Clot *et al.* 1996). One of the consequences of the alkylation by HER of liver proteins is the stimulation of an immune response characterized by the generation of antibodies specifically recognizing HER-derived epitopes (Moncada *et al.* 1994). These antibodies are detectable in the sera of both chronically ethanol-fed rats (Albano *et al.* 1996) and patients with ALD (Clot *et al.* 1995). Human anti-HER IgG recognize as antigen several HER-modified liver microsomal proteins and, particularly, HER–CYP2E1 adducts (Clot *et al.* 1996). This finding demonstrates that in human liver HER are mostly generated as a result of CYP2E1-dependent ethanol oxidation. Consistently, in both rats and human subjects the presence of anti-HER antibodies is closely correlated with CYP2E1 activity (Albano *et al.* 1996; Dupont *et al.* 1998). In particular, a minority of heavy drinkers who do not display CYP2E1 induction, when assayed by the chlorzoxazone oxidation test, have titres of anti-HER IgG comparable with those of non-drinking controls and lower than those of drinkers with normally-induced CYP2E1 activity (Dupont *et al.* 1998).

Lowering of antioxidant defences and ethanol-induced oxidative stress

The possible contribution of impaired antioxidant defences to ethanol-induced oxidative stress has been extensively investigated. Early studies have shown that a decrease in the liver content of reduced GSH, independent of nutritional status or the extent of liver disease, is a common feature in ethanol-fed animals as well as in patients with alcoholism (for review, see Albano, 2002). The importance of GSH homeostasis in preventing alcohol-mediated oxidative injury is supported by the observation that the stimulation of GSH re-synthesis in rats by supplementation with either of the GSH precursors L-2-oxothiazolidine-4-carboxylic acid or N-acetylcysteine prevents liver injury in the enteral alcohol model (Iimuro *et al.* 2000; Ronis *et al.* 2005). Although chronic alcohol intake does not appreciably affect the hepatocyte cytosolic GSH content, it lowers the mitochondrial GSH (mtGSH) content by 50–85% (Fernandez-Checa *et al.* 1991; Hirano *et al.* 1992). Such an effect is more evident in the centrilobular hepatocytes (Garcia-Ruiz *et al.* 1994) and precedes the development of mitochondrial dysfunctions and lipid peroxidation (Hirano *et al.* 1992; Garcia-Ruiz *et al.* 1994). The selective depletion of the mtGSH pool is the consequence of a defect in GSH transport from the cytosol to

the mitochondrial matrix (Fernandez-Checa & Kaplowitz, 2005). According to a recent report (Coll *et al.* 2003) a decreased fluidity of mitochondrial membranes associated with cholesterol accumulation interferes with the activity of the 2-oxoglutarate carrier as well as that of another, as yet unidentified, high-affinity GSH carrier. The action of ethanol on mtGSH homeostasis might play an important role in the development of ALD, as it favours oxidative mitochondrial damage and impairs hepatocyte tolerance to TNF- α (Fernandez-Checa & Kaplowitz, 2005).

Vitamin E (α -tocopherol) plays an important role as the main lipid-soluble antioxidant in the liver. In both human subjects and rodents chronic alcohol intake decreases liver and plasma levels of vitamin E, and the lowering of the α -tocopherol levels is inversely correlated with the detection of lipid peroxidation markers (Bell *et al.* 1992; Clot *et al.* 1994; Sadrzadeh *et al.* 1994a; Rouach *et al.* 1997). The mechanisms responsible for the lowering of vitamin E associated with alcohol intake have not yet been completely elucidated. Liver microsomes from ethanol-fed rats show a decrease in α -tocopherol: α -tocopheryl quinone (the main oxidation product of α -tocopherol; Kawase *et al.* 1989), suggesting that an increased oxidation of α -tocopherol might be responsible for the lowering of hepatic vitamin E. Although vitamin E-deficient rats have an increased susceptibility to alcohol toxicity (Sadrzadeh *et al.* 1994a), α -tocopheryl acetate supplementation fails to protect against liver injury (Sadrzadeh *et al.* 1995). This discrepancy might be explained by the poor bioavailability of some tocopheryl esters in rodents or by ethanol interfering with the enteral absorption of vitamin E. It is noteworthy that when alcohol feeding is discontinued the administration of vitamin E contributes to a reduction in the severity of hepatic lesions (Nanji *et al.* 1996). However, a recent randomized placebo-controlled clinical trial of patients with mild or moderate alcoholic hepatitis (Mezey *et al.* 2004) has not confirmed a marked effect of vitamin E supplementation in human subjects.

Several studies have investigated the effects of ethanol on the enzymes involved in the detoxification of ROS or lipid peroxidation products. However, the results are rather inconclusive (Nordmann *et al.* 1992). More recent investigations using the enteral alcohol model (Rouach *et al.* 1997; Polavarapu *et al.* 1998) have shown a marked decline in both the enzymic activity and the immuno-reactive protein concentrations of liver Cu,Zn superoxide dismutase (SOD; SOD-1), catalase and GSH peroxidase. The changes in these ROS enzyme activities are inversely correlated with the extent of both lipid peroxidation and hepatic injury (Polavarapu *et al.* 1998). Interestingly, the increase in mRNA expression for both liver GSH peroxidase and catalase is appreciable in the same experimental model (Nanji *et al.* 1995b), suggesting that ethanol might interfere at the post-transcriptional level with the synthesis of antioxidant enzymes or might stimulate their intracellular degradation. A further insight into the role of SOD in alcohol toxicity has been derived from the use of SOD-1-knock-out mice that show increased lipid peroxidation, nitrotyrosine accumulation, extensive centrilobular necrosis and inflammation with moderate ethanol consumption (Kessova *et al.* 2003). Conversely, rodents over-expressing

SOD-1 or the mitochondrial form Mn-SOD (SOD-2) are protected against the liver injury associated with enteral administration of a large amount of alcohol (Wheeler *et al.* 2001*a, b*). Ebselen, an organoselenium compound that mimics GSH peroxidase activity (Kono *et al.* 2001*a*), also protects against ethanol toxicity and oxidative stress. About 25% of Caucasians carry a genetic polymorphism that causes an Ala16Val substitution in the leader amino acid sequence that is responsible for the mitochondrial localization of SOD-2. Although the Ala-SOD-2 variant might translocate to the mitochondria less efficiently than the Val-SOD-2 variant (Sutton *et al.* 2003), a study performed in 281 patients with advanced ALD and 218 heavy drinkers without liver disease or with steatosis only (Stewart *et al.* 2002) does not show any prevalence of SOD-2 polymorphisms in ALD. Moreover, neither the severity of liver injury nor the detection of oxidative stress markers is increased in Ala-SOD-2 homozygotes. Similarly, a large case-control survey (Brind *et al.* 2004) has failed to confirm any association between the susceptibility to ALD and several polymorphisms of GSH S-transferase isoenzymes. Altogether, these latter results cast some doubt on the actual importance of alcohol-induced changes in liver antioxidant enzymes to human pathology.

Mechanisms of free radical damage in alcoholic liver disease

The histological features of human ALD include a variety of lesions comprising macro- or microvesicular steatosis, ballooning degeneration of hepatocytes, hyaline necrosis, Mallory's bodies, inflammatory infiltrates and various extents of fibrosis up to cirrhosis (Ishak *et al.* 1991). In recent years the contribution of oxidative damage to causing some of the histological lesions associated with ALD has been discerned from experimental studies showing a positive correlation between the extent of alcohol-induced liver pathology and the detection of oxidative stress markers (Kamimura *et al.* 1992; Nanji *et al.* 1994*a, b*; Albano *et al.* 1996; Rouach *et al.* 1997; Polavarapu *et al.* 1998). Moreover, in animal studies supplementation with antioxidants or treatments that moderate oxidative stress have been reported to lower the extent of focal necrosis and inflammation, and in some cases to ameliorate steatosis (Nanji *et al.* 1994*b*; Sadrzadeh *et al.* 1994*b*; Albano *et al.* 1996; Sadrzadeh & Nanji, 1998; Arteel, 2003; Ronis *et al.* 2005). However, the mechanisms by which oxidative stress contributes to the development of the histological lesions associated with ALD are still poorly understood.

Oxidative mechanisms and mitochondrial damage by ethanol

Morphological and functional abnormalities of mitochondria represent one of the earliest manifestations of hepatocyte injury by alcohol (Ishak *et al.* 1991). As discussed earlier, ethanol promotes ROS formation in the mitochondria and causes a selective decrease in mtGSH content that makes these organelles more susceptible to oxidative damage (Fernandez-Checa & Kaplowitz, 2005). The loss

of mtGSH during chronic ethanol feeding precedes the impairment of ATP synthesis, which in rats can be partially prevented by restoring mtGSH by supplementation with S-adenosyl-L-methionine (Fernandez-Checa & Kaplowitz, 2005). Consistently, ethanol-stimulated lipid peroxidation has been linked to the impairment of mitochondrial oxidative phosphorylation and the appearance of megamitochondria (Matsushashi *et al.* 1998). Mitochondria obtained from both acute and chronic alcohol-treated rats show oxidative modifications of mitochondrial DNA (Hoek *et al.* 2002). Single or multiple deletions of mitochondrial DNA are also 8-fold more frequent in the liver of patients with alcoholism as compared with age-matched controls (Mansouri *et al.* 1997). These alterations are probably responsible for the reduction in mitochondrially-encoded subunits of the electron transport chain observed in experimental animals following chronic ethanol treatment, and together with the alkylation of cytochrome c oxidase by lipid peroxidation products they contribute to the impairment of hepatic respiratory activity caused by alcohol (Bailey & Cunningham, 2002). Factors affecting the efficiency of NADH oxidation by the mitochondria have also been proposed to contribute to the metabolic imbalances responsible for hepatic steatosis (Fromenty & Pessayre, 1995). Supporting this view, mitochondrial DNA deletions show a very high prevalence (about 85% of the cases) in patients with alcoholism who have hepatic microvesicular steatosis (Fromenty *et al.* 1995; Mansouri *et al.* 1997), which is a lesion that is ascribed to impaired mitochondrial β -oxidation of fatty acids (Fromenty & Pessayre, 1995). Although supplementation with antioxidants such as ebselen, polyphenolic extracts or allopurinol (Arteel, 2003), but not with the 21-aminosteroid, tirilazad mesylate and N-acetylcysteine (Sadrzadeh & Nanji, 1998; Ronis *et al.* 2005), ameliorates the fat accumulation induced by enteral feeding of alcohol, the mechanisms involved have not been clarified. The observation that mice deficient in TNF receptor 1 or endotoxin binding-signalling molecules develop less steatosis than wild-type animals (Arteel, 2003) suggests the possible involvement of oxidative mechanisms in the signals leading to Kupffer cell activation by endotoxins and/or in the metabolic effects of TNF- α (Dandona *et al.* 2004). Nonetheless, it cannot be excluded that oxidative stress might also contribute to the effects of ethanol on the regulation of lipid metabolism by either the PPAR α or the sterol regulatory element-binding protein 1 (You & Crabb, 2004).

The induction of oxidative stress within liver mitochondria is associated with the collapse of mitochondrial membrane potential and the onset of mitochondrial permeability transition (MPT) (Hoek *et al.* 2002). MPT is characterized by the opening of a megachannel in the mitochondrial membrane as a result of the assembly of a protein complex that includes a voltage-dependent anion channel, an adenine nucleotide translocator and cyclophilin D (Bernardi *et al.* 2001). According to Adachi *et al.* (2004) ethanol-induced oxidative stress promotes MPT by favouring the translocation to the mitochondria of the proapoptotic factor Bax that forms a complex with a voltage-dependent anion channel. Extensive MPT leads to mitochondrial swelling as a result of the influx of ions and

water, and is critical in the onset of hepatocyte death by necrosis. In addition, by promoting the permeabilization of the outer mitochondrial membrane MPT triggers the release of cytochrome c and the induction of apoptosis (Green & Kroemer, 2004). Ethanol addition to HepG2 cells over-expressing the human *CYP2E1* gene promotes the collapse of mitochondrial membrane potential, MPT and apoptosis, which can be prevented by CYP2E1 inhibitors and antioxidants, as well as by the MPT inhibitor cyclosporine A (Caro & Cederbaum, 2004). Similarly, cultured hepatocytes exposed *in vitro* to ethanol undergo apoptotic changes in association with the formation of ROS and MTP (Adachi & Ishii, 2002). In both hepatocytes and HepG2 cells ethanol-induced apoptosis is prevented by antioxidants and by the over-expression of the anti-apoptotic protein Bcl-2 (Adachi & Ishii, 2002; Caro & Cederbaum, 2004). Thus, oxidative mitochondrial damage can be regarded as one of the mechanisms responsible for the hepatocyte apoptosis associated with advanced ALD (Natori *et al.* 2001; Ziol *et al.* 2001). Nonetheless, even when ethanol-induced mitochondrial damage is not sufficiently extensive to be the primary cause of cell death, the impairment of ATP production might be harmful to the liver by enhancing its susceptibility to alcohol-induced centrilobular hypoxia (French *et al.* 1984).

Oxidative stress in the modulation of hepatic inflammatory reactions induced by alcohol

One important contribution to the understanding of the pathogenesis of human ALD has originated from studies in the late Ron Thurman's laboratory concerning the role played by inflammatory responses in the mechanisms of alcohol hepatotoxicity (Thurman, 1998). The current view is that ethanol ingestion increases the translocation of gut-derived endotoxins to the portal circulation where they stimulate intrahepatic Kupffer cells by interaction with the surface receptor CD14 (Rao *et al.* 2004). Activated Kupffer cells are then responsible for the synthesis and release of proinflammatory cytokines, particularly TNF- α , eicosanoids, ROS and NO (Hines & Wheeler, 2004). In accordance with this interpretation plasma TNF- α concentrations are increased in patients with ALD and the values correlate with disease severity and mortality (Felver *et al.* 1990). Moreover, experiments in rats receiving ethanol enterally (Thurman, 1998) have shown that the administration of antibiotics to reduce endotoxaemia or the inactivation of Kupffer cells with GdCl₃ prevent liver injury. CD14-knock-out or CD14-coupled Toll-like receptor 4-knock-out mice, which produce less TNF- α , are also resistant to alcohol toxicity (Uesugi *et al.* 2001; Yin *et al.* 2001). Oxidative stress probably contributes to this proinflammatory action of alcohol, as lipid peroxidation is associated with a marked up-regulation of TNF- α and cyclooxygenase-2 mRNA expression (Nanji *et al.* 1997), while blocking CYP2E1 induction with chlormethiazole down regulates ethanol-stimulated expression of TNF- α and IL-1 β mRNA in the liver of alcohol-fed rats (Fang *et al.* 1998).

TNF- α is implicated as being an important cause of alcohol hepatotoxicity by the observation that rats

receiving anti-TNF- α antibodies as well as TNF receptor 1-knock-out mice are protected against the liver damage induced by enteral administration of alcohol (Hines & Wheeler, 2004). It is now well established that interaction between TNF- α and TNF receptor 1 induces cell apoptosis by activating several signal pathways that promote caspase activation, ceramide release, MPT and the stimulation of Jun-N-terminal kinase and p38 mitogen-activated kinase (Wajant *et al.* 2003). However, hepatocytes, like many other cells, are resistant to the pro-apoptotic action of TNF- α because of the concomitant induction of anti-apoptotic signals involving both NF- κ B-dependent gene transcription and phosphatidylinositol-3-kinase/protein kinase B activation (Wajant *et al.* 2003). As Hoek & Pastorino (2004) point out, the way in which ethanol affects the balance between the pro- and anti-apoptotic signals triggered by TNF- α is different, with oxidative stress playing a role in causing the imbalance. Indeed, in HepG2 cells over-expressing CYP2E1 the sensitivity to TNF- α is abolished by the antioxidant ebselen, while in hepatocytes obtained from rats chronically fed ethanol selective depletion of mtGSH is associated with increased susceptibility to the cytotoxic effects of TNF- α (Hoek & Pastorino, 2004; Fernandez-Checa & Kaplowitz, 2005). At present, the mechanisms by which ethanol-induced oxidative stress enhances TNF- α toxicity are not fully understood. However, likely targets might be represented by an increased susceptibility of mitochondria to TNF- α -induced MPT, as well as the enhanced activation of apoptosis signalling kinase-1 as a result of the oxidation of its binding protein thioredoxin (Hoek & Pastorino, 2004). Furthermore, it cannot be excluded that oxidative stress might contribute to ethanol-mediated down-regulation of the anti-apoptotic signals involving phosphatidylinositol-3-kinase/protein kinase B (Shulga *et al.* 2005). These observations suggest that the interplay between oxidative stress and TNF- α might represent an important cause of the extensive hepatocyte apoptosis that characterizes alcohol-related hepatitis (Natori *et al.* 2001).

Free radical mechanisms in immune reactions associated with alcoholic liver disease

Although chronic ethanol exposure increases the Kupffer cell response to endotoxins (McClain *et al.* 2004) and renders hepatocytes susceptible to TNF- α toxicity (Hoek & Pastorino, 2004), Kupffer cell activation by endotoxins might not be the only factor responsible for maintaining the chronic inflammatory response in the liver, since continuous exposure to endotoxins promotes tolerance (Ziegler-Heitbrock, 1995), while chronic administration of ethanol in combination with endotoxins fails to increase alcohol hepatotoxicity (Jarvelainen *et al.* 1999). Moreover, rats enterally fed alcohol show liver injury, inflammation and increased TNF- α mRNA expression, even in the absence of appreciable elevations in plasma endotoxins (Ronis *et al.* 2004).

The immune system is known to have a key role in regulating the inflammatory processes associated with chronic liver diseases (Kita *et al.* 2001). Early studies (Neuberger *et al.* 1984; Izumi *et al.* 1985) have shown the

presence of circulating antibodies targetting alcohol-altered hepatocytes in patients with ALD. Furthermore, histological examination reveals that liver infiltrates characteristic of alcoholic hepatitis contain both CD8+ and CD4+ T lymphocytes, and their presence correlates with the extension of piecemeal necrosis, intralobular inflammation and regenerating nodules (Chedid *et al.* 1993). Liver-associated lymphocytes isolated from alcohol-consuming rats have an increased capacity to secrete proinflammatory cytokines (Batey *et al.* 2002), which suggests that they may contribute to the orchestration of inflammation during the evolution of ALD. Moreover, alcohol consumption increases the activity of natural killer T-cells present in the liver, which may be a causative factor in hepatocyte apoptosis by both TNF- α - and FAS-mediated mechanisms (Minagawa *et al.* 2004). Further evidence for a role of the immune response in favouring the progression of liver damage towards fibrosis has been provided by a recent report (Safadi *et al.* 2004) that lymphocyte destruction by sublethal irradiation reduces liver fibrosis in mice treated with CCl₄ or thioacetamide, while the transfer of CD8+ lymphocytes from CCl₄-treated mice to immuno-deficient SCID mice leads to the fibrogenic activation of hepatic stellate cells (HSC).

The first indication of the possible contribution of oxidative stress in promoting immune reactions in ALD was the observation that antibodies specifically recognizing HER-derived epitopes are detectable in both rats chronically fed ethanol (Albano *et al.* 1996) and patients with ALD (Clot *et al.* 1995). Subsequent studies (Mottaran *et al.* 2002) have shown that elevated titres of circulating IgG towards epitopes derived from the modification of proteins by lipid peroxidation products (MDA, 4-hydroxynonenal and oxidized arachidonic acid) are also present in a large percentage (55–70) of patients with biopsy-proven alcoholic hepatitis and/or cirrhosis, but only in a few (8–13%) subjects with fatty liver only, irrespective of the magnitude and the duration of alcohol intake. In addition, the combined reaction of MDA and acetaldehyde with the ϵ -NH₂ group of protein lysine residues generates condensation products, termed MDA–acetaldehyde adducts, that are also antigenic (Thiele *et al.* 2004) and stimulate immune reactions in advanced ALD (Rolla *et al.* 2000). The presence of MDA–acetaldehyde adducts could also favour autoimmune processes during the development of alcohol-related liver injury, as mice immunized with MDA–acetaldehyde adduct-containing proteins produce antibodies that recognize not only the MDA–acetaldehyde adduct epitopes but also the carrier proteins (Thiele *et al.* 2004).

The reasons why alcohol-induced oxidative damage promotes the formation of such a variety of antibodies are still unclear. In order to initiate an antibody response, peptide antigens should be presented by professional antigen-presenting cells to CD4+ T lymphocytes in conjunction with molecules of the class II MHC and accessory signals. In the liver professional antigen-presenting cells are represented by hepatic dendritic cells, Kupffer cells and sinusoidal endothelial cells. However, exposure to pro-inflammatory cytokines induces the expression of both class II MHC and co-stimulatory B7.1 (CD80) molecules in hepatocytes that can then function as antigen-presenting

cells to CD4+ T lymphocytes (Herkel *et al.* 2003). Thus, in the course of ALD the modification of protein by HER or lipid peroxidation products within the hepatocytes might directly trigger the activation of CD4+ T-cells infiltrating the liver and promote antibody production by the T-helper 2 immune response. It is noteworthy that in about 35% of patients with advanced ALD (but only 9% of heavy drinkers without major hepatic damage) the presence of antibodies towards MDA-derived antigens is accompanied by a proliferative response of CD4+ T lymphocytes to the same antigens, indicating the involvement of free radical mechanisms in promoting both the humoral and cellular immune reactions associated with ALD (Stewart *et al.* 2004b).

In addition to developing antibodies directed against a variety of allo-antigens, patients with ALD can often show signs of autoimmune reactions (for review, see McFarlane, 2000). In this context, it has been observed that about 40% of patients with advanced ALD have circulating IgG directed against CYP2E1 (Vidali *et al.* 2003). These auto-antibodies recognize at least two distinct conformational epitopes present in the C-terminal portion of the CYP2E1 molecule (Vidali *et al.* 2004). One of the mechanisms proposed to explain the formation of anti-CYP auto-antibodies postulates that the binding of reactive drug metabolites to CYP promotes a humoral immune response against the modified protein and concomitantly favours the activation of normally-quiescent auto-reactive lymphocytes, which leads to the production of antibodies to the native CYP molecules (Van Pelt *et al.* 1995). Patients with ALD who have anti-HER antibodies have a 4-fold increased risk of developing anti-CYP2E1 auto-reactivity as compared with patients without anti-HER IgG, which indicates that CYP2E1 alkylation by HER is critical for the development of anti-CYP2E1 auto-reactivity in ALD (Vidali *et al.* 2003). Nonetheless, genetic factors are also involved. In particular, it has been observed that the presence of an A→G base exchange at position 49 in exon 1 of the gene encoding cytotoxic T lymphocyte-associated antigen-4 (a membrane receptor that down regulates T-cell-mediated immune responses; Egen *et al.* 2002) increases by 3.8-fold the risk of developing anti-CYP2E1 IgG without influencing the formation of antibodies towards HER antigens. Interestingly, the risk of developing anti-CYP2E1 auto-reactivity in patients with ALD who have both anti-HER IgG and the mutant cytotoxic T lymphocyte-associated antigen-4 G allele is 23-fold higher than that in subjects negative for both these factors (Vidali *et al.* 2003). Thus, antigenic stimulation by HER-modified CYP2E1 in combination with impaired control of T-cell proliferation as a result of the cytotoxic T lymphocyte-associated antigen-4 mutation promotes the development of anti-CYP2E1 auto-antibodies. The contribution of both allo- and auto-reactivity involving CYP2E1 to hepatic injury by alcohol is suggested by the observation that anti-HER IgG recognize HER–CYP2E1 adducts on the outer layer of the plasma membranes of ethanol-treated hepatocytes where they activate antibody-dependent cell-mediated cytotoxicity (Clot *et al.* 1997). Interestingly, an epidemiological prospective survey has associated the presence of antibodies towards

alcohol-modified hepatocytes with an increased risk of developing alcoholic liver cirrhosis (Takase *et al.* 1993). Antibody-dependent cytotoxicity would also result from the recognition of plasma membrane CYP2E1 by anti-CYP2E1 auto-antibodies. Indeed, preliminary data (Stewart *et al.* 2004a) indicates that high titres of anti-CYP2E1 auto-antibodies correlate with the extension of lymphocyte infiltration and the number of apoptotic hepatocytes.

Anti-phospholipid antibodies are among the auto-antibodies most frequently associated with ALD, being detectable in $\leq 80\%$ of patients with alcoholic hepatitis or cirrhosis (McFarlane, 2000). It has been observed (Rolla *et al.* 2000) that oxidative stress markers are increased in patients with ALD who have anti-phospholipid antibodies and that these antibodies are largely directed towards oxidized phospholipids. The current view (Savill *et al.* 2002) is that impaired macrophage clearance of apoptotic corpses is one of the causes of the development of anti-phospholipid auto-reactivity. Indeed, the production of anti-phospholipid antibodies can be induced by the immunization of mice with syngenic apoptotic lymphocytes, but not with viable cells (Levine *et al.* 1999). The possibility that defective clearance of apoptotic hepatocytes might contribute to the development of anti-phospholipid reactions during the progression of ALD is suggested by the observations that: (1) chronic alcohol consumption affects the capacity of hepatocytes to scavenge apoptotic bodies through interaction with the asialoglycoprotein receptors (McVicker *et al.* 2002); (2) anti-phospholipid antibodies from patients with ALD recognize apoptotic, but not living, hepatic cells by specifically targeting oxidized phosphatidylserine on the outer layer of apoptotic bodies (Vay *et al.* 2006). This specificity is consistent with recent reports (Kagan *et al.* 2003) showing that phosphatidylserine is oxidized during apoptosis by interaction with cytochrome c and is then exposed on the outer layer of cell plasma membranes. The actual relevance of the association between severe ALD and anti-phospholipid immune reactivity is still poorly understood (McFarlane, 2000). According to recent evidence (Kagan *et al.* 2003; Gregory & Devitt, 2004), the phagocytosis of apoptotic bodies is important in the termination of inflammation, and the presence of phosphatidylserine, or more likely oxidized phosphatidylserine (Kagan *et al.* 2003), on the surface of apoptotic cells represents a key signal for stimulating macrophages to secrete the anti-inflammatory cytokine transforming growth factor $\beta 1$ and IL-10 (Gregory & Devitt, 2004). It is proposed that the masking of these recognition sites by IgG targeting oxidized phosphatidylserine (Vay *et al.* 2006) would not only interfere with such an anti-inflammatory response, but might also favour the proinflammatory activation of phagocytes that recognize the apoptotic bodies through the IgG Fc receptors. Thus, in the presence of anti-phospholipid antibodies ethanol-induced hepatocyte apoptosis might represent a fuel for the perpetuation of inflammation. Indeed, in human and experimental alcoholic hepatitis apoptotic hepatocytes co-localize with neutrophil infiltration, which correlates with the severity of tissue damage (Jaeschke, 2002). Moreover, Gores' group (Canbay *et al.* 2003) has

shown that the engulfment of Kupffer cells by apoptotic bodies stimulates the expression of TNF- α and Fas ligand.

Together these observations indicate that alcohol-induced oxidative stress triggers the production of a variety of allo-antibodies and, in combination with genetic predisposition, can favour the breaking of self-tolerance in the liver. Oxidative stress-mediated immune responses might represent one of the mechanisms by which alcohol abuse promotes and maintains inflammatory processes during the evolution of ALD. In support of this possibility Ronis *et al.* (2005) have reported that in an endotoxin-free enteral alcohol model lipid peroxidation directly correlates with both TNF- α mRNA expression and the extent of inflammatory infiltrates, while supplementation with N-acetylcysteine reduces hepatic inflammation along with the immune response triggered by HER and lipid peroxidation products.

Oxidative mechanisms in the onset of alcohol-induced liver fibrosis

Liver fibrosis and cirrhosis represent the terminal stage of ALD and are one of the main causes of death among patients with alcohol abuse. Research performed in recent years (Friedman, 2003; Battaler & Brenner, 2005) has given new insights into the mechanisms responsible for liver fibrosis, showing that HSC (perisinusoidal fat-storing cells or Ito cells) under the influence of transforming growth factor $\beta 1$, platelet-derived growth factor and macrophage-stimulating factor 1 transform into myofibroblast-like cells that produce collagen and extracellular matrix components. Oxidative stress contributes to HSC activation by different means. Intracellular redox changes in Kupffer cells activate NF- κ B and activator protein-1, which trigger the transcriptional up-regulation of the genes encoding for fibrogenetic cytokines that stimulate HSC (Parola & Robino, 2001). Moreover, *in vitro* studies (Caro & Cederbaum, 2004) show that oxidative stress directly promotes collagen synthesis in HSC that over-express the *CYP2E1* gene. This effect is probably mediated by aldehydic products of lipid peroxidation, as concentrations of 4-hydroxynonenal comparable with those detected *in vivo* act as a potent profibrogenic stimulus for activated human HSC, up regulating both procollagen I and tissue inhibitor of metalloproteinase-1 expression (Parola & Robino, 2001; Zamara *et al.* 2004). In addition, interaction between MDA-modified proteins and the CD36 scavenger receptors on HSC membranes is also capable of stimulating the synthesis of fibronectin and collagen (Schneiderhan *et al.* 2001). Despite the difficulty of reproducing alcoholic fibrosis in animal models of ALD, several studies have shown that detection of MDA and 4-hydroxynonenal in the liver precedes the appearance of the initial signs of fibrosis (Kamimura *et al.* 1992; Kamimura & Tsukamoto, 1995; Niemelä *et al.* 1995) and is associated with transforming growth factor $\beta 1$ production by Kupffer cells (Kamimura & Tsukamoto, 1995). Ethanol-stimulated transforming growth factor $\beta 1$ synthesis is particularly evident in the perivenous regions and is abolished by CYP2E1 inhibition with chlormethiazole (Fang *et al.* 1998). Using the enteral alcohol model Tsukamoto *et al.* (1995) have also observed

that ethanol-induced liver fibrosis is exacerbated by the combined administration of carbonyl Fe. This effect is closely associated with the promotion of MDA and 4-hydroxynonenal formation as well as with an increase in transforming growth factor $\beta 1$ and procollagen- $\alpha - 1$ mRNA expression in both the whole liver and freshly-isolated HSC (Tsukamoto *et al.* 1995). In human subjects elevated titres of antibodies towards lipid peroxidation adducts or oxidized phospholipids are prevalent in heavy drinkers with fibrosis or cirrhosis, irrespective of the magnitude and the duration of alcohol intake (Rolla *et al.* 2000; Mottaran *et al.* 2002). These antibodies are also higher in patients with severe cirrhosis (Child's (Child & Turcotte, 1964) grade B and C) as compared with those with the milder disease (Child's grade A), suggesting the possibility that an immune response involving lipid peroxidation antigens might also have a role in the progression of alcohol-induced liver damage to fibrosis. Furthermore, the presence of high titres of anti-MDA antibodies in patients with non-alcoholic fatty liver disease increases by 3-fold the risk of developing advanced fibrosis or cirrhosis as compared with patients whose antibody titres are within the control range (Albano *et al.* 2005). Although these results are far from being conclusive, they strongly suggest the possible contribution of ethanol-induced oxidative stress to the progression of ALD to liver fibrosis.

Conclusions

In conclusion, experimental and clinical studies not only demonstrate the association between free radical-mediated oxidative damage and alcohol hepatotoxicity, but also begin to unravel the cellular mechanisms involved. Growing evidence indicates that oxidative mitochondrial damage directly causes hepatocyte death and favours alcohol-induced sensitization to the pro-apoptotic action of TNF- α . Moreover, by regulating gene expression oxidative events probably contribute to the development of hepatic fibrosis. Immunological reactions against antigens originating from HER or lipid oxidation products can be regarded as an additional mechanism in the pathogenesis of alcoholic liver injury, as they might contribute to the perpetuation of chronic inflammatory processes. Together these observations provide a rationale for the possible clinical application of antioxidant compounds to reduce the progression of ALD.

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