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Review

Oxidative mechanisms in the pathogenesis of alcoholic liver disease

Emanuele Albano *

Department of Medical Sciences, University "Amedeo Avogadro" of East Piedmont, Via Solaroli 17, 28100 Novara, Italy

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Abstract

Although the capacity of ethanol to induce oxidative stress in the liver is well established, the mechanisms by which oxidative damage contributes to the pathogenesis of alcoholic liver disease (ALD) is still incompletely understood. Recent reports have implicated oxidative mechanisms in the onset of alcoholic steatosis and in the formation of Mallory's bodies. Moreover, by inducing mitochondrial alterations, oxidative stress promotes hepatocyte necrosis and contributes to alcohol-induced sensitization of hepatocyte to the pro-apoptotic action of TNF-α. Oxidative mechanisms play also a role in the progression of liver fibrosis by triggering the release of pro-fibrotic cytokines and activating collagen gene expression in hepatic stellate cells. Finally, immune responses towards antigens originating from the reactions of lipid peroxidation products with hepatic proteins might represent one of the mechanisms that contribute to perpetuate chronic hepatic inflammation in ALD. Altogether these observations give a rationale to the possible clinical application of antioxidants in the therapy of ALD.

Keywords: Ethanol; Lipid peroxidation; Free radicals; Steatosis; Liver fibrosis; Inflammation

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Abbreviations: ALD, alcoholic liver disease; ROS, reactive oxygen species; CYP2E1, cytochrome P4502E1; NO, nitric oxide; mtGSH, mitochondrial GSH; MPT, mitochondria permeability transition; NAFLD, non alcoholic fatty liver disease; IRS-1, insulin receptor substrate protein-1; PPARα, proliferator-activated receptor-α; SREBP-1, sterol regulatory element binding protein 1; TLR-4, the tool-like receptor 4; NFkB, nuclear transcription factor kB; TNF-R1, TNF-receptor 1; ERK1/2, extracellular signal-regulated kinases 1/2; ASK-1, apoptosis signalling kinase-1; PI3K/PKB, phosphatidylinositol-3-kinase/protein kinase B; MDA, malonildialdehyde; MAA, malonildialdehyde–acetaldehyde adducts; aPL, anti-phospholipid antibodies; HCS, hepatic stellate cells.

* Tel.: +39 0321 660642; fax: +39 0321 620421.
E-mail address: albano@med.unipmn.it

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1. Introduction

Alcoholic liver disease (ALD) is a common consequence of long-term alcohol abuse and represents a major cause of morbidity and mortality worldwide. ALD encompasses a broad spectrum of morphological features ranging from steatosis with minimal injury to more advanced liver damage, including steato-hepatitis and fibrosis/cirrhosis. Moreover, approximately 15% of the patients with established alcoholic cirrhosis develop hepatocellular carcinoma (Yip and Burt, 2006). It is now well accepted that the progression of ALD to more severe disease is a multifactorial process that involves a number of genetic, nutritional and environmental factors (Day, 2006). Among the mechanisms implicated in the pathogenesis of ALD free radical-mediated oxidative stress has received growing interest. This review focalize on the most recent insides concerning the contribution of oxidative mechanisms to liver damage by alcohol.

The involvement of oxidative injury in ethanol toxicity has emerged from a number of reports showing that alcohol-fed experimental animals as well as in patients with ALD have increased liver and blood content of lipid peroxidation products (see Albano, 2006 for review). However, the most convincing evidences for a role of oxidative damage in the pathogenesis of alcohol-induced liver injury came from experiment using the enteral alcohol-feeding procedure. This experimental model has shown that preventing lipid peroxidation by modulating the dietary content of unsaturated fatty acids or by the animal supplementation with antioxidants or inhibitors of free radical generation reduces focal necrosis and inflammation and, in some case, ameliorates steatosis (Arteel, 2003; Nanji, 2004 for review). Oxidative stress associated with alcohol toxicity is mainly caused by reactive oxygen species (ROS) generated by the mitochondrial respiratory chain, the ethanol metabolizing cytochrome P4502E1 (CYP2E1) of hepatocytes and the NADPH oxidase of Kupffer cells and liver infiltrating granulocytes. Nonetheless, hydroxyethyl radicals produced during ethanol oxidation by CYP2E1 and nitric oxide (NO) originating from Kupffer cell NO synthetase are also involved in causing oxidative injury (see Albano, 2006; Dey and Cederbaum, 2006 for review). In addition, an impairment of liver antioxidant defenses and alterations of hepatic iron homeostasis may further contribute to oxidative damage associated to alcohol hepatotoxicity (Albano, 2006). In this latter respect, recent evidence indicates that oxidative stress induced by ethanol is responsible for promoting hepatic iron accumulation by down-modulating the liver expression of hepcidin (Harrison-Findik et al., 2006), a 25 amino acid peptide that regulates the absorption and the body distribution of iron (Ganz and Nemeth, 2006).

2. Mechanisms of free radical damage in alcoholic liver disease

2.1. Oxidative mechanisms in mitochondrial damage and hepatocyte apoptosis

Morphological and functional abnormalities of mitochondria represent one of the earliest manifestations of hepatocyte injury by alcohol (Yip and Burt, 2006). Ethanol promotes ROS formation within the mitochondria and decreases mitochondrial GSH (mtGSH) content making these organelles more susceptible to oxidative damage (Bailey and Cunningham, 2002; Fernandez-Checa and Kaplowitz, 2005). Ethanol-stimulated lipid peroxidation is linked to the impairment of mitochondrial oxidative phosphorylations (Bailey and Cunning-

ham, 2002). Moreover, alcohol-treated rats show oxidative modifications of mitochondrial DNA (mtDNA) (Hoek et al., 2002). Single or multiple deletions of mtDNA are also eightfold more frequent in the liver of alcoholic patients as compared to age-matched controls (Mansouri et al., 1997). The alterations of mtDNA are responsible for the lowering of mitochondrially-encoded sub-units of the electron transport chain observed following chronic ethanol treatment and contribute to the impairment of the hepatic respiratory activity caused by alcohol. Oxidative mitochondrial damage is a recognized cause of the collapse of the mitochondrial membrane potential and the onset of mitochondria permeability transition (MPT) (Hoek et al., 2002). According to Adachi et al. (2004) ethanol-induced oxidative stress promotes MTP by favouring Bax translocation to the mitochondria. Extensive MPT leads to mitochondrial swelling and precipitates hepatocyte necrosis, while, by releasing cytochrome c, transient MPT triggers apoptosis (Green and Kroemer, 2004). HepG2 cells over-expressing human CYP2E1 gene and cultured rat hepatocytes exposed *in vitro* to ethanol undergo MTP and apoptosis (Adachi and Ishii, 2002; Caro and Cederbaum, 2004). In these experimental settings, ethanol-induced apoptosis is prevented by antioxidants, the MTP inhibitor cyclosporine and by the over-expression of the anti-apoptotic protein Bcl-2 (Adachi and Ishii, 2002; Caro and Cederbaum, 2004). Thus, oxidative mitochondrial damage can be regarded as one of the mechanisms responsible for hepatocyte apoptosis/necrosis in ALD. Interestingly, *S*-adenosylmethionine supplementation prevents oxidative alterations of mitochondria in alcohol-treated rats (Bailey et al., 2006), suggesting a possible rationale for the use of this compound in the therapy of ALD.

2.2. Oxidative stress in lipid and protein metabolism: role in alcohol-induced steatosis and Mallory's bodies formation

Intracellular accumulation of lipids is the most frequent liver lesion in heavy drinkers. Alcoholic fatty liver has long been considered benign. However, increasing evidence supports the idea that steatosis may contribute to the progression of hepatic injury (Powel et al., 2005). Although the supplementation with antioxidants ameliorates steatosis in the enteral alcohol feeding model (Arteel, 2003), the actual contribution of oxidative mechanisms is still incompletely clarified. The impairment of mitochondrial lipid oxidation has been proposed as one of the mechanisms responsible for fat accumulation (Pessayre and Fromenty, 2005). Deletions of mtDNA show a very high prevalence (about 85%) in alcoholics with hepatic microvesicular steatosis (Fromenty et al., 1995), a lesion that is ascribed to impaired mitochondrial β-oxidation of fatty acids. Pan et al. (2004) have recently reported that lipid peroxidation reduces lipoprotein secretion by enhancing the intrahepatic degradation of newly synthesized ApoB100. Such an effect, in combination with oxidative alterations of lipoprotein glycosylation in the Golgi apparatus (Albano, 2006), might contribute to macrovesicular alcoholic steatosis. Growing evidence from studies concerning non-alcoholic fatty liver disease (NAFLD) indicates that insulin resistance is important in the pathogenesis of hepatic steatosis (Bugianesi et al., 2005). In this contest, the activation of stress-responsive kinases by reactive oxygen species has been shown to impair the correct transduction of insulin-mediated signals through the induction of serine/threonine phosphorylation of the insulin receptor substrate protein-1 (IRS-1) and the concomitant down-modulation of IRS-1 tyrosine phosphorylation (Houstis et al., 2006; Bloch-Damti and Bashan, 2005). IRS-1 serine/threonine phosphorylation is also evident in cell over-expressing CYP2E1 (Shattemberg et al., 2005), suggesting a possible link between alcohol-mediated oxidative stress and hepatic insulin resistance. Nonetheless, it can not be excluded that oxidative stress might also interfere with the regulation of lipid synthesis by the peroxisome proliferator-activated receptor-α (PPARα) and the sterol regulatory element binding protein 1 (SREBP-1) (Crabb and Liangpunsakul, 2006).

Several studies have reported that alcohol affects the catabolism of hepatic proteins by interfering with their degradation by the proteasome (Donohue, 2002). CYP2E1-mediated ROS production and protein alkylation by lipid peroxidation products are important in this respect (Dey and Cederbaum, 2006). An elegant works by French's group (Bardag-Gorce et al., 2006) has shown that one of the consequences of oxidative proteasome impairment in CYP2E1 expressing Hep2 cells is the formation of insoluble protein aggregates containing cytokeratins 8 and 18. This indicates a possible contribution of oxidative mechanisms in the generation of Mallory's bodies that are a one of the features of alcoholic hepatitis (Yip and Burt, 2006) and characteristically contain cytokeratins 8 and 18.

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

The importance of inflammation in the pathogenesis of ALD is now well recognized (Hines and Wheeler, 2004). Current view suggests that ethanol ingestion increases the translocation of gut-derived endotoxins to the portal circulation where they stimulate intrahepatic Kupffer cells through the interaction with CD14 and the tool-like receptor 4 (TLR-4) (Rao et al., 2004). Activated Kupffer are then responsible the release of pro-inflammatory cytokines/chemokines, particularly TNF-α, eicosanoids, ROS and NO (Hines and Wheeler, 2004). According to this interpretation, the administration of antibiotics to reduce endotoxemia or the inactivation of Kupffer cells with gadolinium chloride prevent liver injury in enteral alcohol-fed rats. CD14 knockout or TLR-4-deficient mice produces less TNF-α and are resistant to alcohol toxicity (Hines and Wheeler, 2004). Kupffer cell activation and liver infiltration by neutrophyls significantly contribute to oxidative damage in ALD (Jaeschke, 2002; Arteel, 2003). On their turn, ROS produced by CYP2E1 and NADPH oxidase promote the activation of the nuclear transcription factor kB (NFkB) and the phosphorylation of the stress activated kinases ERK1/2 and p38 MAPK that amplify Kupffer cell production of TNF-α in response to endotoxin (Nagy, 2003; Cao et al., 2005; Thakur et al., 2006). Conversely, the block of CYP2E1 induction with chlormethiazole or the supplementation with the phenolic antioxidant curcumin reduce ethanol-stimulated mRNA expression of pro-inflammatory cytokine/chemokines in alcohol-fed rats (Fang et al., 1998; Nanji et al., 2003).

The implication of TNF-α as cause of alcohol hepatotoxicity steams from the observations that rats receiving anti-TNF-α antibodies as well as TNF-receptor 1 (TNF-R1) knockout mice are protected against liver damage induced by enteral alcohol administration (Hines and Wheeler, 2004). Hepatocytes, as many other cells, are resistant to the pro-apoptotic action TNF-α because TNF-α induces survival signals involving the transcription of NFkB-dependent genes and the activation of protein kinase cascades (Schwabe and Brenner, 2006). However, HepG2 cells over-expressing CYP2E1 are sensitive to TNF-α, while the selective depletion of mtGSH has been associated with the increased susceptibility to TNF-α killing of hepatocytes obtained from chronically ethanol-fed rats (Hoek and Pastorino, 2004; Fernandez-Checa and Kaplowitz, 2005). This indicates that ethanol-induced oxidative stress alters the balance between the pro- and anti-apoptotic signals triggered by TNF-α. As pointed out by Hoek and Pastorino (2004), an increased susceptibility of mitochondria to TNF-α-induced MPT as well as an enhanced activation of the apoptosis signalling kinase-1 (ASK-1) upon the oxidation of its binding proteins thioredoxin might contribute in enhancing hepatocyte susceptibility to apoptosis by TNF-α. Moreover, Sampey et al. (2007) have recently reported that the lipid peroxidation products 4-hydroxynonenal affects the activity and the nuclear translocation of hepatocyte extracellular signal-regulated kinases 1/2, that are responsible for trasducing cell survival signals. Oxidative stress might also contribute to ethanol-mediated down-modulation of other anti-apoptotic signals involving phosphatidylinositol-3-kinase/protein kinase B (PI3K/PKB) (Shulga et al., 2005). Altogether, these observations indicate that the interplay between oxidative stress and TNF-α might represent an important cause for the extensive hepatocyte apoptosis that characterizes alcoholic hepatitis (Natori et al., 2001).

2.4. Free radical mechanisms in immune reactions associated with alcoholic liver disease

The increase in circulating endotoxins might not be the only factor by which alcohol fuels chronic hepatic inflammation, as enteral alcohol-fed rats show liver injury, inflammation and increased TNF-α mRNA expression even in the absence of appreciable elevations in plasma endotoxins (Ronis et al., 2004). Moreover, chronic administration of ethanol in combination with endotoxins fails to increase alcohol hepatoxicity (Jarvelainen et al., 1999). The immune system is known to have a key role in regulating inflammatory processes associated to chronic liver diseases (Kita et al., 2001). Histology reveals that liver infiltrates contains both $CD8^+$ and $CD4^+$ T lymphocytes are detectable in about 40% of ALD patients and correlates with the extension of intralobular inflammation peacemeal necrosis and septal fibrosis (Chedid et al., 1993; Colombat et al., 2002). Liver-associated lymphocytes isolated from alcohol consuming rats have an increased capacity to secrete pro-inflammatory cytokines (Batey et al., 2002), suggesting their possible contribution to orchestrate inflammation during the evolution of ALD. The possible role of oxidative stress in promoting immune reactions in ALD has first emerged from the observation that antibodies specifically recognizing hydroxyethyl

radical-derived epitopes are detectable in chronically ethanol-fed rats as well as in ALD patients (Albano, 2006). More recently, we have reported that elevated titres of circulating IgG towards epitopes derived from the modification of proteins by lipid peroxidation products such as malonildialdehyde (MDA), 4-hydoxynonenal and oxidized arachidonic acid are also prevalent in patients with advanced ALD (55–70%), as compared to subjects with fatty liver only (8–13%) (Mottaran et al., 2002). In addition, the reaction of MDA and acetaldehyde with protein lysine residues generates condensation products, named malonildialdehyde–acetaldehyde adducts (MAA), that are also antigenic (Thiele et al., 2004) and stimulate immune reactions in ALD patients (Rolla et al., 2000). About 35% of the patients with advanced ALD also show $CD4^+$ T-lymphocyte responses towards malonildialdehyde-derived antigens, indicating that oxidative mechanisms promotes both humoral and cellular immune responses (Stewart et al., 2004). Beside developing antibodies directed against allo-antigens, ALD patients not rarely have signs of auto-immune reactions (see McFarlane, 2000 for review). Anti-phospholipid antibodies (aPL) are among the auto-antibodies most frequently associated with ALD, being detectable in up to 80% of patients with alcoholic hepatitis or cirrhosis (McFarlane, 2000). We have observed that aPL in ALD patients specifically target oxidized phospholipids, namely oxidized cardiolipin and phosphatidylserine (Rolla et al., 2001; Vay et al., 2006). These auto-antibodies are particularly interesting because recognize apoptotic, but not to living, cells by specifically targeting oxidized phosphatidylserine expressed on the cell surface (Vay et al., 2006). Such a specificity is consistent with recent reports showing that phosphatidylserine is oxidized during apoptosis and is exposed on the outer layer of cell plasma membranes (Kagan et al., 2003). According to recent evidence, the phagocytosis of apoptotic bodies is important in the termination of inflammation because stimulates macrophages to secrete the anti-inflammatory cytokines TGF-β1 and IL-10 (Gregory and Devitt, 2004). Oxidized phosphatidylserine on the surface of apoptotic cells is a key signal for the recognition of apoptotic bodies (Greemberg et al., 2006). Thus, we propose that the hiding of these recognition sites by aPL targeting oxidized phosphatidylserine (Vay et al., 2006) might impair anti-inflammatory responses associated with the clearance of apoptotic hepatocytes. Accordingly, in alcoholic hepatitis, apoptotic hepatocytes co-localize with granulocyte infiltration (Jaeschke, 2002). The actual contribution of oxidative stress-mediated immune responses to maintain inflammation in ALD is supported by the observation that, in an endotoxin-free enteral alcohol feeding model, lipid peroxidation derived antibodies are associated with both the TNF-α mRNA expression and the extent of inflammatory infiltrates, while the supplementation with *N*-acetylcysteine reduces hepatic inflammation and the immune response triggered by lipid peroxidation (Ronis et al., 2005). Moreover, among heavy drinkers the prevalence of elevated serum TNF-α is fivefold higher in the subjects with oxidative stress-induced IgG than in those with these antibodies within the control range (Vidali et al., 2007 submitted for publication).

2.5. Oxidative mechanisms in the onset of alcohol-induced liver fibrosis

Liver cirrhosis represents the terminal stage of ALD and one of the main cause of death among alcohol abusers. In spite the difficulty to reproduce alcoholic fibrosis in the animal models of ALD, several studies have shown that liver lipid peroxidation precedes the initial signs of fibrosis and is associated with increased production of the pro-fibrogenic cytokine TGF-β1 by Kupffer cells (Tsukamoto and Lu, 2001). Moreover, Nieto (2007) has recently reported that ethanol-induced lipid peroxidation trigger the NFkB transactivation of collagen α2(1) gene promoter in hepatic stellate cells (HCS) through the stimulation of kinase cascade involving PKC, PI3K and PKB/Akt. These observations are consistent with the role of 4-hydroxynonenal as a pro-fibrogenic stimulus for collagen production in human HSC (Parola and Robino, 2001) as well as with *in vitro* studies showing that oxidative stress directly promotes collagen synthesis in HCS over-expressing the CYP2E1 gene (Caro and Cederbaum, 2004). In humans we have observed that oxidative stress-induced immune responses are prevalent in heavy drinkers with fibrosis/cirrhosis, irrespective of the magnitude and the duration of alcohol intake (Rolla et al., 2000; Mottaran et al., 2002). Patients with non-alcoholic fatty liver disease (NALFD) with high titres of anti-MDA antibodies also show a threefold increased risk of developing advanced fibrosis/cirrhosis than patients whit these antibody within the control range (Albano et al., 2005), suggesting that immune responses towards lipid peroxidation antigens might have a role in the progression of fibrosis. Such a possibility is consistent with a recent report showing that lymphocyte destruction by sub-letal irradiation reduces liver fibrosis in mice treated with CCl_4 or thioacetamide, while the transfer of

$CD8^+$ lymphocytes from CCl_4-treated mice to immunodeficient SCID mice leads to the fibrogenic activation of HCS (Safadi et al., 2004).

3. Conclusions

In conclusion, experimental and clinical studies increasingly show that the oxidative damage induced by ethanol contribute in many ways to the pathogenesis of alcohol hepatoxicity. Oxidative stress-mediated interference with mitochondrial functions, protein degradation, insulin signalling and lipoprotein secretion likely contribute to the development of alcoholic steatosis. Moreover, oxidative mitochondrial damage represents a directly cause hepatocyte death and favours alcohol-induced sensitization to the pro-apoptotic action of TNF-α. By modulating gene expression in hepatic stellate cells, oxidative stress play also a role in the progression of fibrosis. Immunological reactions against antigens originating from hydroxyethyl free radical or lipid oxidation products can be regarded as an additional mechanism in the pathogenesis of alcoholic liver injury, as they might contribute to perpetuate chronic inflammation associated with ALD. Altogether these observations give a rationale to the possible clinical application of antioxidant therapies to reduce the progression of ALD.

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