Chapter 10
Oxidative Stress in Alcoholic Liver Disease

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10.1 Introduction

The excessive consumption of alcoholic beverages is an important cause of death and disease worldwide. In developed countries alcohol-related mortality is estimated to range from 7.9 to 14.3 per 100.000 habitants representing the third cause of death [1]. However, in the recent years alcohol abuse has become a leading cause of disease also in the developing countries of Central and South America and East Asia [2]. Although ethanol can damage several organs, alcoholic liver disease (ALD) is the most common medical consequence of excessive alcohol intake. ALD accounts for 8 % of newly diagnosed liver diseases and for more than 50 % of chronic liver diseases [3]. In addition, heavy alcohol consumption is often a comorbility factor in hepatic injury caused by viral infections or by metabolic disorders [4, 5]. ALD encompasses a broad spectrum of histological features ranging from lipid accumulation within the hepatocytes (fatty liver or steatosis) with minimal parenchymal injury to more advanced liver damage, including steatohepatitis and fibrosis/cirrhosis. Almost all heavy drinkers develop steatosis with variable degree of necro-inflammation; however, overt alcoholic hepatitis is diagnosed in only 10–35 % of cases [5]. The progression of ALD to alcoholic cirrhosis is evident in about 8–20 % of alcohol abusers [5]. Even so, only in US alcohol accounts for about 44 % of the 30,000 deaths caused every year by hepatic cirrhosis [2]. Similar figures hold for Europe, where the prevalence of alcoholic cirrhosis in the different countries correlates quite well to the national per capita alcohol consumption [6, 7]. At the individual level, the risk of developing ALD shows a dose–effect relationship with daily alcohol intake [8]. However, such an association is not linear as only 6 %

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of the subjects consuming more than 80–90 g of ethanol a day show clinical sings of cirrhosis [9]. Moreover, prospective studies in heavy drinkers suggest that the mortality for alcoholic cirrhosis has a threshold, but not a dose–response effect [10]. It is also noteworthy that chronic alcohol consumption increases by about fivefold the risk of hepatocellular carcinoma (HCC) and that about 15 % of the patients with alcoholic cirrhosis develop HCC [11, 12]. At present, the reasons for the large inter-individual variability in the risk of alcohol-induced liver injury are still poorly characterized. A gender difference is well evident with women being twice more sensitive to alcohol-mediated hepatotoxicity than men. They develop ALD at lower intake of alcohol and with shorter duration of consumption as compared to males [5]. Ethnic differences are also appreciable with African–Americans and Hispanic males showing incidence rates of alcoholic cirrhosis higher than Caucasian males, irrespective of the alcohol intake [5]. Thus, the duration of alcohol abuse, the drinking patterns, the type of alcoholic beverages along with metabolic and genetic factors likely influence the risk of ALD progression independently from the amount of ethanol consumed [5, 13]. Furthermore, in spite of the fact that from the clinical point of view steatosis, steatohepatitis, and fibrosis/cirrhosis represent the evolution of alcohol-induced hepatic injury, it is increasingly clear that the mechanisms leading to each of these lesions can be quite different.

10.2   Ethanol Metabolism in the Liver

Ethanol is largely (90–98 %) metabolized in the liver. Such a specificity justifies why ethanol toxicity mostly involves this organ. In human hepatocytes ethanol is converted to acetaldehyde by the action of alcohol dehydrogenase (ADH), microsomal cytochrome P450, and to minor extent by catalase [14, 15]. The major pathway for hepatic ethanol oxidation involves ADH, a NAD-dependent zinc metalloenzyme present in human tissues in five different iso-enzyme classes [14, 15]. These enzymes originate from the association of eight different subunits into active dimeric molecules. The hepatic alcohol metabolism largely relies on the Class I ADH (ADH1A/B/C) that has high affinity for ethanol. Microsomal ethanol oxidation mainly relies on the action of cytochrome P4502E1 isoenzyme (CYP2E1) with a minor contribution of other isoforms (CYP1A2 and CYP3A4) [14]. CYP2E1 is mainly expressed in centrilobular hepatocytes, but small amounts are detectable also in hepatic macrophages obtained from alcohol-fed rats [16]. CYP2E1 K_m for ethanol is about tenfold higher than that of ADH, and at low alcohol concentration, microsomal ethanol oxidation accounts for about 10 % of the overall alcohol elimination [16]. However, chronic alcohol exposure increases by 5- to 20-fold CYP2E1 activity through both enzyme stabilization and increased gene expression [16]. Thus, CYP2E1 may have a major role in ethanol elimination in alcohol abusers. However, the evaluation of CYP2E1 activity in humans through the measure of the oxidation of the myorelaxant drug chlorzoxazone reveals that an appreciable CYP2E1 up-regulation occurs already following moderate alcohol consumption [17]. There is also a large inter-individual variability in CYP2E1 induction with a minority of heavy drinkers who

do not show appreciable increase in CYP2E1 activity [18]. Recent evidence indicates that CYP2E1 induction not only involves the microsomal form of the enzyme, but also two CYP2E1 variants that are present in the mitochondrial matrix (mtCYP2E1s). These mtCYP2E1s consist of a highly phosphorylated form and a truncated form lacking of the hydrophobic part at the NH_2-terminus [16]. At difference from the microsomal CYP2E1 that uses NADPH and cytochrome P450 reductase for electron transfer, the electron suppliers of mtCYP2E1s are adrenodoxin and adrenodoxin reductase [16]. Overall, mtCYP2E1s account for 30–40 % of the microsomal enzyme activity, and because of their localization, have important implications in alcohol hepatotoxicity [16]. Finally, a small fraction of CYP2E1 (about 10 % or the microsomal content) is also expressed on the outer layer of the hepatocyte plasma membranes, where it is transported from the Golgi apparatus via the secretory vesicles [19]. Plasma membrane CYP2E1 is catalytically active, but its importance in alcohol toxicity mainly relies on the fact that it is the target for allo- and auto-immune reactions (see below). Finally, catalase has been implicated in ethanol oxidation [14, 15]. Although such a reaction might have a role in brain alcohol metabolism, its role in human liver appears to be negligible [14].

Acetaldehyde that originates from ethanol oxidation is largely detoxified by the action of NAD-dependent aldehyde dehydrogenases (ALDH) with the formation of acetate. ALDHs are tetramers or dimers formed by the same subunits [20]. Among the 19 ALDHs characterized in humans, only a few are involved in acetaldehyde oxidation, and of these, the mitochondrial ALDH2 and the cytosolic ALDH1A are the most effective. These enzymes are highly efficient (Km values of about 1 M) and detoxify more than 99 % of the acetaldehyde generated within the hepatocytes [20]. However, low amounts of acetaldehyde can escape detoxification interacting with proteins and nucleic acids [20]. Acetaldehyde binding to DNA is regarded as an important factor in the carcinogenic effects of alcohol [12]. It is noteworthy that ALDHs play also an important role in the detoxification of a variety of aldehydic compounds originating from the peroxidation of unsaturated lipids during oxidative stress [21].

During acute ethanol intoxication, large amount of NADH are generated and the increased NADH/NAD ratio can affect hepatic enzymes involved in lipid, carbohydrate, and uric acid metabolism [4]. In particular, the excess of NADH decreases fatty acid oxidation and enhances lipogenesis leading to triglyceride accumulation within the hepatocytes, while the block of pyruvate conversion to glucose increases lactic acid production causing alcoholic hypoglycaemia and acidosis [4]. These metabolic disturbances are, however, rapidly reversible and seem to be attenuated during chronic ethanol ingestion.

10.3   Oxidative Stress in the Pathogenesis of ALD

It is now widely accepted that ALD has a multifactorial pathogenesis and that the disease evolution is the result of the interaction between many factors including oxidative damage, metabolic disturbances, endoplasmic reticulum stress, and

inflammatory responses. The role of oxidative stress in alcohol liver injury has received attention since the early 1960s when Di Luzio first reported the detection of lipid peroxidation in the livers of alcohol-treated rats [22]. During the following three decades, the capacity of ethanol to trigger oxidative stress was further supported by the demonstration that ethanol increases the hepatic production of free radical species in both parenchimal and non-parenchimal cells and by the detection of oxidative modifications in hepatic constituents following both acute and chronic ethanol administration to experimental animals [23, 24]. In line with these findings, many reports have demonstrated an increase in lipid peroxidation products, protein carbonyls, and other oxidative stress biomarkers in both the liver and the serum of patients with alcohol abuse, particularly in those with advanced ALD [25–28]. The relevance of these observations to the human disease has also been confirmed by the immunohistochemical detection of proteins adducted by lipid peroxidation products in liver biopsies from alcohol abusers where these adducts are particularly evident in the areas of focal necrosis and around fibrotic septa [29]. However, rodent aversion for alcohol has hampered for many years the possibility to verify experimentally the actual role of oxidative stress in the pathogenesis ALD and only the introduction of rodent enteral nutrition with an alcohol-containing liquid diet has now partially overcome this limitation. By using this experimental approach, it has been possible to show that rats receiving ethanol in combination with a diet rich in highly oxidizable unsaturated fatty acids from corn or fish oil develop extensive hepatic lipid peroxidation and parenchymal damage [30, 31], while minor effects are evident when a similar amount of ethanol is given together with poorly oxidizable medium chain triglycerides [32]. Furthermore, replacing fish oil with less-oxidizable palm oil while continuing ethanol administration improves oxidative stress and ameliorates already established liver damage [33]. On the same line, several other studies have demonstrated that supplementing alcohol-fed rodents with a variety of antioxidants and free radical scavengers reduces oxidative stress and hepatic injury [34–36]. Protection against oxidative stress and hepatotoxicity caused by the enteral alcohol administration is also evident in mice over-expressing antioxidant enzymes such as cytosolic (Cu–Zn)-superoxide dismutase (SOD-1) or mitochondrial (Mn)-superoxide dismutase (SOD-2) [37, 38]. Conversely, SOD-1 knockout mice show increased lipid peroxidation, extensive centrilobular necrosis, and inflammation upon moderate ethanol consumption [39]. In a similar manner, mice deficient for the transcription factor erythroid-2-related factor (Nfr2) which regulates the gene expression of antioxidant enzymes are more sensitive to alcohol hepatotoxicity undergoing extensive liver injury and an increased mortality when exposed to low amounts of ethanol otherwise well-tolerated by wild-type animals [40].

10.4 Mechanisms Responsible for Alcohol-Induced Oxidative Stress in the Liver

The occurrence of oxidative stress during ethanol intake is the result of the combined action of alcohol in increasing the generation of oxidizing species such as reactive oxygen species (ROS) and nitric oxide (NO) and in lowering

intracellular antioxidant defenses [12]. In addition, ethanol itself can generate free radical intermediates, known as hydroxyethyl-free radicals.

10.4.1   Reactive Oxygen Species

During alcohol intake the mitochondrial respiratory chain and CYP2E1-dependent microsomal monoxygenase system are the main intracellular sources of ROS in hepatocytes, while extracellular ROS generated by activated inflammatory cells also significantly contribute to the pro-oxidant action.

The capacity of ethanol to promote ROS generation by the mitochondria has emerged from the observation that both acute and chronic ethanol exposure increase the oxidation of mitochondrial proteins and DNA (mtDNA) and deplete the organelle pool of reduced glutathione (mtGSH) [39, 40]. During acute ethanol intoxication, an excess of O_2^- results from an enhanced leakage of electrons from complexes I and III of the mitochondrial respiratory chain in relation to an increased availability of NADH [41, 42]. Conversely, the stimulation in mitochondrial ROS production that characterizes chronic alcohol administration is the consequence of an impaired synthesis of mitochondria-encoded constituents of the respiratory chain [41, 42]. Indeed, single or multiple mtDNA deletions are frequent in the liver of alcohol-treated rats as well as hepatic biopsies from alcoholic patients [42]. On their turn, these mtDNA alterations contribute to the selective lowering of mitochondrial respiratory chain components evident in alcohol-treated rodents [43]. Additional mechanisms that can contribute to mitochondrial ROS generation during alcohol exposure can involve the interaction of the complexes II and III with N-acetylsphingosine (C_2-ceramide) released by hepatocytes in response to tumor necrosis factor α (TNF-α) [44] and the development of hepatic hypoxia.

As mentioned above, CYP2E1-dependent microsomal monoxygenase system represents an important biotransformation pathway of ethanol, particularly in the presence of high alcohol intake [16]. CYP2E1 has a especially high NADPH oxidase activity leading to the production of large quantities of O_2^- and H_2O_2. In liver microsomes from either humans or alcohol-fed rodents, CYP2E1 content positively correlates with NADPH-oxidase activity as well as with the extent of lipid peroxidation. Recent studies have also implicated mitochondrial CYP2E1s (mtCYP2E1s) as additional sources of ROS [16, 45]. In fact, cells over-expressing mtCYP2E1s undergo oxidative damages upon ethanol exposure [46]. Thus, the high efficiency of CYP2E1 in reducing oxygen to O_2^- and H_2O_2 is regarded as key contributor to oxidative stress during chronic exposure to alcohol. Indeed, the addition of ethanol to HepG2 hepatoma cells stably transfected with the *CYP2E1* gene increases ROS production and causes oxidative stress-mediated cell injury [16]. In these cells as well as in the liver of heavy drinkers, CYP2E1 levels also correlate with the amounts of DNA adducts with the lipid peroxidation products 4-hydroxynonenal (4-HNE) [16, 47]. These 4-HNE-DNA adducts involve the codon 249 of human p53 gene, a unique mutational hot spot in hepatocellular carcinoma [48], suggesting CYP2E1-

dependent oxidative injury as a relevant factor in alcohol-induced hepatocarcinogenesis. In line with these findings, experiments performed using enteral alcohol-fed rats have demonstrated that the induction of CYP2E1 by ethanol enhances hepatic lipid peroxidation, while compounds interfering with CYP2E1 expression significantly decrease oxidative stress and hepatic damage [49]. However, CYP2E1 knockout mice are not protected from alcohol toxicity [50] in spite of the fact that protein carbonyls and oxidized DNA products are lower in CYP2E1-null mice than in wild-type animals [51]. Such a discrepancy can be explained considering that, differently from rats and humans, CYP2E1 represents less than 5 % of the total hepatic cytochrome P450 content in ethanol-fed mice making its contribution to alcohol toxicity easily blunted by other factors [51]. On the other hand, ethanol administration to CYP2E1 transgenic mice over-expressing CYP2E1 causes more liver injury than in naïve mice and this effect is associated with a potentiation of oxidative damage and centrilobular hypoxia [52]. Interestingly, following alcohol feeding mice expressing human instead of murine CYP2E1 show higher hepatic CYP2E1 content and increased oxidative stress and hepatic injury than similarly treated wild-type mice [53], confirming the importance of the monoxygenase system in alcohol hepatotoxicity.

As discussed in the next paragraph, inflammation plays an important role in alcohol hepatotoxicity. Lobular inflammation associated to both acute and chronic alcohol intoxication is an important source of extracellular ROS that are generated by Kupffer cells and liver-infiltrating leucocytes through the activation of NADPH oxidase. Phagocyte NADPH oxidase is a multimeric transmembrane enzymatic complex that comprises the catalytic subunit (NOX2), regulatory subunits (p22^{phox}, p40^{phox}, and p47^{phox}), and the Rho small GTPase Rac1/2 [54]. The contribution of phagocyte-derived ROS to alcohol-induced oxidative stress has emerged from the observation that macrophage depletion with gadolinium chloride lowers the hepatic production of O_2^- following ethanol infusion and decreases both liver injury and lipid peroxidation markers in chronic enteral alcohol-fed rats [55]. A similar protection is also evident in mice knockout for NADPH oxidase p47^{phox} [56] or in animals deficient for ICAM-1, an endothelial adhesion molecule required for the recruitment of leucocytes into inflammatory sites [57]. Nonetheless, following alcohol administration, the extent of DNA oxidation induced is comparable in both p47^{phox} knockout and naïve mice, whereas CYP2E1-null mice are instead protected from such damages [51]. Altogether, these results indicate a different contribution of intracellular and extracellular ROS sources in ethanol-induced oxidative stress.

10.4.2   Hydroxyethyl-Free Radicals

One peculiarity of CYP2E1-mediated alcohol metabolism is represented by the conversion of ethanol itself to 1-hydroxyethyl-free radical (CH$_3$C$^\bullet$HOH; HER) [58]. The mechanisms responsible for HER formation involve the presence of ferric-CYP2E1-oxygen complexes as well as of hydroxyl radicals [58]. HERs are

produced by rat liver microsomes at a rate 10 times lower than the ethanol conversion to acetaldehyde [59]. Nonetheless, because of their high reactivity, they are mainly responsible for the alkylation of hepatic constituents including CYP2E1 itself [58]. One of the consequences of protein alkylation by HER is the stimulation of an immune response characterized by the generation of antibodies specifically recognizing HER-derived epitopes [59]. These antibodies are detectable in the sera of both chronically ethanol-fed rats and patients with ALD where they strictly correlate with CYP2E1 activity [58, 60], indicating that HER generation actually takes place during "in vivo" alcohol metabolism in humans. Interestingly, heavy drinkers who do not display CYP2E1 induction have titers of anti-HER IgG comparable to non-drinking controls and significantly lower than drinkers with normally induced CYP2E1 activity [18].

10.4.3   Nitric Oxide

An additional contribution to alcohol-induced oxidative stress may originate from the interaction of nitric oxide (NO) with O_2^- to generate highly oxidizing peroxynitrite ($ONOO^-$). During alcohol intoxication, NO is mainly generated in activated phagocytes by inducible NO synthetase (iNOS) that increases by threefold following chronic ethanol exposure [61]. Accordingly, signs of ONOO-mediated nitrosative stress are evident in alcohol-treated rodents [62]. One of the targets of the increased NO formation are mitochondria that shows a NO-dependent decrease in the respiratory chain functions [63]. Consistently, the selective iNOS inhibitor N-(3-aminomethyl)benzyl-acetamindine (1,400 W) or iNOS genetic deficiency reduce oxidative stress, mitochondrial respiratory impairment, and hepatic injury induced by chronic alcohol feeding [34]. Nonetheless, the actual role of NO in the pathogenesis of alcohol-mediated oxidative stress awaits further investigations, as the treatment with the non-selective NO-inhibitor, N-nitro-L-arginine methyl ester (L-NAME), worsens alcohol liver damage [64]. This suggests that NO generated by phagocyte iNOS might be harmful in ALD, while NO produced by endothelial (eNOS) and possibly other NO sources can prevent alcoholic injury. According to this view, the stimulation of NO production within the hepatocytes increases their resistance to ethanol-induced oxidative stress by lowering intracellular low molecular weight iron content [65].

10.4.4   Iron Overload

Alteration of iron homeostasis is a well-recognized cofactor in the promotion of oxidative stress in many liver diseases, in relation to the capacity of this metal to stimulate the breakdown of lipid hydroperoxides and to catalyze the generation of hydroxyl radicals. A common feature of chronic alcohol intake in humans is the

accumulation of an excess of iron within the hepatocytes [66]. However, an increased liver iron deposition is also evident even after moderate alcohol consumption [67]. Growing evidence suggests that ethanol induces liver iron overload by interfering with the functions of hepcidin [68], a liver-produced peptide that regulates the circulating iron levels by inhibiting ferroportin-mediated iron release from enterocytes and macrophages [69]. Alcohol-mediated oxidative stress has been proposed to affect hepatocyte hepcidin synthesis by acting on its transcription factor CCAAT/enhancer-binding protein-α (C/EBP-α) [70]. Nonetheless, enhanced activity of hypoxia-inducible factors (HIF-1/2α) as a consequence of hepatocyte hypoxia can also contribute in down-modulating C/EBP-α expression during chronic alcohol intake [71]. This latter effect can be related to CYP2E1 activity as modulation of intrahepatic CYP3E1 content influence in a similar manner liver hypoxia and HIF-1α activity in alcohol-treated mice [72]. In line with these findings, hepatic hepcidin mRNA levels are low in alcoholic patients [73], while ferroportin expression is increased in macrophages from alcohol-exposed rats [74]. In this scenario, low hepatocyte hepcidin production would favor an increased iron release by enterocytes and macrophages and its concomitant storage within the liver. On its turn, an increased hepatic content of intracellular low molecular weight non-protein iron exacerbates ethanol-induced oxidative damage in parenchymal cells of animals receiving alcohol [23] and stimulates macrophage ROS production [74]. Accordingly, iron supplementation of enteral alcohol-fed rats enhances lipid peroxidation and worsens liver pathology [75].

10.4.5 Interference with Liver Antioxidant Defenses

Beside alcohol capacity to stimulate free radical generation, the impairment of liver antioxidant defenses substantially contributes to oxidative injury in ALD. Alcohol action on antioxidant defenses involves a lowering of small molecular weight antioxidants, such as reduced glutathione (GSH) and α-tocopherol (Vitamin E), and an impairment in antioxidant enzymes. Early studies have shown that a decrease in the liver GSH content is common in ethanol-fed animals as well as in alcoholic patients independently from the nutritional status or the degree of liver disease [23]. On the other hand, the stimulation of GSH re-synthesis by rat supplementation with the GSH precursors L-2-oxothiazolidine-4-carboxylic acid or N-acetylcysteine prevents oxidative stress in enteral alcohol-feed rats [36, 76]. Hepatic GSH lowering can be regarded as one of the consequences of the impairment of S-adenosylmethionine (SAMe) production, as SAMe is a precursor of cysteine that is required for GSH synthesis [77]. SAMe is generated in an ATP-dependent reaction catalyzed by the enzyme methionine adenosyltransferase isoenzymes MATI and MATIII [77]. Beside to be a cysteine precursor, SAMe acts as the principal methyl group donor for the methylation reactions involving DNA, RNA biogenic amine, histones, and phospholipids [77]. These latter reactions generate homocysteine that is converted back to methionine by the action of methionine synthetase using methyl groups

supplied by the combined action of methyltetrahydrofolate and vitamin B12 [77]. A lowering of hepatic SAMe content is evident in experimental animals chronically treated with alcohol as well as in patients with alcoholic hepatitis [78]. Alcohol affects SAMe formation either directly by impairing MAT and methionine synthetase activities, or indirectly by interfering with the turnover of folate, a key cofactor in methyl group transfer [78]. Consistently, SAMe administration attenuates alcohol-induced GSH depletion and oxidative injury in rats and mini-pigs [78]. It is noteworthy that during chronic alcohol intake, hepatic GSH depletion mainly involves the mitochondrial pool (mtGSH) of centrilobular hepatocytes [79]. Such a selective mtGSH depletion is partially due to an enhanced mtGSH oxidation in response to mitochondrial ROS production as the expression of mtCYP2E1 in HepG2 cell selectively depletes mtGSH [16]. In addition, defects in the GSH transport from cytosol to the mitochondrial matrix further contribute to affect mtGSH homeostasis. In fact, alcohol increases the hepatic synthesis of cholesterol and cholesterol unbalance in mitochondrial membranes interferes with the activity of GSH carrier proteins [79]. The action of ethanol on mtGSH homeostasis favors oxidative mitochondrial damage and enhances hepatocyte susceptibility to TNF-α-mediated cytotoxicity [79, 80]. Beside the effects on GSH, in both humans and rodents chronic alcohol intake decreases the liver and the plasma levels of vitamin E. Vitamin E depletion contributes to oxidative injury as in humans vitamin E levels inversely correlate with the extent of lipid peroxidation [25]. Moreover, vitamin E-deficient rats show an increased susceptibility to alcohol-induced oxidative stress and hepatotoxicity [81], while upon discontinuation of alcohol feeding the administration of vitamin E reduces the severity of hepatic lesions [32].

The effects of alcohol on antioxidant enzymes are less well-characterized. In one hand, ethanol increases the liver expression of the nuclear factor erythroid-2-related factor (Nfr2), which regulates the expression of antioxidant enzyme genes [81]. Consistently, the mRNA levels of liver glutathione peroxidase and catalase are increased following chronic alcohol administrations [82, 83], whereas Nfr2 deficiency greatly enhances mice susceptibility to alcohol-induced oxidative stress [38]. On the other hand, alcohol hepatotoxicity is associated with a significant decline in the hepatic content and enzymatic activity of (Cu–Zn)-superoxide dismutase (SOD-1), catalase, and glutathione peroxidase and the loss of these antioxidant enzymes inversely correlates with the extent of both lipid peroxidation and hepatic injury [31]. Similarly, mice ethanol feeding rapidly reduces the hepatic content of thioredoxin-1 (Trx-1), a redox-sensitive protein implicated in the reduction of oxidized proteins [33]. Little is known about the mechanisms responsible for such contrasting effects, but it is possible that ethanol might stimulate the intracellular degradation of antioxidant enzymes or interfere with their post-transcriptional regulation. On this latter respect, alcohol feeding in mice has been recently shown to modulate several epigenetic systems in the liver including the expression of microRNAs (miRNAs) that might influence the synthesis regulation of a variety of proteins [84]. Although in rodents manipulations of antioxidant enzymes such as Trx-1, SOD-1, and SOD-2 strongly influence alcohol-induced oxidative injury and hepatotoxicity [35, 37–39], human studies investigating the possible role of genetic polymorphisms

of antioxidant genes in ALD have been inconclusive [13]. One of the most frequent of those genetic variants is a [16]alanine/valine substitution in the leader amino acid sequence (about 25 % prevalence in Caucasians) that is responsible for the mitochondrial localization of SOD-2 [13]. The Ala-SOD-2 variant translocates less efficiently to the mitochondria than the Val-SOD-2 [13], but Ala-SOD-2 does not appear to influence oxidative damage in ALD [85].

10.5 Role of Oxidative Stress in the Pathogenesis of Alcohol-Induced Liver Injury

As previously mentioned, ALD is characterized by a variety of histological lesions including steatosis, hepatocyte death by either necrosis or apoptosis, the presence of Mallory's bodies, lobular inflammation, and fibrosis/cirrhosis that implicate to different extent oxidative stress.

10.5.1 Alcoholic Steatosis

An increase in the hepatic content of triglycerides represents the most common histological and biochemical feature of excessive alcohol intake. Alcoholic steatosis mainly consists in the presence of a medium-sized/large fat droplet in hepatocyte cytoplasm (macrovesicular steatosis) with lateral displacement of the nucleus. Fat accumulation mostly involves all hepatic acinus, but may be prominent in the centrilobular areas [4]. Microvesicular steatosis, consisting in liver cell filling by small fat droplets, is relatively rare in ALD (0.8–2.3 %), but it is often associated with a more severe evolution of the hepatic injury [4]. Although steatosis is mostly asintomatic and often reversible, it is presently regarded as an important contributor in the progression of liver damage to fibrosis [86]. Alcoholic steatosis results from the combination of increased triglyceride synthesis, lowered fatty acids oxidation, and impaired lipoprotein secretion. These effects are the consequence of ethanol interferences with the nuclear transcription factors, sterol regulatory element-binding protein-1 (SREBP-1) and peroxisome proliferator-activated transcription factor-α (PPAR-α) controlling lipid metabolizing enzymes, as well as from direct damage of mitochondria and endoplasmic reticulum [87, 88]. Oxidative stress might contribute to the pathogenesis of alcoholic steatosis through actions on transcription factors regulating lipid metabolism, mitochondrial injury, and endoplasmic reticulum stress.

Mitochondrial β-oxidation of fatty acids represents a key pathway in hepatic lipid metabolism. During chronic alcohol intake, an important cause of steatosis relays on the lowering in fatty acid oxidation due the impairment of mitochondrial functions as consequence of oxidative stress-mediated mtDNA mutations or oxidation of mitochondrial proteins [42, 89]. The prevalence of mtDNA deletions is

particularly high in alcoholics with microvesicular steatosis (about 85 % of the cases), indicating that the loss of mitochondrial respiratory capacity may represent the main cause of this lesion [90].

Hepatocyte endoplasmic reticulum (ER) is the site of protein folding, lipid and sterol synthesis, and intracellular calcium storage. The perturbation of ER functions activates several sensor proteins that trigger a signal network collectively termed the unfolded protein response (UPR) [91]. UPR counteracts ER alterations by reducing protein synthesis and promoting protein re-folding and/or degradation. Furthermore, ER stress stimulates the transcription of cytoprotective genes under the control of Nfr2 transcription factor and activates autophagy [91]. Increasing evidence indicates that alcohol causes ER stress in the liver. Oxidative stress is implicated in this process, as sulphydril redox unbalance in the ER, CYP2E1-mediated ROS production, and protein alkylation by lipid peroxidation products cause protein unfolding [92]. In particular, 4-HNE binding to heat shock proteins 70 and 90, protein sulphide isomerase, and the fatty acid-binding protein L-FABP have been recently detected in the liver of alcohol-fed rodents [93]. Moreover, ethanol can also contribute to hepatic ER stress by affecting protein degradation by the ubiquitin-proteasome system and autophagy [94, 95]. On its turn, ER stress is a stimulus for the proteolytical cleavage of SREBP-1 that translocates to the nucleus inducing the expression of genes encoding for enzymes involved in fatty acid synthesis, namely fatty acid synthetase, acyl-CoA carboxylase, and ATP citrate lysase [87, 89]. An increased SREBP-1 activation is evident in mice receiving an ethanol-containing diet, while SREBP-1 knockout mice are protected against ethanol-induced steatosis [87]. Consistently, betaine supplementation of alcohol-fed mice prevents ER stress, SREBP-1 activation, and fatty liver [96]. These observations suggest that alcohol-induced ER may stimulate intrahepatic lipid accumulation by triggering SREBP-1-dependent lipogenetic enzymes. In addition, ethanol interferes with PPAR-α action [87, 89] and oxidative stress mediated by CYP2E1 induction has been proposed to contribute to this effect [97]. PPAR-α regulates several genes responsible for the mitochondrial transport of fatty acids and both mitochondrial and peroxisomal fatty acid oxidation [89], making its down-modulation an important contributor in the development of alcoholic steatosis.

10.5.2   Lobular Inflammation

The transition from steatosis to steato-hepatitis is characterized by the appearance of mixed lobular inflammation featuring scattered infiltration by polymorphonuclear leucocytes and mononucleated cells [4, 88]. The persistence of mild-moderate parenchymal injury and inflammation is now recognized to be the main factor in the progression of ALD to cirrhosis. Furthermore, extensive lobular inflammation characterizes alcoholic steatohepatitis and contributes to impair liver functions [4, 88]. As previously discussed, inflammatory cell activation represents a relevant source of ROS and NO, leading to oxidative and nitrosative injury in ALD. In its

turn, oxidative stress significantly contributes to the mechanisms that promote hepatic inflammation during alcohol abuse. In fact, growing evidence indicates that oxidized lipids and the adducts between proteins and end products of lipid peroxidation can act as damage-associated molecular patterns (DAMPs), promoting the activation of inflammatory cells through the interaction with soluble and cell-associated pattern recognition receptors such as Toll-like receptors 2 and 4 (TLR-2, TLR-4) and lectin-like oxidized LDL receptor-1 (LOX-1) [98, 99]. Recent studies have also implicated oxidative mechanisms in inflammasome activation, as intracellular ROS formation stimulates Nod-like receptor protein 3 (NLPR3) to trigger caspase-1-mediated release of interleukins (IL-1β and IL-18) [100]. Interestingly, caspase-1-dependent release of IL-1β has been recently shown to be required for the onset of hepatic inflammation in alcohol-treated mice, while the supplementation with IL-1β receptor antagonist ameliorated ethanol hepatotoxicity [101]. Furthermore, ethanol-triggered redox signals in liver macrophages amplify proinflammatory responses by modulating TLR-4 transduction pathway, ERK1/2 and p38 MAPK kinase signal cascades and TNF-α transcriptional control [102].

An increased translocation of bacterial lipopolysaccharides (LPS) to the portal circulation is presently recognized as an important cause for hepatic inflammation during chronic alcohol intake [88, 103]. In chronic alcohol-fed rats as well as in ALD patients, plasma LPS content increases several fold over physiological levels and correlates with the circulating TNF-α levels and the severity of alcoholic hepatitis [103]. Interestingly, CYP2E1-mediated oxidative stress in the gut and increased intestinal NO production have been shown to enhance the permeability of enteral mucosa to LPS in chronic alcohol-treated rats [104, 105], while prevention of oxidative damage ameliorates endotoxemia associated with ethanol intake [105, 106].

It is increasingly evident that, beside the effect on innate immunity, oxidative stress induces adaptive immune responses [107]. In fact, oxidized phospholipids and proteins modified by lipid peroxidation are recognized as important antigens in autoimmune disease and in atherosclerosis [108, 109]. In line with these observations, we have reported that advanced ALD is characterized by the detection of elevated titres of circulating antibodies recognizing epitopes derived from protein modification by malonyldialdehyde (MDA), 4-hydroxynonenal (4-HNE), and lipid hydroperoxides [107] as well as by malonyldialdehyde-acetaldehyde (MAA) condensation products [110]. In about 35 % of the ALD patients, the presence of these antibodies is also associated with the detection of CD4+ T-lymphocytes recognizing malonyldialdehyde-derived antigens, indicating that oxidative stress promotes both humoral and cellular immune responses [111]. Finally, patients with alcoholic hepatitis or cirrhosis often have high titers of anti-phospholipid antibodies targeting oxidized phospholipids, namely oxidized cardiolipin and phosphatidylserine [112]. This latter observation is consistent with a recent report demonstrating increased levels of oxidized phospholipids in the plasma of alcohol-fed mice and patients with alcoholic hepatitis [28]. These clinical observations are supported by data generated in enteral alcohol-fed rats in which the development of lipid peroxidation-derived antibodies is associated with a sustained increase of TNF-α and IL-12 and histological evidence of necro-inflammation [113]. In these animals the supplementation

with the antioxidant N-acetylcysteine ameliorates oxidative stress, hepatic inflammation, and the immune response triggered by lipid peroxidation [36]. Consistently, heavy drinkers with elevated titers of IgG targeting lipid peroxidation-induced antigens have a fivefold higher prevalence of elevated plasma TNF-α levels than the subjects with these antibodies within the control range irrespective of alcohol intake [114]. The risk of advanced ALD also increases by 11-fold in the heavy drinkers with the combination of high TNF-α and lipid peroxidation-induced antibodies as compared to the subjects with high TNF-α, but no immune responses [114]. Furthermore, the combination of steatosis and high titers of antibodies against lipid peroxidation-derived adducts is an independent predictor of advanced fibrosis/cirrhosis in alcohol-consuming patients with chronic hepatitis C [115]. Interestingly, antibodies towards oxidative stress-derived antigens are also a risk factor for severe lobular inflammation and fibrosis in both children and adults with non-alcoholic steatohepatitis (NASH) [116, 117]. In this latter respect, using an experimental model of NASH, it has been possible to show that liver injury and lobular inflammation parallel with the development of IgG against MDA- and 4-HNE-derived antigens and the hepatic recruitment of CD4$^+$ and CD8$^+$ T-lymphocytes responsive to the same antigens [117]. In this setting, mice immunization against MDA-adducted proteins further stimulates transaminase release and lobular inflammation and increases hepatic macrophage activation by promoting the Th-1 activation of CD4$^+$ T-lymphocytes [118]. These findings are in line with early studies showing that, in alcoholic hepatitis and active alcoholic cirrhosis, liver inflammatory infiltrates contain CD8$^+$ and CD4$^+$ T lymphocytes [119] and that CD4$^+$ T-cells respond to T-cell receptor stimulation by producing Th-1 cytokines such as interferon-γ (IFN-γ) and TNF-α [120]. More recently, IL-17-producing T helper (Th-17) lymphocytes have also been found to increase in hepatic inflammatory infiltrates of patients with alcoholic hepatitis/cirrhosis [121]. Although still indirect, these evidences suggest that the development of humoral and cellular immunity against oxidative stress-derived antigens may contribute to hepatic inflammation during the evolution of ALD.

A further contributor to adaptive immunity involves the antibody responses against HER-derived antigens. Human anti-HER IgG selectively recognize HER-CYP2E1 adducts [60]. Furthermore, CYP2E1 alkylation by HER favors the breaking of the self-tolerance towards CYP2E1, leading to the development of anti-CYP2E1 auto-antibodies that are detectable in about 40 % of patients with advanced ALD [122]. Anti-CYP2E1 autoimmune responses are more frequent in individuals carrying a genetic polymorphism in the locus coding for the cytotoxic T lymphocyte-associated antigen-4 (CTLA-4), a membrane receptor that down-modulates T-cell activation [122]. Thus, the combination of the antigenic stimulation by HER-modified CYP2E1 and an impaired T cell control due to CTLA-4 mutation act synergically in promoting the development of anti-CYP2E1 auto-antibodies. Both allo- and auto-reactivity involving CYP2E1 may contribute to alcohol hepatotoxicity, as anti-HER IgG recognize HER-CYP2E1 adducts present on the outer layer of the plasma membranes of ethanol-treated hepatocytes and activate antibody-dependent cell-mediated cytotoxicity [60].

The involvement of B- and T-cell responses in ALD is not in contrast with the observations concerning the role of innate immunity in alcohol-induced hepatic inflammation. Indeed, the activation of the innate immunity is an important stimulus for lymphocyte activation towards oxidative stress-derived antigens. On their turn, lymphocyte-derived cyto/chemokine might provide a stimulus for phagocyte recruitment and activation maintaining chronic hepatic inflammation in ALD.

10.5.3   *Hepatocellular Injury*

Hepatocyte death by either necrosis or apoptosis characterizes parenchymal injury in ALD and significantly contributes to the progression of the disease. In particular, an increase in hepatocyte apoptosis is associated with the severity of liver injury in patients with alcoholic steatohepatitis [123]. At histology, hepatocyte injury is characterized by cell swelling (ballooning) and by the presence of Mallory's bodies, consisting in intracellular protein aggregates mainly containing cytokeratins (CK) 8 and 18 [5]. Serum levels of CK18 and of caspase-cleaved CK18 have been recently proposed as specific markers of hepatocyte death [124]. These markers are increased in heavy drinkers and prominently in patients with alcoholic hepatitis in whom they correlate with the prevalence of Mallory's bodies, hepatocyte ballooning, and fibrosis [124]. Several mechanisms account for the cytotoxic action of ethanol. Alcohol-induced oxidative damage of mitochondria causes the collapse of mitochondrial membrane potential and the onset of mitochondria permeability transition (MPT) [42]. In this contest, the mitochondrial translocation of the pro-apoptotic factor Bax favors MTP by complexing the voltage-dependent anion channel, (VDAC) [125]. According to this view, ethanol addition to HepG2 over-expressing human CYP2E1 collapses mitochondrial membrane potential causing MPT and apoptosis that can be prevented by CYP2E1 inhibitors, antioxidants as well as by the over-expression of the anti-apoptotic protein Bcl-2 [16]. Similarly, antioxidants block ethanol-induced apoptosis in isolated hepatocytes [125]. On the other hand, ethanol-fed SOD-1-deficient mice show extensive hepatocellular damage, mitochondrial depolarization, and increase in MTP [126]. A further mechanism contributing to alcohol-induced hepatocyte apoptosis involves the stimulation ER stress and the subsequent production of the pro-apoptotic CHOP protein [127]. Intracellular accumulation and aggregation of oxidized proteins is also implicated in the formation of Mallory's bodies [94]. In fact, CYP2E1-expressing HepG2 cells exposed to alcohol form insoluble protein aggregates containing CK8 and 18 as a result of oxidative proteasome impairment [128].

A further important factor in hepatocellular damage concerns altered hepatocyte responses to inflammation. As discussed above, ALD is characterized by an increased production of pro-inflammatory cytokines including TNF-α and elevated plasma TNF-α levels correlate with the severity and the mortality of alcoholic hepatitis [88]. Experiments using enteral alcohol feed rodents have shown that the treatment with anti-TNF-α antibodies as well as TNF-α receptor 1 (TNF-R1) deficiency protect against liver damage [88]. At difference of other parenchymal cells, hepato-

cytes are resistant to the pro-apoptotic action of TNF-α, because TNF-α interaction with TNF-R1 stimulates pro-survival responses through the activation of anti-apoptotic NF-κB-dependent genes and PI3K/PKB/Akt (protein kinase B)-mediated signals [129]. However, CYP2E1-expressing HepG2 cells or CYP2E1 induction in mouse liver sensitize hepatocytes to the cytotoxic action of TNF-α [15], suggesting that alcohol may alter the balance between pro- and anti-apoptotic signals. More detailed investigations in hepatocytes from chronically ethanol-fed rats have shown that the selective depletion of mtGSH enhances the susceptibility to TNF-α-induced killing, without interfering with NF-κB response [79]. Such an effect involves cardiolipin oxidation in the mitochondrial membranes that favors Bax-induced MPT and cytochrome c release [130]. Finally, oxidative stress-dependent mechanisms can interfere at different levels with the signal network controlling hepatocyte pro-survival responses. In fact, ethanol activates the apoptosis signaling kinase-1 (ASK-1) by oxidizing its binding proteins thioredoxin [131], while lipid peroxidation products affect hepatocyte ERK1/2, a kinase responsible for transducing anti-apoptotic signals [132]. Furthermore, chronic alcohol treatment inhibits hepatocyte AMP-activated protein kinase (AMPK) that is an important regulator of cell responses to pro-apoptotic signals consequent to ER stress, oxidative injury, and mitochondrial damage [133]. This latter effect appears to be mediated by the direct interaction of AMPK with 4-HNE generated by lipid peroxidation [134]. Thus, by inducing mitochondrial damage, ER stress, and by interfering with hepatocyte pro-survival signals, oxidative stress contributes to hepatocyte killing in alcohol-exposed livers.

Beside its hepatotoxic action, oxidative stress is also implicated in causing liver cell senescence, a condition characterized by the block of proliferative capacity, morphological changes, the expression of senescence-associated (SA) ß-galactosidase, the up-regulation of p53-dependent cycline-dependent kinase inhibitors p21, p16, and the production of inflammatory cytokines [135]. This action has been implicated in reducing the regenerative capacity of mature hepatocytes by activating p38 MAPK and p21 [136]. Concomitantly, oxidative damage promotes the proliferation and accumulation of hepatic progenitor cells (HPCs) that are the precursors of both hepatocytes and biliary duct epithelial cells [137]. It has been observed that in both ethanol-treated mice and patients with severe alcoholic hepatitis, the increase in HPCs within liver midzonal and perivenular areas is associated with a higher mortality [138]. Conversely, a recent report has shown that acute alcohol intoxication promotes liver regeneration after partial hepatectomy. This apparent paradox is due to the induction of aldehyde dehydrogenase 2 (ALDH2) that can effectively metabolizes lipid peroxidation-derived aldehydes such as 4-HNE that are generated by oxidative injury associated to liver surgical resection [139].

Altogether these data indicate that in response to chronic ethanol hepatotoxicity, HPCs can sustain hepatocyte turnover in an attempt to compensate for the impaired proliferation of mature cells. This reaction disturbs the normal parenchymal structure contributing to hepatic dysfunction associated with the evolution of cirrhosis. It is also possible that the expansion of immature cell populations, together with the mutagenic effects induced by oxidative stress products [25], might be important in the development of hepatocellular carcinomas in alcoholic cirrhotics.

10.5.4   Alcohol-Induced Fibrosis and Cirrhosis

Liver cirrhosis represents the terminal stage of ALD and is one of the main causes of death among patients with alcohol abuse. Fibrosis develops primarily in the pericentral areas, where thin bundles of fibrotic tissue surround groups of hepatocytes and thicken the space of Disse, in a "chicken wire" fashion [4]. However, during the progression of alcohol liver damage, parenchymal injury along with unresolved inflammation promotes further deposition of collagen-rich extracellular matrix (ECM), causing the extension of fibrosis to periportal areas and the formation of fibrous septa that encircle islets of hepatic parenchyma, eventually leading to micronodular cirrhosis [5]. As in other chronic liver diseases, alcohol-induced fibrosis is the consequence of a wound healing response mediated by hepatic stellate cells (HSCs), which under the local influence of transforming growth factor β1 (TGF-β1), platelet-derived growth factor (PDGF), and the chemochine CCL2 (MCP-1), trans-differentiate into myofibroblast-like cells (HSC/MSs) producing collagen and ECM components [140]. Furthermore, decreased hepatic matrix degradation due to a reduced production of matrix metalloproteases (MMPs) and/or an increased production of matrix metalloprotease inhibitors might also contribute to collagen accumulation [140]. Although hepatic macrophages are responsible for the secretion of HSC-activating cyto/chemokines, HSCs themselves can sustain through paracrine mechanisms liver wound-healing responses [140, 141]. In this contest, ROS and aldehydic end products of lipid peroxidation are well-recognized pro-fibrogenic stimuli triggering intracellular signals, leading to myofibroblast transition [54, 142]. In particular, redox changes activate multiple signal pathways involving the kinases INK/AP-1 and Janus kinases 1/2 as well as the transcription factors NF-κB and C/EBPß, which promote pro-collagen α1(I) gene expression, stimulate HSC proliferation, and enhance HSC migration [142]. Similarly, NADPH-oxidase generation of intracellular ROS in HSC/MFs sustains pro-fibrogenic signals in response to PDGF-BB, angiotensin II, and leptin [143]. In this latter respect, microarray analysis has shown that the gene expression of non-phagocitic components of NADPH-oxidase (NOX4, DUOX1 and DUOX2) is markedly elevated in the livers of patients with alcoholic hepatitis as compared to healthy controls where these mRNAs are barely detectable [144]. From the functional point of view, the relevance of oxidative stress-related mechanisms in the development of alcohol-induced hepatic fibrosis is suggested by several observations. Studies *in vitro* have shown that ROS directly triggers collagen synthesis in ethanol-treated HSCs over-expressing human CYP2E1 [145] as well as in HSCs isolated from ethanol-fed mice livers [146]. Interestingly, such an effect is also evident when ethanol is added to cocultures of HSC and CYP2E1-expressing HepG2 cells [147], indicating that oxidative stress-derived mediators generated within alcohol-metabolizing hepatocytes may diffuse out and signal to neighboring HSC. Consistently, *in vivo* experiments reveal that alcohol-induced lipid peroxidation is associated with macrophage production of TGF-1ß and precedes the appearance of fibrosis [148]. Ethanol-stimulated TGF-ß1 synthesis is particularly evident in the perivenous regions and is abolished

by CYP2E1 inhibition with chlormethiazole [149]. Furthermore, ethanol-induced liver fibrosis is exacerbated by the combined administration of carbonyl iron that greatly enhances oxidative stress as well as TGF-ß1 and procollagen-α1 mRNA expression in either the whole liver and freshly isolated HSCs [75]. Animal experiments have also shown that binge whisky administration to rats causes oxidative stress, p38 MAP kinase activation, and collagen deposition only in the livers with moderate steatosis induced by a choline-deficient diet [150]. On the same line, the combination of oxidative stress and steatosis is a risk factor for severe fibrosis in hepatitis C patients consuming alcohol [115], suggesting that alcoholic steatosis might worsen oxidative stress-dependent mechanisms of fibrogesis. Nonetheless, it should be considered that the pathogenesis of alcoholic fibrosis/cirrhosis is complex, and beside oxidative stress, chronic inflammation, impaired NK cell functions, and alterations in adipokine and endocannabinoid secretion might also specifically contribute to the disease evolution [140].

10.6 Conclusions

A large body of experimental and clinical data strongly implicates free radical formation and oxidative injury in several aspects of alcohol hepatotoxicity such as mitochondrial failure, hepatocyte apoptosis, amplification of alcohol-dependent pro-inflammatory signals, stimulation of immune responses, liver cell sensitization to the cytotoxic action of TNF-α, and activation of hepatic stellate cells to matrix production. Altogether, these observations give a rationale for the possible clinical use of antioxidant compounds to reduce the progression of ALD. However, despite experimental studies have shown that antioxidants can ameliorate alcohol liver injury in rodents [3, 32], the available clinical reports on the use of antioxidants in the therapy of human ALD give negative or inconclusive results [151–154]. On the same line, meta-analyses concerning the effects of milk thistle and S-adenosyl-L-methionine in alcoholic hepatitis were rather inconclusive [155, 156]. There are several possible explanations for such discrepancies: (1) alcohol-dependent liver injury in humans has a complex multifactorial pathogenesis and oxidative stress represents one of the factors influencing the disease evolution; (2) large inter-individual variability might influence the actual contribution of oxidative stress-dependent factors; (3) most of the clinical studies were performed in patients with severe alcoholic steatohepatitis or cirrhosis in which the lowering of oxidative stress would likely minimally modify already advanced hepatic lesions. Furthermore, to our knowledge, in none of the studies evaluating antioxidant therapy in ALD the actual effects on oxidative damage were monitored. Nonetheless, new hopes on the possible use of antioxidants in ameliorating hepatic damage in ALD come from recent evidences indicating that antioxidant treatments might be effective in improving hepatic damage in non-alcoholic steatohepatitis (NASH) [154, 157], which also implicate oxidative stress in the disease pathogenesis [158].

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