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POINTS OF VIEW

Hepatocyte oxidant stress and alcoholic liver disease

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RESUMEN

El consumo agudo y crónico de alcohol aumenta la producción de especies reactivas de oxígeno (ERO) y potencia la peroxidación de los lípidos, las proteínas y ADN. El mecanismo por el que el alcohol produce lesión celular no está del todo claro, pero se piensa que las ERO y los productos de la peroxidación lipídica intervienen de forma decisiva. Se cree que muchos mecanismos participan en el proceso por el que se induce estrés oxidativo, como los cambios de estado redox, la producción de acetaldehído, el daño mitocondrial, la lesión en la membrana, la apoptosis, la hipoxia inducida por etanol, los efectos sobre el sistema inmune y la producción alterada de citoquinas, el aumento de los niveles de endotoxina y la activación de las células de Kupffer, la movilización del hierro, la modulación de la defensa antioxidante, especialmente del glutatión (GSH) mitocondrial, la oxidación monoelectrónica del etanol al radical 1-hidroxi-etilo y la inducción de la CYP2E1. Estos mecanismos no son excluyentes entre sí y es probable que sean varios, probablemente muchos, los sistemas que contribuyan a la capacidad del etanol de inducir un estado de estrés oxidativo.

Palabras clave: Apoptosis. Muerte celular. Hepatocito. Hepatopatía alcohólica. Especies reactivas de oxígeno.

ABSTRACT

Acute and chronic alcohol consumption increases the production of reactive oxygen species (ROS), and enhances lipid peroxidation of lipids, proteins, and DNA. The mechanism by which alcohol causes cell injury is still not clear but a major role for ROS and lipid peroxidation-end products is considered. Many pathways have been suggested to play a role on how ethanol induces a state of "oxidative stress", including redox-state changes, acetaldehyde production, damage to the mitochondria, membrane injury, apoptosis, ethanol-induced hypoxia, effects on the immune system and altered cytokine production, increased endotoxin levels and activation of Kupffer cells, mobilization of iron, changes in the antioxidant defense, particularly mitochondrial glutathione (GSH), one electron oxidation of ethanol to 1-hydroxy-ethyl radical, and induction of CYP2E1. These pathways are not exclusive of one another and it is likely that several, indeed many systems contribute to the ability of ethanol to induce a state of oxidative stress.

Key words: Apoptosis. Cell death. Hepatocyte. Alcoholic liver disease. Reactive oxygen species.

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PATHOPHYSIOLOGY OF ALCOHOLIC LIVER DISEASE

Liver disease related to alcohol consumption can be classified into different categories: Fatty liver, alcoholic hepatitis, and cirrhosis. Fatty liver, which occurs after prolonged alcohol intake, is normally reversible with abstinence and does not predispose to any chronic form of liver disease provided that abstinence and/or moderation are maintained (1). Alcoholic hepatitis is an acute form of alcohol-induced liver injury that covers a spectrum of severity ranging from an asymptomatic unbalance of liver biochemistry to liver failure and death. The development of alcoholic hepatitis generally involves consumption of a large amount of alcohol for a long period of time, sometimes years (2). Cirrhosis implies replacement of the normal hepatic parenchyma with collagen fibers along with insufficient extracellular matrix remodeling, leading to clinical manifestations of portal hypertension and liver failure (3).

The main sites for alcohol metabolism are the liver and, to a lesser extent, the gastrointestinal tract (4). Within the liver, the alcohol dehydrogenase (ADH) and cytochrome p450 2E1 (CYP2E1) are the main pathways of

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alcohol metabolism. ADH is a hepatocyte cytosolic enzyme that metabolizes alcohol to acetaldehyde (5). CYP2E1 is a microsomal membrane protein which converts alcohol to acetaldehyde when alcohol levels are high enough to reach ADH saturation. Acetaldehyde in turn is transformed to acetate via the mitochondrial matrix enzyme acetaldehyde dehydrogenase (6).

Liver injury occurs through several interrelated pathways. ADH and acetaldehyde dehydrogenase cause the reduction of NAD^+ to NADH. The altered ratio of NAD^+/NADH promotes fatty liver via inhibition of gluconeogenesis and fatty acid oxidation (7). CYP2E1, which is up-regulated in chronic alcohol consumption and stabilized by alcohol itself, generates free radicals through the oxidation of NADPH to $NADP^+$ (8). In addition, chronic alcohol exposure activates Kupffer cells to generate tumor necrosis factor-α (TNF-α), which subsequently induces production of reactive oxygen species in the mitochondria (ROS) (9). Oxidative stress induces hepatocyte necrosis and apoptosis (10), both elevated in alcoholic patient with low antioxidants such as glutathione (GSH) and vitamin E (11). ROS promote lipid peroxidation, which induces inflammation and fibrosis (12). Inflammation is also initiated by acetaldehyde which, when bound covalently to cellular proteins, forms antigenic adducts (13). The earliest changes at the histological level in alcoholic hepatitis are located predominantly around the central vein. Alcohol generates a gradient of hypoxia from the portal vein to the central vein, indicating that the hypoxia induced by chronic alcohol intake may contribute to hepatic injury (14).

HEPATOCYTES AND ROS PRODUCTION

Oxidative stress results from the imbalance between pro-oxidant and antioxidant mechanisms leading to cell injury, and it appears to be involved in liver disease such as chronic viral hepatitis, alcoholic hepatitis, non-alcoholic steatohepatitis (NASH), cirrhosis, and chronic cholestasis (15,16). ROS include a variety of species such as superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($HO^{\cdot}$). Some of these species (e.g. $O_2^{\cdot-}$ and $HO^{\cdot}$) are free radical species as they contain unpaired electrons and therefore are extremely unstable, while others like H_2O_2 are highly diffusible and relatively stable. Endogenous sources of ROS in hepatocytes include mitochondrial damage, xanthine oxidase, cytochrome P450 metabolism, peroxisomes, and NADPH oxidase; many of which are present in the hepatocytes.

Energy sources such as glucose are initially metabolized in the cytoplasm. The products are imported into the *mitochondria* which continues catabolism through metabolic pathways such as the Krebs cycle, fatty acid oxidation, and amino acid oxidation (17). The end result of these pathways is the production of two energy-rich electron donors, NADH and FADH2. Electrons from these donors are transferred through an electron transport chain to O_2, which is reduced to water (18,19). This is a multistep redox process that occurs in the mitochondrial inner membrane (20-22). The enzymes that catalyze these reactions have the remarkable ability to simultaneously create a proton gradient across the membrane (23). Although electron transport occurs with great efficiency, a small percentage of electrons are prematurely leaked to O_2, resulting in the formation of the toxic free radical, $O_2^{\cdot-}$ (23). Under normal conditions, $O_2^{\cdot-}$ will hardly diffuse into the cytosol and will undergo dismutation to generate H_2O_2 which can cross the mitochondrial membrane (23). During liver injury, however, due to damage to the mitochondrial membrane, $O_2^{\cdot-}$ may diffuse into the cytosol triggering the subsequent cascade or ROS-mediated reactions (24).

Xanthine oxidase is a cytosolic molybdenum and iron containing hydroxylating enzyme involved in the degradation of purine-like nucleotides. Xanthine oxidase catalyzes the oxidation of hypoxanthine to xanthine and can further catalyze the oxidation of xanthine to uric acid (25) generating ROS in the process.

Many pathways have been suggested to contribute to the ability of ethanol to induce oxidative stress. One central pathway is the induction of CYP2E1, a member of the cytochrome P450 mixed-function oxidase system. CYP2E1 is of interest because of its ability to metabolize and activate many toxicological substrates, including ethanol, to more reactive toxic products (12,26-30). Levels of CYP2E1 are elevated under a variety of physiological and pathophysiological conditions, and after acute and chronic alcohol treatment. CYP2E1 is also an effective generator of ROS such as $O_2^{\cdot-}$ radical and H_2O_2, and in the presence of iron catalysts, it produces powerful oxidants such as hydroxyl radical and 1-hydroxy ethyl radical (31).

Peroxisomes contain oxidative enzymes, such as catalase, D-amino acid oxidase, and uric acid oxidase. Certain enzymes within the peroxisome, by using O_2, remove H atoms from specific organic substrates in an oxidative reaction to produce H_2O_2. Catalase uses H_2O_2 to oxidize other substrates, including phenols, formic acid, formaldehyde and alcohol, thus eliminating the H_2O_2 in the process (32). This reaction is important in hepatocytes where peroxisomes detoxify various toxic substances that enter the blood stream. About 25% of the ethanol is oxidized to acetaldehyde in this way (33). In addition, when excess H_2O_2 accumulates in the cell, catalase converts it into H_2O through this reaction. A major function of the peroxisome is the β-oxidation of fatty acids whereby fatty acids are broken down by two carbons at a time, converted to Acetyl-CoA, which is then shuttled back to the cytosol for further use (34). β-oxidation can occur in the mitochondria as well.

The *NADPH oxidase* complex although not described in hepatocytes it is highly expressed in macrophages, Kupffer cells, stellate cells, and neutrophils, all of which play major roles in alcoholic liver disease (ALD) (35). The NADPH oxidase complex is normally latent and it is

activated to assemble in the membranes during the respiratory burst. It generates O_2^- by transferring electrons from NADPH inside the cell across the membrane and coupling these to O_2 to produce the O_2^-. ROS can also be produced in hepatocytes by exogenous substances, including environmental toxins, xenobiotics, radiation, ultraviolet light, metal ions and in drug metabolism.

Oxidant stress can be counterbalanced by the hepatocyte antioxidant defense which induces both enzymatic and non-enzymatic mechanisms. Among the enzymatic antioxidant defense are: a) *Superoxide dismutase* (SOD) of which SOD1 is localized in the cytosol and in the mitochondrial inter-membrane space, SOD2 is localized in the mitochondria, and SOD3 is extracellular and interacts with matrix components (36). All three isoforms dismutate O_2^- into H_2O_2 and O_2; b) *Catalase* is an iron-containing enzyme found in peroxisomes whose role is to remove H_2O_2 generating H_2O and O_2; (37); and c) *Glutathione peroxidase* and *Glutathione reductase* using the cofactor NADPH are able to decompose H_2O_2 while oxidizing glutathione (37).

Non enzymatic mechanisms of antioxidant defense include: a) *Glutathione* (GSH), a tri-peptide (γ-glutamylcysteinylglycine) synthesized in the cytosol, in a two-step energy consuming process, and distributed in different organelles, such as endoplasmic reticulum, cytosol, and mitochondria. Glutathione is found almost exclusively in its reduced form, since the enzyme which converts it from the oxidized form (GSSG) to the reduced form (GSH), glutathione reductase, is constitutively active and inducible upon oxidative stress. In fact, the ratio of GSH to GSSG within cells is often used as a measurement of cellular toxicity. GSH detoxifies ROS produced in the mitochondrial electron transport chain. Mitochondrial GSH depletion may compromise mitochondrial function and sensitizes cells to oxidant-induced toxicity, leading to cell death (38); b) *Metal-binding proteins* help iron and copper to remain in a non-reactive state and avoid the formation of hydroxyl radicals. Transferrin and lactoferrin bind iron whereas albumin binds copper; and c) *Vitamins* such as vitamin C (ascorbate), vitamin E (α-tocopherol), and carotenoids (vitamin A precursors) act as free-radical scavengers (12,39). Tocopherols and flavonoids inhibit peroxidation by acting as chain-breaking peroxyl-radical scavengers. Finally, other molecules like bilirubin, melatonin, and uric acid are natural antioxidants (40,41).

HEPATOCYTE CELL DEATH IN LIVER DISEASE

During acute and chronic liver disease, hepatocytes are exposed to increased levels of ROS, cytokines, and bile acids. Even though hepatocytes have good detoxifying capacity, over-exposure to high levels of ROS may disrupt their redox state resulting in cell death (necrosis and/or apoptosis). While necrosis is a passive mechanism involving ATP depletion, rupture of the plasma membrane, and drop out of the cellular content triggering inflammation (42); in contrast, apoptosis, or programmed cell death (42-44), is an active process characterized by mitochondrial swelling, chromatin condensation, formation of apoptotic bodies, and eventually activation of caspases (45-47). Apoptosis represents a regulated form of cell death and it is important in processes such as cell selection during development, immunologic responses and homeostasis. Regulation of apoptotic cell death allows therapeutic intervention strategies. The most important modes of apoptotic response in hepatocytes under ROS stimulation are: a) the death receptor-mediated apoptosis; and b) the mitochondrial-mediated apoptosis.

Death receptor-mediated apoptosis. In death receptor-mediated apoptosis, death ligands on effector cells, such as Fas ligand (FasL, CD95, Apo1), TNFα, or TNF-related apoptosis-inducing ligand (TRAIL), bind to death receptors expressed on the surface of the target cell. Upon death receptor binding, intracellular adaptor molecules are recruited, and these molecules can, in turn, associate with initiator caspases through death effector domain (DED) or caspase recruitment domain interactions leading to their activation, thereby starting the caspase cascade with the final end of the cell as a result.

Hepatocytes express Fas (CD95) but not Fas ligand. The expression of Fas is markedly increased in the livers of patients with non-alcoholic steatohepatitis (NASH) (48) or in fat-laden mouse hepatocytes (49). Furthermore, selected drugs, alcohol abuse, and Wilson's disease, which elevate ROS production, cause Fas ligand expression in hepatocytes, leading to apoptosis (50). Hepatocytes also express Tumour necrosis factor Related Apoptosis Inducing Ligand-receptor-1 (TRAIL-R1), TRAIL receptor-2 (TRAIL-R2) and tumour necrosis factor-receptor type-1 (TNFα-R1) (51). Unlike Fas or TRAIL-R1 and TRAIL-R2, TNF-R1-mediated intracellular signalling is more complex as it activates both apoptotic and survival signals. Patients with NASH have both high hepatic TNF-α mRNA levels and high TNF-R1 expression (52). Upon activation by TNF-α, trimerization of TNF-R1 is followed by recruitment of the adaptor protein TNF-Receptor Associated protein with Death Domain (TRADD). TRADD recruits Fas Associated Death Domain (FADD) and it is also capable of activating pathways like Nuclear Factor kappa B (NF-κB) and Mitogen-activated protein kinases (MAPKs). FADD contains a death effector domain, which, through Death-Inducing Signalling Complex (DISC), mediates the recruitment of caspases-8 and -10 activating the death signalling cascade. Active caspase-8 is involved in the cleavage and activation of effector caspase-3, the central executioner molecule as it cleaves various proteins thereby disabling important cellular structural and repair processes.

Mitochondria and apoptosis. Release of toxic proteins form the inter-membrane space of the mitochondria triggered by permeabilization of the outer mitochondrial membrane constitutes a "point of no return" in most cases of apoptosis. Members of the Blc-2 family control this

process tightly: upon apoptotic signals, pro-apoptotic Bcl-2 proteins such as Bax and Bak are activated, resulting in an increase in the outer mitochondrial membrane permeabilization (53-55). In contrast, anti-apoptotic Bcl-2 family members, such as Bcl-2 and Bcl-X_L, can prevent this occurrence by heterodimerization with Bax-like proteins. Other pro-apoptotic Bcl-2 proteins which contain only the BH3 domain (e.g., Bad, Bid, Bim, Bmf, and Noxa) act by opposing the inhibitory effect of Bcl-2 or Bcl-X_L, or by activating Bax-like proteins by direct binding (56).

A second mechanism of permeabilization of the outer mitochondrial membrane is the opening of a permeability transition pore in the inner mitochondrial membrane. This allows water and small molecules (up to 1.5 kDa) to pass through the pore, leading to swelling of the intermembrane space and rupture of the outer mitochondrial membrane. The first protein released from the mitochondria upon apoptotic stimuli is cytochrome c, an essential component of the respiratory chain. Upon release into the cytoplasm, it forms, in the presence of ATP, the so-called "apoptosome" together with Apaf-1 and caspase 9, triggering the classic apoptotic cascade, and leading to apoptotic cell death (57-60). The catalytic function of cytochrome c is safeguarded by members of the inhibitor of apoptosis proteins family, which are in turn controlled by two other mitochondrial proteins, Smac/DIABLO and OMI/HtrA2 (61). In this way, OMI/HtrA2 plays a role in caspase-dependent cell death, but it can also act as an effector protein in necrosis-like apoptosis. Apoptosis inducing factor (AIF) is a mitochondrial protein that plays a pivotal role in apoptosis, it is normally retained in the inter-membrane mitochondrial space, acting as an oxidoreductase. Similar to the bi-functional role of cytochrome *c*, AIF induces cell death when it is released to the cytosol; it then translocates to the nucleus and triggers, possibly together with endonuclease G, peripheral chromatin condensation and high molecular weight (50 kb) DNA loss. The lethal effects of AIF are controlled by the anti-apoptotic protein heat shock protein 70 that interacts with AIF and protects against its apoptotic effects (61).

HEPATOCYTE SURVIVAL PATHWAYS IN LIVER DISEASE

Among the signalling pathways activated in response to oxidant injury heme oxygenase, ERK1/2, p38, PI3K, and NF-κB signalling pathways are considered survival pathways whereas JNK is usually related to apoptosis.

Nuclear factor kappa B (NF-κB) signalling pathway. NF-κB is an ubiquitous heterodimeric transcription factor that is sequestered in the cytoplasm by proteins of the IκB family (62). IκB, is regulated by a protein complex that includes two kinases IKKα and IKKβ, both capable of phosphorylating IκB, and a regulatory subunit IKKγ (NEMO). Phosphorylation and degradation of IκB frees NF-κB and exposes a nuclear localization sequence, leading to the translocation of NF-κB to the nucleus. Once in the nucleus, NF-κB binds to κB binding sites in promoters of target genes, inducing their transcription. NF-κB is activated by inflammatory cytokines, such as TNF-α and IL-1β, oxidative stress (63), endotoxin (LPS) (64), protein kinase C (PKC) and phosphatidylinositol-3 kinase (PI3K). NF-κB signalling pathway has been described to antagonize hepatocyte cell death by influencing the balance between pro- and anti-apoptotic signals. NF-κB inhibits TNF-α-induced accumulation of ROS that normally mediate prolonged c-Jun N-terminal kinase (JNK) activation and cell death (65). Indeed, inhibition of NF-κB activity induces apoptosis in hepatocytes, suggesting its role in the transcription of anti-apoptotic genes (66).

Mitogen-activated protein kinases (MAPKs) signaling pathways. The MAPK cascade includes a mitogen-activated protein kinase kinase kinase (MAPKKK), mitogen-activated protein kinase kinase (MAPKK) and MAPK. In the large MAPK family, three subgroups have been identified: the c-Jun N-terminal kinase (JNK), p38 MAPK and the extracellular signal-regulated kinase (ERK1/2), which have been shown to be activated by ROS, and affect cell survival (67). ERK1/2 and p38 MAPK have been associated to cell survival, whereas JNK has been linked to cell death (10,68). The balance between ERK1/2, p38, and JNK activation is crucial in determining cell fate between death and survival. Inhibition of JNK activation, using specific inhibitors or dominant-negative mutants for JNK, suppresses apoptosis.

Phosphoinositide 3-kinase (PI3K)/Akt signaling pathway. The PI3-kinase family is a super family including three different classes of enzymes that are linked with cell survival. Class I enzymes have been characterised and subdivided into two groups of PI3-kinases IA and IB. The catalytic subunit of class IA interacts with adaptor proteins and it is involved in activation by growth factor receptors (e.g. the epidermal growth factor receptor: EGF-R), while class IB is required for G-protein-coupled receptor systems (69). Class I PI3-kinase reside mainly in the cytosol until recruited into active signalling complexes in the plasma membrane, where they are involved in the generation of 3'-phosphorylated phosphoinositides, that function as signalling intermediates in signal transduction cascades. Targets of PI3K, such as the serine kinase Akt, also known as protein kinase B, have been associated with the inhibition of apoptosis in a variety of ways (70,71). The PI3K/Akt pathway transduces survival signals through phosphorylation processes and regulates pro- and anti-apoptotic factors, such as BAD, caspase-9, and IKKα. It has been reported that Akt activates ERK1/2, NF-κB and inhibits JNK and Bax phosphorylation and thus protects against mitochondria disruption and apoptosis (72,73). Crosstalk between pro- and anti-apoptotic pathways is described, such as PI3K/Akt and JNK MAPK pathways, modulating the balance between cell survival and cell death.

Src family signaling pathways. Src-family protein-tyrosine kinases are intermediate regulatory proteins that play important roles in differentiation, motility, proliferation and survival. Src activates the anti-apoptotic PI3K/Akt pathway in human colon tumour cell lines (74). In addition, Src increases Bcl-XL expression in rat intestinal epithelial cells (75). Transforming growth factor-β (TGF-β regulates hepatocyte growth, inhibiting proliferation and inducing apoptosis, it also activates PI3-k/Akt pathway in hepatocytes by a mechanism dependent on EGF receptor and c-Src activity (76).

Heme oxygenase. Heme oxygenase (HO) catalyzes the oxidation of heme to form equimolar amounts of ferrous iron, carbon monoxide (CO) and biliverdin, which is rapidly converted into bilirubin by NAD(P)H:biliverdin reductase. Three different isoforms of HO have been described (77). These isozymes are products of different genes and differ in their tissue distribution and molecular properties. The HO-2 isoform is constitutively expressed and is present in high levels in brain and testes (78). HO-3 has catalytic activity and functions as a heme-binding protein (79). In contrast, HO-1 is ubiquitously distributed and is highly inducible by a variety of stimuli, most of them associated with oxidative stress (80). HO-1 may act as an inducible defense system against oxidative stress, e.g. in models of inflammation, ischemia-reperfusion, hypoxia and hyperoxia-mediated injury (81). In the liver, HO-1 induction protects against ischemia/reperfusion injury (82,83) and endotoxemia (84, 85). In addition, overexpression of HO-1 by gene transfer has been shown to protect against hyperoxia induced by lung injury (86) and to from immune-mediated apoptotic liver damage in mice (87). However, the mechanisms by which HO-1 mediates cytoprotection have not been elucidated yet. Protective effects of both biliverdin and CO (10,88) have been reported and several studies suggest that biliverdin protects against oxidative stress by acting as an anti-oxidant in different models of liver injury (89,90).

In spite of the highly efficient detoxification mechanisms, over-exposure to high level of ROS results in oxidative stress and cell death. Under oxidative stress conditions, the mode of cell death (apoptosis or necrosis) is primarily dependent on the variety of ROS and the cell type. In chronic liver diseases, such as alcoholic and viral hepatitis (16,91,92), NASH (93-95) and cholestasis, hepatocytes are invariably exposed to oxidative stress from different sources, which induces cell damage and subsequently hepatocyte cell death and loss of liver function. Therefore, further knowledge on the cellular mechanisms controlling liver cell death is of clinical and scientific relevance to identify targets for the development of novel therapies to treat liver disease.

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