Liver Research 3 (2019) 191–205

Contents lists available at ScienceDirect

Liver Research

journal homepage: http://www.keaipublishing.com/en/journals/liver-research

Review Article

Lysosome and proteasome dysfunction in alcohol-induced liver injury☆

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ARTICLE INFO

Article history:
Received 10 July 2019
Received in revised form
23 October 2019
Accepted 13 November 2019

Keywords:
Alcohol
Lysosome
Proteasome
Ethanol
Liver
Proteostasis
Lipostasis
Alcohol-induced liver disease (AILD)

ABSTRACT

The review describes research findings on the influence of alcohol consumption on two crucial catabolic systems in hepatocytes: the lysosome and the ubiquitin-proteasome system (UPS). The lysosome is a membrane-bound organelle that degrades all aging and/or damaged organelles and hydrolyzes all forms of macromolecules. The UPS is mostly proteolytic. It carries out the majority of its functions in the soluble portion of the cytoplasm (cytosol) and degrades nearly all intracellular proteins, particularly those with short half-lives, so that their levels are tightly controlled. Our review will briefly discuss the epidemiology of alcohol abuse and the spectrum of alcohol-induced liver disease (AILD). We will explain why ethanol (EtOH) metabolism, but not EtOH alone, is hepatotoxic. Then, we will summarize how heavy drinking alters hepatic catabolic systems, resulting in liver enlargement that develops from hepatocyte swelling due, in part, to aberrant accumulation of undegraded lipid droplets (steatosis) and undegraded proteins (proteopathy). Our detailed description of each catabolic system will highlight its discoverer(s) and emphasize each system's characteristics. Most important, we will review the evidence that chronic EtOH consumption disrupts hepatic lysosome biogenesis and inhibits the UPS by impeding hepatic proteasome activity. It will become evident that each of these EtOH-induced defects has far-reaching functional consequences. Finally, we will describe current and potential therapeutic interventions for alleviating EtOH-induced liver injury. The most effective intervention is the cessation of EtOH consumption. However, there are other potential approaches using natural or synthetic compounds that activate autophagy or the proteasome to enhance the degradation of accumulated lipid droplets or proteins, respectively, which could alleviate AILD. These approaches, now in their early stages of investigation, will also be discussed in this review.

1. Introduction

For those readers, who are unacquainted with alcohol research, the connection between alcohol-induced liver injury and macromolecular catabolism may seem remote, even far-fetched. However, it is generally acknowledged that fully functional macromolecular degradative systems, namely lysosomes and proteasomes, are necessary for cell survival. This is because inhibition of either or both systems can hasten cell death. Conversely, activation of these degradative systems can prolong cell viability.[1,2] Continuous ethanol (EtOH) consumption and its hepatic oxidation disrupts lysosome biogenesis and inhibits proteasome activity. An early result of these latter changes is intrahepatocyte lipid droplet (LD) accumulation, causing fatty liver (steatosis), which is followed soon after by hepatic protein accumulation or proteopathy. The latter intracellular changes shorten hepatocyte lifespan

☆ Edited by Yuxia Jiang, Peiling Zhu and Genshu Wang.

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https://doi.org/10.1016/j.livres.2019.11.001

because if drinking continues, they activate cell death signals that propagate liver injury. This review focuses on how chronic drinking down-regulates lysosome biogenesis, autophagy and the ubiquitin-proteasome system (UPS) and how hindrance of these catabolic systems contributes to the pathogenesis of alcohol-induced liver disease (AILD).

2. Alcohol-induced liver injury

2.1. Epidemiology

Excessive alcohol consumption is a significant healthcare problem, worldwide. In 2016, alcohol abuse contributed to an estimated 3 million deaths (5.3% of all deaths) globally and caused 132.6 million disability-adjusted life years (DALYs) which represented 5.1% of all DALYs in that year.[3] More than 20 million Americans have substance use disorders, of which alcohol abuse accounts for an estimated economic cost of $249 billion.[4] It is expected that the total per capita alcohol consumption will increase from 8 to 8.4 L by 2025.[3] This is despite the well-known fact that excessive drinking over decades damages nearly every organ in the body. The liver, however, sustains the earliest and the greatest degree of tissue injury from excessive drinking because it is the primary site of EtOH metabolism, with resultant AILD causing significant morbidity and mortality.[5]

2.2. Spectrum of liver injury

AILD is comprised of a spectrum of liver injury. These include steatosis, steatohepatitis, fibrosis, cirrhosis and hepatocellular carcinoma (HCC). The earliest manifestation of chronic EtOH abuse is steatosis, which is characterized by the deposition of fat in hepatocytes. Nearly all patients who heavily imbibe alcohol have some degree of steatosis. Continued heavy alcohol consumption leads, in a subset of patients, to the development of steatohepatitis, characterized by polymorphonuclear cell infiltration and hepatocellular damage. Sustained alcohol abuse can ultimately cause progression to fibrosis and cirrhosis which are characterized by the aberrant deposition of extracellular matrix (ECM) proteins that are mostly produced by activated hepatic stellate cells. There is progressive architectural distortion of normal liver parenchyma during cirrhosis development with a gradual loss of vital liver function. The main pathological feature of cirrhosis is the formation of regenerative nodules of hepatic parenchyma surrounded by fibrous septa. Cirrhosis development progresses from a compensated phase, when undamaged parts of the liver functionally compensate for the damaged tissue, to a decompensated phase, in which scar tissue fully envelops the organ. Patients chronically abusing alcohol with underlying cirrhosis are predisposed to developing alcoholic hepatitis which is one of the deadliest diseases in clinical hepatology, with a mortality rate of 30–50% at 3 months.[6] Despite the well-characterized AILD progression trajectory, alcohol-related liver injury worldwide is rarely detected at early stages compared with liver diseases due to other etiologies.[7]

3. EtOH metabolism

Most of the toxic effects of ethyl alcohol or EtOH, the active ingredient in alcoholic beverages, have been attributed to the metabolic conversion of EtOH to its toxic intermediates and associated metabolic stresses. More than 90% of ingested EtOH is metabolized by oxidative pathways in the liver, as this organ expresses the highest levels of the major EtOH-oxidizing enzymes. The non-oxidative pathways constitute a minor fraction of total EtOH metabolism.[8]

3.1. Oxidative EtOH metabolism

EtOH is chiefly metabolized in hepatocytes. The first step in the oxidative pathway is the conversion of EtOH to acetaldehyde, catalyzed mainly by cytosolic alcohol dehydrogenase (ADH) and to a lesser extent by microsomal cytochrome P450 2E1 (CYP2E1).[9] ADH is the most catalytically efficient EtOH metabolizing enzyme. The Km of most ADH isozymes for EtOH is low (around 0.5–2 mM), well below levels that are considered legally intoxicating (17.4 mM).[10] The EtOH elimination process proceeds at maximal velocity even at these low EtOH concentrations.[9] While the catalytic efficiency of CYP2E1 is considerably slower than that of ADH, CYP2E1 has a 10-fold higher capacity for binding EtOH, becoming half-saturated at 46–92 mg per deciliter (10–20 mM). Importantly, CYP2E1 is an inducible enzyme; its hepatocellular content rises during chronic EtOH consumption.[11,12] This occurs because of a ~6-fold decrease in the rate of CYP2E1 degradation by the UPS.[13,14] Hepatocytes also express very high levels of catalase, a peroxisomal enzyme, that normally catalyzes detoxification of hydrogen peroxide forming water and oxygen. However, when EtOH is present, catalase has an accessory role in EtOH metabolism by utilizing hydrogen peroxide to oxidize EtOH to acetaldehyde. EtOH oxidation by catalase is a relatively minor pathway in the liver, but it has a larger EtOH-oxidizing function in the brain.[15]

Acetaldehyde is subsequently oxidized to acetate in a reaction catalyzed by aldehyde dehydrogenase (ALDH) which irreversibly oxidizes acetaldehyde to acetate. The latter is released into the circulation or is further metabolized. Major ALDH isoforms exist in the mitochondrial, microsomal, and cytosolic compartments. Mitochondria contain a low Km ALDH in the matrix space and a high Km ALDH in the outer membrane. Microsomes contain a high Km ALDH, while the cytosol contains an intermediate and a high Km ALDH.[9]

Acetaldehyde can also be oxidized by aldehyde oxidase, xanthine oxidase, and CYP2E1, but these are insignificant pathways.[9] Genetic polymorphisms of several ALDHs have been identified in humans; the best known is the *ALDH2* gene, which encodes a low Km mitochondrial enzyme primarily responsible for oxidizing EtOH-derived acetaldehyde.[16] Circulating levels of acetaldehyde are usually elevated in problem drinkers because of increased acetaldehyde production and/or its decreased removal because of lower ALDH2 activity and/or impaired mitochondrial function.[9]

3.2. Non-oxidative EtOH metabolism

There are several non-oxidative routes of EtOH metabolism that result in the enzymatic conjugation of EtOH to endogenous metabolites such as glucuronic acid, sulfate, phospholipids and fatty acids. The most abundant conjugates detected are ethyl glucuronide, ethyl sulfate, phosphatidyl-EtOH and fatty acid ethyl esters.[17] Even though these non-oxidative pathways constitute a minor fraction of total EtOH metabolism in the liver, some of these metabolites have proven to be reliable biomarkers for the detection of heavy EtOH consumption, even after several months of abstinence. This occurs because these conjugates have slower elimination rates, and hence, persist in body fluids, hair and other tissues.[17]

3.3. EtOH metabolism generates intermediates and stressors

EtOH metabolism generates toxic intermediate metabolites and metabolic stressors as detailed below:

(i) Acetaldehyde and reactive oxygen species (ROS) generation: Acetaldehyde, generated by ADH, CYP2E1 and catalase, is a

major pro-oxidant, implicated in many of the pathogenic effects of alcohol. Acetaldehyde is highly electrophilic and can covalently bind to reactive residues bearing free amino groups, such as in phospholipids and nucleic acids and the epsilon amino groups of lysine residues in proteins.[18–21]

(ii) Induction of CYP2E1 by EtOH also puts the liver cell in metabolic peril, as more CYP2E1 enzyme produces not only more acetaldehyde, but, because of its broad substrate specificity, it also produces greater amounts of other ROS, including hydroxyethyl radicals, superoxide anions and hydroxyl radicals. Continuous generation of these reactive molecules in problem drinkers eventually creates the condition known as oxidant stress (a.k.a. oxidative stress). Here, the rate of ROS generation exceeds the liver's capacity to neutralize them with natural antioxidants such as glutathione and vitamins E, A, and C, or to remove them, using antioxidant enzymes.[22] Animal studies reveal that chronic EtOH consumption decreases the activities and/or amounts of several antioxidant enzymes, which exacerbates the hepatocyte's oxidant burden. Oxidant stress is further increased when the aforementioned ROS undergo secondary reactions with proteins and unsaturated lipids, the latter forming lipid peroxides, such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). These lipid peroxides, themselves, form adducts with proteins. Studies show that MDA has a special ability to enhance the stable binding of acetaldehyde to proteins, resulting in the generation of novel and distinct hybrid adducts designated as MDA-acetaldehyde (MAA) or MAA adducts, which are detectable in hepatocytes of EtOH-fed rats.[23] These adducts are highly immunogenic and can generate an immune response in human alcoholics and in animal models of alcohol toxicity.[24]

(iii) Decreased redox ratio: Both ADH- and ALDH-catalyzed oxidations utilize oxidized nicotinamide adenine dinucleotide (NAD^+) as a cofactor to generate reduced NAD (NADH), which decreases the normal intrahepatocyte ratio of NAD^+/NADH. This ratio, reflects the hepatic redox state and causes significant metabolic shifts from oxidative metabolism toward reductive synthesis, favoring the formation of fatty acids and impairing fatty acid oxidation, thereby contributing to fatty liver development.[25] The decreased NAD^+/NADH redox ratio also impairs other important reactions, including glycolysis, citric acid cycle, pyruvate dehydrogenase and gluconeogenesis, causing significant metabolic perturbations in hepatocytes.[9]

4. Catabolic changes in the liver after heavy drinking

4.1. Fatty liver (steatosis)

A rise in hepatic lipid content, particularly triglycerides (TGs) and cholesteryl esters, is the most rapid change that occurs in nearly all cases of excessive drinking.[26,27] Steatosis is regarded as the "first hit" in the spectrum of alcohol-induced pathologies that, if sustained, contributes to liver enlargement (hepatomegaly), while propagating hepatocyte injury by enhancing oxidant stress. Intracellular fats are sequestered inside lipid droplets (LDs). These are dynamic, flexible, balloon-like organelles that, if drinking continues, increase in number and expand in size within hepatocytes.[28] Alcohol-induced LD accumulation transforms the liver from a fat-burning to a fat-storing organ. Compared with adipose tissue, which normally stores body fat, the liver is ill-adapted for lipid storage. Alcohol-induced steatosis arises from alterations in gene expression, metabolism and organelle biogenesis that result in, not only enhanced lipogenesis,[29,30] but also impaired very low-density lipoprotein (VLDL) secretion,[31] retarded lipophagy, and inhibited fatty acid oxidation. VLDL synthesis and secretion depend on the breakdown of LD-TG stores, as 70% of VLDL-TGs are derived from lipolysis and re-esterification of LD-derived fatty acids. Chronic alcohol consumption slows lipophagy, the lysosomal degradation of LDs and retards subsequent lipolysis, by cytosolic and lysosomal lipases, of LD-derived TGs.[28,32–36] Fatty acid oxidation, which occurs inside the mitochondrion, is disrupted by both alcohol-induced mitochondrial damage and defective regulation of genes that encode enzymes of fatty acid oxidation.[37] As described in a recent review, EtOH-induced deficiencies in lipid and LD catabolism in lysosomes contribute significantly to the pathogenesis of alcohol-induced fatty liver.[35]

4.2. Proteopathy

Cellular proteins comprise the most diverse class of macromolecules. Protein possess widely varied primary structures and perform vital functions by catalyzing countless biochemical reactions, facilitating transport, driving cell motility, and transducing cellular signals. Intracellular proteins continuously undergo turnover (synthesis and degradation), each at a different, but essentially constant rate. Proteins with shorter half-lives with rapid rates of degradation generally have high rates of synthesis. The converse is true of proteins with low rates of turnover.[38] Such constancy is essential for cell survival, not only because the proteins, themselves, are sources of cellular energy, but also because certain proteins with very short half-lives (*e.g.*, transcription factors) must be used and then rapidly degraded to prevent their "inappropriate re-use", which would prolong the expression of certain genes.[39,40] Proteins, are also easily oxidized, when their constituent amino acid residues react with intracellular oxidants, including superoxide, hydroxyl radicals and by adduct formation with other ROS, including MDA, peroxynitrite (PN), 4-HNE, acetaldehyde and MAA (see Section 3.3). Such reactions can render proteins biologically inactive, by causing conformational disruptions which, in turn, trigger their rapid degradation. Interestingly, the total cellular protein content in most tissues remains essentially constant, a sign that tissue proteostasis is maintained and protein metabolism is normal. However, deviations from this "normal" value signify intracellular changes that, if sustained, become pathological consequences, during which proteostasis is supplanted by proteopathy. Therefore, besides fat buildup, (described in Section 4.1), alcohol-induced liver enlargement reflects hepatic protein accumulation. Lipids and proteins each contribute about half the additional liver mass in alcohol-fed rats.[41]

4.3. Sources of proteopathy

Our early investigations examined hepatic protein synthetic activity *ex vivo* (in liver slices), and *in vivo* (in live animals).[42,43] Both studies clearly indicated that hepatic protein synthetic activity in EtOH-fed rats is unchanged or is even lower than in control animals. Hence, protein accumulation in livers of EtOH-fed rats is not caused by enhanced protein synthesis, as reported in earlier work.[44] However, it should be noted that the latter-cited study, showed that chronic EtOH consumption causes hepatic retention of secretory proteins destined for export. This was a key finding that prompted further research, which established that acute and chronic EtOH consumption disrupt hepatic protein secretion (exocytosis) as well as intracellular trafficking events, including endocytosis and autophagy.[45–49]

Early investigations with alcohol-fed rats, reported that hepatic microsomes from these animals have longer half-lives than those of controls.[50] Later studies confirmed that chronic EtOH administration retards the catabolism of long-lived hepatic proteins by 37%,

resulting in a 26% rise in total hepatic protein mass.[48] Long-lived proteins possess half-lives of 24–72 h. Many of these are degraded in lysosomes.[51,52] Later studies confirmed that EtOH-fed rats exhibit slower rates of hepatic proteolysis, associated with lower volume densities of (*i.e.*, fewer) autolysosomes, as assessed by electron microscope morphometry.[49] Subsequent studies revealed that purified hepatic lysosomes from EtOH-fed rats have lower capacities for degrading their endogenous proteins.[53] The same lysosome preparations also exhibit faulty degradation of exogenous radiolabeled ribonuclease, a protein with a chaperone-recognition motif that targets it to the lysosome for chaperone-mediated autophagy (CMA).[54] The latter findings were found to be due, in part, to lower hepatic intralysosomal contents of, cathepsins B and L, in EtOH-fed rats than in controls.[53] These and other findings (given in the next two sections) clearly show that EtOH-induced hepatic protein accumulation arises from defective proteolysis by lysosomes and by impaired proteasome activity.[55] Fig. 1 summarizes the EtOH-induced changes in oxidant levels, which impact these catabolic systems.

5. Lysosome structure and function

5.1. Early work by Christian De Duve and Yoshinori Oshumi

"Lysosome", Greek for "digestive body" is the term coined by Christian De Duve to describe the organelle he discovered, which contains a pool of acid hydrolases.[56,57] De Duve's early work addressed the subcellular distribution of metabolic enzymes in rat liver.[58] During these investigations, De Duve and his colleagues followed the distribution of a non-specific acid phosphatase and found that the enzyme performed a "vanishing act", as it exhibited abnormally low activity in rat liver homogenates, but when it was re-assayed after 5 days of cold storage, its activity was higher. From this, De Duve concluded that acid phosphatase was enclosed in "membrane sacs" that prevented its access to the substrate. However, conditions such as "ageing" at low but above-freezing temperatures, allowed the enzyme to diffuse through the damaged membranes, leading to a rise in its activity in the same homogenates. This prompted De Duve to isolate the "membrane sacs" using differential centrifugation. This effort, culminated in the discovery that the "membrane sacs", which later were called "lysosomes", are major sites for intracellular macromolecular digestion.

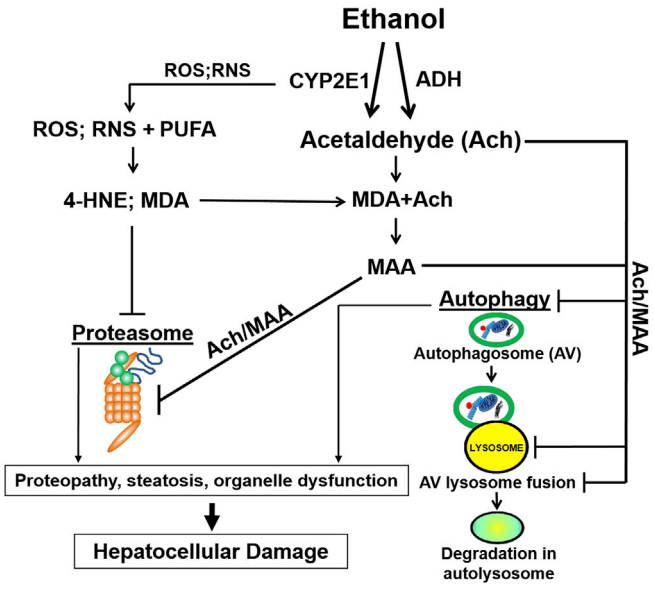

Fig. 1. Ethanol metabolism and hepatic oxidant stress. Ethanol is oxidized in hepatocytes by ADH, CYP2E1, and catalase (not shown), each forming acetaldehyde (Ach). Ach covalently binds to proteins or can react with MDA, to form MAA which also forms adducts with proteins (see text). Ethanol exposure induces the content of CYP2E1, which produces greater quantities of Ach, ROS and reactive nitrogen species (RNS). The latter two species can undergo secondary reactions with polyunsaturated fatty acids (PUFA), producing lipid peroxides, including 4-HNE and MDA. Both these can also form adducts with proteins. Ach (and MAA) can form adducts with tubulin to inhibit its polymerization. Such inhibition impairs the movement of autophagosomes (AVs) to lysosomes that degrade AV cargo during autophagy. Further, Ach and MAA can interfere with lysosome biogenesis, thereby exacerbating autophagy slowdown. Ach, MAA, MDA and 4-HNE may also form adducts with proteasome subunits, causing a decline in its ability to degrade proteins. Thus, the decline in lysosome number, vesicular movement, and proteasome activity contribute to protein accumulation (proteopathy), and LD accumulation (steatosis), ultimately resulting in organelle dysfunction and hepatocellular damage. Abbreviations: ADH, alcohol dehydrogenase; CYP2E1, cytochrome P450 2E1; MDA, malondialdehyde; MAA, malondialdehyde-acetaldehyde; ROS, reactive oxygen species; 4-HNE, 4-hydroxynonal.

Lysosomes are the terminal degradative compartment of the endocytic and autophagic pathways, found in nearly all mammalian cells. The lysosomal matrix (interior) is acidic, with a pH of ~4.7.[62] It contains about 60 specialized acidic hydrolases, including phosphatases, nucleases, glycosidases, proteases, peptidases, sulphatases and lipases that digest most cellular macromolecules. Ultrastructurally, lysosomes appear as dense bodies in the cytoplasm. They are morphologically heterogeneous, due to variations in the content of internalized and/or partially degraded material. Therefore, they appear spherical, ovoid or occasionally tubular in shape. Their size differs, depending on the cell type. In most cells, including hepatocytes, lysosomes are typically between 0.2 and 0.8 μm in diameter, but, in macrophages, they can exceed several microns. Depending on the cell type, the number of lysosomes also varies between 50 and 1000 lysosomes/cell. Their combined volume accounts for 0.5–15% of the total cell volume.[63]

Lysosomes are limited by a single 7–10 nm phospholipid-bilayer.[63] Their membranes are rich in carbohydrate, due to heavily glycosylated lysosomal membrane proteins. Some of the most abundant lysosomal membrane proteins include lysosome-associated membrane proteins (LAMP)-1, LAMP-2, lysosomal integral membrane protein-2, and CD63.[64]

5.3. Lysosome biogenesis

Proteins destined for placement into the lysosome are synthesized as glycoprotein precursors in the rough endoplasmic reticulum (ER) and transported to the *trans*-Golgi network (TGN) from which, they follow the direct intracellular pathway to reach the

As part of describing lysosomal function, De Duve also coined the term "autophagy" (self-eating) to distinguish degradation by lysosomes of intracellular components from "heterophagy" (eating others) which is lysosomal degradation of extracellular substances. While De Duve was instrumental in proposing the cellular process of autophagy, it was Yoshinori Oshumi's efforts using genetics to characterize the major proteins that drive autophagy (Fig. 2) and that led to major breakthroughs in autophagy regulation, contributing to further understanding of the functional role of autophagy in health and disease.[60,61]

5.2. Morphology of lysosomes

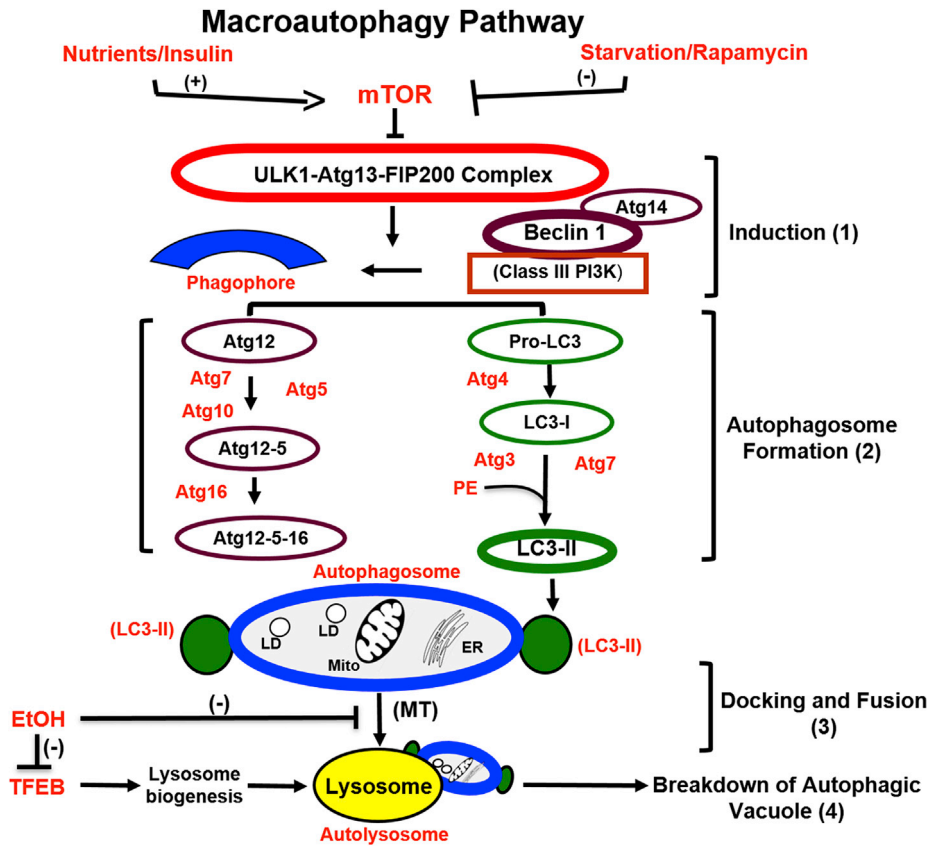

Fig. 2. Macroautophagy pathway. The mammalian target of rapamycin (mTOR) suppresses autophagy when nutrient supply is adequate. Starvation or rapamycin treatment block mTOR and release the suppression of autophagy causing its activation **(1)** by formation of the ULK1-Atg13-FIP200 complex, which, in the presence of the phagophore (the autophagosome precursor membrane) triggers initiation of **(2)** autophagosome formation. This involves the formation of coordinated complexes among beclin 1, Atg 14 and class III phosphatidylinositol 3-kinases (PI3K) conjugation systems that enable lipidation with phosphatidylethanolamine (PE) of microtubule-associated protein 1 light chain 3 (LC3I) to form LC3II. Proteins and organelles (LDs, endoplasmic reticulum (ER), and mitochondria (Mito)) are sequestered within the autophagosome. The latter undergoes fusion with lysosomes (generated by lysosome biogenesis) **(3)** to form an autolysosome, which is the degradative organelle. The contents of the autolysosome are acidified and then degraded by lysosomal hydrolases **(4)**. Fig. 2 also shows specific points in the pathway inhibited by chronic EtOH consumption, as shown by "EtOH". These are lysosome biogenesis and the movement of autophagosomes to lysosomes via microtubules (MT). Abbreviations: ULK1, unc-51 like autophagy activating kinase 1; Atg-13, autophagy-related protein 13; FIP200, focal adhesion kinase family interacting protein of 200 kD.

lysosome compartment.[65] The best-characterized pathway is the clathrin–coated vesicular transport of lysosomal precursors by the mannose-6-phosphate receptor (M6PR).[58,66] Upon arrival in the TGN, most lysosomal precursors are phosphorylated on their mannose moieties to form mannose-6-phosphate (M6P) residues. These are recognized by M6PR in the TGN. Two types of M6PRs, a 46 kDa cation-dependent and 300 kDa cation-independent M6PR, both bind to and segregate newly-synthesized lysosomal precursor proteins from the secretory pathway and re-route them to the lysosomal biogenetic pathway. The M6PR-precursor complexes, sequestered into clathrin-coated vesicles, exit the TGN and fuse with lysosomal structures. Although it is not clear whether the transport of lysosomal enzyme precursors merges with the endocytic pathway, it is believed that TGN-derived transport vesicles may also deliver cargo to late endosomes (LE) either directly or through the early endosomes. The transport from LE to lysosomes is thought to occur in several different ways: some possibilities are that the LE themselves or a portion of them mature into lysosomes, or that lysosomal precursors from the LE may be packaged into transport vesicles, which may then fuse with pre-existing lysosomes. Alternatively, LE and pre-existing lysosomes may either transiently fuse to exchange contents or fuse permanently to form a transient hybrid organelle which may re-form into dense-core lysosomes. In any case, the acidic pH of the endosomes (specifically LE) and lysosomes facilitate the dissociation of M6PR from its ligand and the M6PR recycles to the TGN for the next round of transport of lysosomal precursor molecules.[67]

5.4. Lysophagy: breakdown of lysosomes by lysosomes

Lysosomal membrane rupture releases considerable amounts of cathepsins, protons or calcium from the lysosomal compartment into the cytoplasm. Such leakage of lysosomal components reportedly induces inflammation and cell death.[68,69] Numerous endogenous and exogenous factors induce lysosomal membrane damage. These include oxidant stress, intralysosomal Fenton reaction, proteases, photodamage, silica or urate crystals, lysosomotrophic drugs, bacterial or viral toxins, certain lipid species and phytochemicals.[68] Cells have evolved protective mechanisms to prevent lysosomal rupture by degrading damaged or obsolete lysosomes by lysophagy. This autophagic mechanism clears not only destabilized and "leaky" lysosomes, but also membrane remnants that result from complete lysosome rupture and that may be harmful by contributing to the inflammatory response.[69]

5.5. Transcription factor EB (TFEB): master regulator of autophagy and lysosome biogenesis

Genes encoding proteins that regulate lysosomal biogenesis and autophagy (Fig. 2) are governed by TFEB. The latter is a member of

the basic-helix-loop-helix leucine-zipper transcription factor microphthalmia transcription factor/transcription factor E (MiTF/TFE) family. It targets promoters of the coordinated lysosomal expression and regulation (CLEAR) motif to activate gene transcription. Some TFEB-regulated lysosomal genes include those encoding LAMP1, LAMP2, lysosomal acid lipase (LAL), cathepsins, β-hexosaminidase, and M6PR.[70–72] Cells that overexpress TFEB have higher lysosome numbers with enhanced hydrolytic capacities. Conversely, TFEB-deficient cells exhibit reduced lysosome numbers and autophagy deficiency, as TFEB also governs the biogenesis of autophagic vacuoles (AVs), their fusion with lysosomes and the rate of autophagic flux. Thus, TFEB is the "master regulator", which coordinates autophagy with lysosome biogenesis. TFEB is transcriptionally inactive after phosphorylation of serines 142 and 211 in its primary structure, by the mechanistic target of rapamycin C1 (MTORC1) and by the mitogen-activated protein kinase, extracellular and signal regulated kinases 1/2 (ERK1/2). Phosphorylated TFEB (P-TFEB) cannot translocate from the cytoplasm to the nucleus. The levels of P-TFEB in the cytoplasm are closely regulated by the UPS, which selectively degrades P-TFEB after it is ubiquitylated by STIP1 homology and U-Box containing protein 1 (STUB1), a chaperone-dependent E3 ubiquitin (Ub) ligase.[73]

5.6. EtOH and lysosome biogenesis

Because EtOH exposure impairs vesicular trafficking in the liver, lysosomes are affected via impaired delivery of both their constituents as well as their cargo as described in Section 4 and in the sections that follow:

5.6.1. Depletion of M6PR

In most cell types, M6PR is the predominant receptor for transport and delivery of lysosomal enzymes into lysosomes. Effective binding by M6PR to lysosomal precursors and adequate amounts of M6PR are crucial for normal delivery of precursors necessary for lysosome maturation. In chronic EtOH studies, we observed that, compared with hepatocytes from control rats, cells from EtOH-fed rats exhibit a 50% decline in ligand binding by the M6PR. This was associated with lower M6PR protein content in cells of alcohol-fed rats.[74] The apparent decrease in receptor content was due to a decline in M6PR synthetic rate compared with control hepatocytes. Interestingly, chronic EtOH feeding did not quantitatively affect the mRNA that encodes M6PR, suggesting that defective translation of M6PR mRNA contributes to reduced receptor content in hepatocytes of EtOH fed rodents. These findings suggest that EtOH-induced downregulation of M6PR activity and content in hepatocytes contributes to impaired processing and delivery of lysosomal precursors necessary for lysosome biogenesis.

5.6.2. Depletion of lysosomal cathepsins

Decreased M6PR synthesis in EtOH fed rodents is associated with a simultaneous decrease in lysosomal cathepsin activities. Cathepsins are intralysosomal proteases that optimally degrade proteins at the acidic pH of the lysosomal matrix. Cathepsins B, L, and H constitute the most abundant proteases in lysosomes. In livers of rats given chronic EtOH feeding, the catalytic activities of all three cathepsins are significantly reduced.[53] Chronic EtOH administration not only decreases cathepsins B and L activities, but also reduces their absolute amounts detected in isolated, purified lysosomes. Our isotopic immunochemical analyses of cathepsin L in rat hepatocytes revealed that the cause of intralysosomal cathepsin L deficiency in EtOH-fed rats was slower trafficking of its procathepsin L precursor to the lysosome compartment and delayed proteolytic maturation of procathepsin L to its active form.[75]

5.7. EtOH and TFEB

TFEB and autophagy are differentially regulated by acute *vs.* chronic EtOH administration. Ding *et al.*[76] reported that acute EtOH gavage to mice enhances autophagy by inhibiting MTORC1 signaling. They also demonstrated that LDs and mitochondria in livers of EtOH-fed mice rather selectively undergo lipophagy and mitophagy, respectively. They later showed that acute alcohol-induced mitophagy is partially regulated by Parkin, an E3 ligase that ubiquitylates mitochondrial membrane proteins prior to their degradation.[77] Ding's group also showed that acute EtOH treatment increased the nuclear translocation of forkhead box O3A (FoxO3a), another transcription factor that activates certain autophagy genes.[78] Our own single-gavage acute ethanol studies confirmed that this treatment augments the nuclear content of TFEB and heightens autophagic indices.[79]

In contrast to acute EtOH treatment, hepatocytes isolated from chronically EtOH-fed rodents exhibit higher autophagosome (AV) numbers and lower lysosome numbers than cells from pair-fed controls.[79] The EtOH-induced decline in lysosome numbers is associated with a reduction in AV-lysosome fusion (Fig. 3). AV-lysosome co-localization events are several-fold lower in hepatocytes of EtOH-fed animals, indicating that higher AV numbers in livers of these animals results from AV accumulation, caused by insufficient lysosome numbers from faulty biogenesis (Fig. 3). Consistent with these findings, higher levels of the adaptor protein, P62/SQSTM1 (P62) are detected in livers of EtOH-fed rodents.[79] P62, which is ultimately degraded in lysosomes, is an indicator of the degree of lysosomal proteolysis. The EtOH-induced rise in P62 indicates that the protein is stabilized due to its decelerated degradation, likely the result of defective lysosome biogenesis.

We have found that total hepatic levels of TFEB are unaffected after continuous (chronic) EtOH feeding with the Lieber-DeCarli diet. Others, using the chronic plus acute binge feeding model, reported that total hepatic TFEB declined after EtOH feeding.[80] This TFEB decline may be due to MTORC1 activation by chronic plus binge EtOH feeding, which could enhance TFEB degradation after its phosphorylation. However, in both EtOH feeding models, hepatic nuclear fractions from EtOH-fed rodents consistently exhibit lower TFEB levels than pair-fed controls. Moreover, lower nuclear TFEB levels in livers of EtOH-fed animals' contrast with a simultaneous rise in cytosolic TFEB, indicating that chronic EtOH administration likely disrupts TFEB nuclear translocation by inducing its phosphorylation. In fact, we reported that chronic EtOH administration activates ERK1/2 in livers of EtOH-fed mice, indicating that ERK1/2 phosphorylates TFEB, thereby blocking its movement from the cytosol to the nucleus, and hindering expression of genes encoding proteins involved in lysosome biogenesis and autophagy. We attribute TFEB cytoplasmic accumulation in EtOH-fed rodents to its stabilization, because the activity of the proteasome, which selectively degrades phosphorylated TFEB, declines in livers of EtOH-fed animals.[79,81] In support of this, we recently reported that seven days after EtOH withdrawal, livers, from rats previously fed EtOH, had normal (control) levels of nuclear and cytoplasmic TFEB, which coincided with restoration of proteasome activity, normalization of lysosomal cathepsins B, and L, and of lysosomal acid lipase (LAL).[82] These findings lead us to suggest that reactivated proteasome degrades phosphorylated TFEB in the cytoplasm, thereby allowing nuclear localization of unphosphorylated TFEB to restore normal lysosome biogenesis and autophagy.

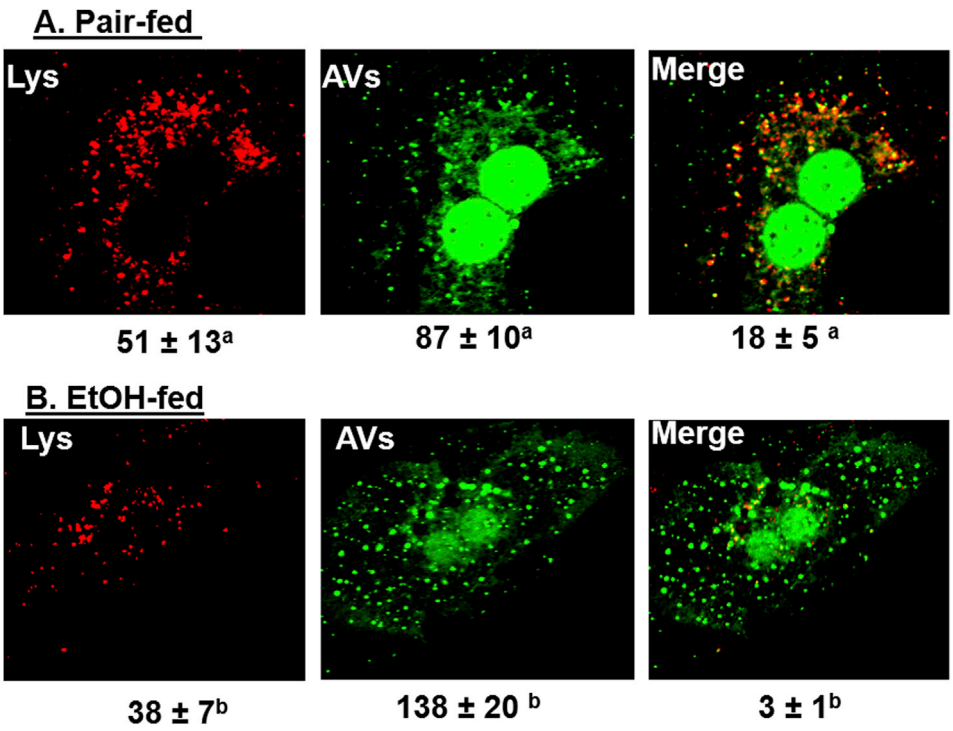

Fig. 3. Photomicrograph of hepatocytes from control and EtOH-fed mice, showing that chronic EtOH feeding increases AV numbers but decreases lysosome (Lys) numbers and AV-Lys fusion events. Micrographs of hepatocytes from chronically pair-fed and EtOH-fed green fluorescence protein (GFP)-LC3 mice were quantified for Lys (left panels), AVs (middle panels) and their co-localization (merged right panels). Numbers below each panel are mean values (±standard error) and are expressed as puncta per nucleus. Values bearing different letter superscripts are significantly different from each other. Values bearing the same letter superscripts are not significantly different. Figure re-used with permission from Thomes *et al.* Alcohol Clin Exp Res. 2015; 12:2354–2363. Abbreviation: AV, autophagosome.

6. The UPS

6.1. Discovery

While lysosomes degrade nearly all macromolecular forms, the UPS is the major pathway that regulates and executes, with precision, the catabolism of ~75% of intracellular proteins in eukaryotic cells.[83] The pathway (Fig. 4) was discovered in the early 1980s by Aaron Ciechanover, Avram Hershko, and Irwin Rose, all recipients of the 2004 Nobel Prize in Chemistry. After lysosomes were discovered by Christian De Duve in the 1950s, it was believed that all cellular proteins are degraded in these organelles. However, other investigations discovered that intracellular protein catabolism is also non-lysosomal. The revelation of the UPS in reticulocytes showed that Ub tagging and proteasome-catalyzed degradation of ubiquitin-conjugated proteins, controls the contents of short-lived cellular proteins including transcription factors, proteins that govern the cell cycle and division, and those that regulate programmed cell death (apoptosis).[83–86]

6.2. Components of the pathway

The UPS has three major components: (i) the Ub enzyme cascade (E1, E2, and E3), in which the three enzymes (each consisting of multiple isozymes with differing specificities) act in sequence to attach one or more Ub molecules to the protein substrate; (ii) two types of proteasome holoenzymes (26S and 20S) that degrade ubiquitylated and unubiquitylated protein substrates, respectively. The two holoenzymes exist in equilibrium; and (iii) multiple de-ubiquitylating enzymes (DUBs) remove Ub from each substrate protein before it is degraded.

By ubiquitylating and degrading many short-lived regulatory proteins, the UPS influences crucial mechanisms of cell survival, including, gene transcription, chromatin organization, apoptosis, endocytosis, signal transduction and immunity.[87] The proteasome is a large multi-subunit, multi-catalytic protease, located in both cytosolic and nuclear compartments of cells. Interestingly, the proteasome was recently reported to exhibit extracellular localization, after being released from cells in extracellular vesicles (EVs). The latter are believed to participate in macromolecular exchange between cells from adjacent organs.[88,89]

6.3. Ubiquitylation of substrate proteins

The 26S proteasome works in conjunction with Ub, an 8.5 kDa polypeptide, which “labels” protein substrates destined for degradation. Ubiquitylation of substrate proteins proceeds by a cascade of three classes of enzymes: E1s are Ub-activating enzymes that activate Ub, using ATP to adenylate Ub and transfer it to an E2, one of the Ub conjugating enzymes. E2 enzymes carry Ub to the protein substrate, where a substrate-specific E3 ligase catalyzes the covalent attachment of the carboxyl-terminal glycine of Ub to either the free amino terminus or to the epsilon-amino group of an internal lysine residue on the substrate protein. Ubiquitylation of substrate proteins allows their recognition by the 26S proteasome (Fig. 4).[90–92] Not all ubiquitylated proteins are proteasome substrates. The 26S proteasome rather selectively recognizes polyubiquitylated substrates on which a chain of Ub monomers is attached to the substrate, with each Ub monomer linked to the next Ub molecule via its lysine 48 in a “K48” configuration degradation signal. Another Ub configuration on proteins is K63, which directs the protein substrate to the autophagy-lysosomal hydrolytic

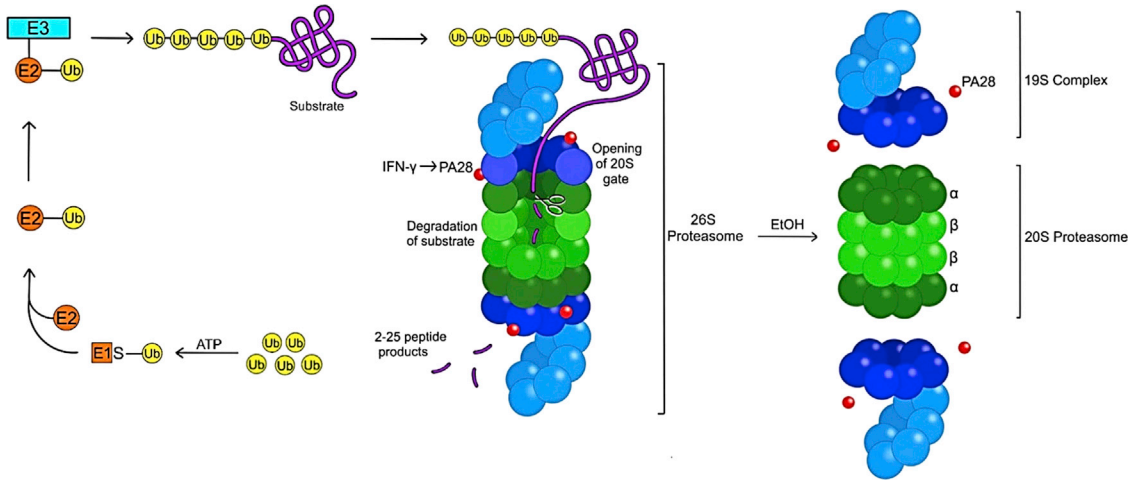

Fig. 4. Schematic representation of the UPS. The proteasome consists of a catalytic core (20S proteasome) and two regulatory caps (19S complexes). The core resembles a barrel–like structure, consisting of four stacked rings with beta (β) subunits, the catalytic sites for peptide bond cleavage. Alpha (α) subunits provide structural support. Two 19S cap particles recognize and bind the ubiquitylated protein substrate destined for degradation and facilitate substrate entry into the center of the catalytic core, while removing ubiquitin chains. Inside the catalytic core, the protein substrate is hydrolyzed to peptides, which can then be further degraded to amino acids by peptidases. Proteins to be degraded by the proteasome first must be marked by the addition of at least four ubiquitin (Ub) molecules linked together in a K48 configuration. Transfer of Ub to the target protein is catalyzed by ubiquitin-activating enzymes (E1), conjugating enzymes (E2), and ligating enzymes (E3). This process requires energy, which is provided in the form of adenosine triphosphate (ATP), the cell's primary energy source. The figure also shows points which are affected by EtOH, interferon gamma (IFN-γ) or the dissociable activator PA28. Abbreviations: UPS, ubiquitin-proteasome system; EtOH, ethanol.

system for degradation. More of these linkage-specific Ub signals are detailed elsewhere and have come to be known as the "Ub code".[93]

6.4. Structures and functions of 20S and 26S proteasomes

The 20S proteasome is a 700 kDa barrel-like hollow structure, which has two outer rings, each with seven alpha (α) protein subunits and two inner rings, each with seven beta (β) protein subunits.[94] Alpha subunits govern proteasome conformation, while β subunits confer catalytic activity. Based on the compositions of their catalytic subunits, proteasomes are classified into constitutive, immunoproteasome and intermediate enzymes. A constitutive, or "standard" 20S proteasome contains two β1 subunits, possessing caspase-like activity, catalyzing peptide bond cleavage after (on the carboxyl side of) acidic amino acids; two β2 subunits, having trypsin-like activity and cleaving after basic amino acids and two β5 subunits with chymotrypsin-like activity, cleaving peptide bonds after hydrophobic residues.[95] Immune cells and hepatocytes exposed to pro-inflammatory cytokines, including interferon gamma (IFN-γ) express the immunoproteasome, which contains β1i (LMP2), β2i (MECL-1) and β5i (LMP7) subunits, which replace the β1, β2 and β5 subunits, respectively. Incorporation of immunoproteasome subunits into the 20S enzyme allows it to hydrolyze antigenic proteins, so that the chain lengths of the antigenic peptide products are uniform and less variable. Bacterial and viral infections cause significant enhancement in LMP7 expression, resulting not only in replacement of constitutive subunits with immuno-subunits, but also increasing overall proteasome abundance within infected tissues.[96] In the absence of infection, the immunoproteasome actively cleaves oxidized and misfolded proteins during periods of oxidant stress. This form of the enzyme exhibits greater susceptibility than the constitutive enzyme to stress-induced regulation of its activity.[97,98] The intermediate proteasome combines the features of both the constitutive and immunoproteasomes. Intermediate proteasomes are expressed in non-immune organs that exhibit multiple immune functions, such as the liver.[99]

6.5. Dissociable protein regulators of proteasome activity

"Professional" protein regulators of the proteasome are those which open the 20S proteasome α-gate to allow the protein substrate to enter the catalytic core.[100] Several such core regulators are as follows:

(i) 19S particle (RP/PA700) regulator is necessary for ATP-dependent degradation of ubiquitylated proteins and has about 19 subunits. The particle binds ubiquitylated proteins, and deubiquitylates them by the specific actions of Usp 14 and Ucb37, which unfold and translocate the substrate into the 20S core.[87] The 20S and 19S particles combine to make up the 26S (2.5 mDa) proteasome. Specific proteins serve to connect the 19S and 20S particles into the 26S enzyme such as the extracellular mutant-29 (Ecm29),[101] Suppressor of exocyst mutation-1 (Sem1) is another protein that confers proteasome stability during oxidative stress.[102]

(ii) The 26S proteasome not only detaches Ub from the substrates for recycling, but also "senses" FAT10-conjugated proteins and degrades them. FAT10 (F-adjacent transcript 10, or Ub D) is a Ub-like modifier that covalently attaches to certain target proteins. FAT10-ylation of a substrate protein requires the same cascade of ubiquitylating enzymes. However, unlike Ub, after FAT10 is attached to the target protein substrate, it is not removed but degraded along with it.[103]

(iii) PA28 (αβ) (a.k.a. 11S αβ activator) binds to the 20S proteasome in the cytosol and increases its peptidolytic activity. PA28 is IFN-inducible and engaged in antigen presentation by regulating cleavage of antigenic proteins by the cytosolic proteasome.[104] Interestingly, the nuclear proteasome is also activated by PA28γ (11Sγ) which works in an IFNγ-independent fashion, exclusively by increasing the trypsin-like activity of the proteasome.[105] Along with 11S αβ, 11Sγ regulates degradation of oxidized proteins.

(iv) Another nuclear proteasome activator, which activates its caspase-like activity is PA200, which activates ATP/Ub-independent degradation of acetylated core histones during

DNA damage and repair in somatic cells.[106] The 20S proteasome usually exists in the form of a "hybrid" enzyme (19S-20S-PA200) in which the 20S enzyme is regulated by both 19 S and PA200.

(v) A putative proteasome inhibitor, PI31, inhibits the hydrolysis of proteins by the 20S proteasome and regulatory activation by 19S and PA28.[107]

6.6. Proteasome-interacting proteins (PIPs)

Besides "professional" 20S proteasome regulators, there are numerous PIPs that are not exclusively proteasome-specific but bind to the enzyme to regulate its activity.[108] These include heat shock proteins (HSP) 70 and 90, which facilitate delivery of aggregation-prone substrates to the proteasome. HSPs are induced by multiple factors (heat, prostaglandins, proteasome inhibitors, and drugs to compensate for decreased proteasome activity.[109–111] Other PIPs include p62, which presents polyubiquitylated proteins to the proteasome; the phosphatase protein phosphatase 2 (PP2A) and protein kinase A which, together, regulate 20S proteasome activity by dephosphorylation/phosphorylation and by binding to its regulatory complexes. In addition, some cytokines, mostly interferons, substantially enhance proteasome activity by activating PIPs.[112]

6.7. EtOH consumption and the UPS

Acute EtOH gavage to rodents has no effect on proteasome activities, but *ex vivo* exposure of precision-cut rat liver slices (PCLS) to 50 mM EtOH for 12 and 24 h suppresses proteasome activity by 1.5–3 fold. This decline is blocked by 4-methylpyrazole, indicating that proteasome activity is inhibited by the products of EtOH metabolism, not by EtOH alone. The same study demonstrated that liver proteasome and autophagosome content in liver cells are reciprocally regulated by EtOH treatment as autophagosomes rise, while proteasome activity falls after EtOH exposure.[81]

Chronic EtOH consumption given by intragastric feeding to rats causes a 35–40% decline in chymotrypsin-like (ChT-L) and trypsin-like (T-L), but not peptidyl-glutamyl-peptide hydrolase proteasome activities. However, voluntary *ad lib* chronic feeding with the Lieber-DeCarli diet did not affect peptidase activities in these earlier studies.[55] However, "second hits", such as dietary supplements, genetic factors or expression of viral proteins, potentiate the effects of EtOH on the proteasome. In this regard, rats given EtOH or isocaloric dextrose-containing high-fat liquid diet containing corn oil, fish oil and other components by intragastric infusion for one month produced a 55–60% decline in the proteasome ChT-L activity which correlated with oxidative stress markers expression and the severity of liver injury.[113]

Another example of a "second hit", which suppresses proteasome activity is infection with hepatotropic viruses, including hepatitis C virus (HCV). Such infection can be mimicked by the expression of viral proteins in livers of transgenic mice that express the non-structural HCV protein, NS5A. The mode of chronic EtOH feeding (*i.e.* in Lieber-DeCarli liquid diet or in the drinking water) affects the magnitude of the decrease in proteasome activity. These transgenic mice fed EtOH in a liquid diet had much higher levels of oxidant stress, which was accompanied by a more potent decline in proteasome ChT-L activity in both liver cytosolic and nuclear fractions, compared with mice given 20% EtOH in the drinking water.[114] In another *in vivo* study with mice expressing HCV structural proteins, 5-week feeding with 20% EtOH in the drinking water potentiated the suppression of ChT-L proteasome activity up to 50% in both crude liver homogenates and purified 26S proteasome, while ChT-L proteasome activity in EtOH-fed HCV-negative littermates declined only 20%.[115] We also observed a similar suppression of proteasome ChT-L activity in EtOH-fed chimeric severe combined immunodeficient (SCID) Alb-uPA mice transplanted with human HCV-infected hepatocytes.[116]

6.8. Mechanisms of the proteasome activity regulation by alcohol

There are multiple mechanisms of proteasome regulation by EtOH.

6.8.1. Bi-phasic regulation of proteasome activity by oxidant stress

In vitro experiments with HepG2 cell lines, that overexpress CYP2E1 and ADH, revealed that EtOH, metabolites, but not EtOH alone, modulate proteasome activities, as EtOH-elicited reduction of proteasome activity is blocked by 4-methylpyrazole, an inhibitor of EtOH oxidation.[117] EtOH metabolism induces oxidant stress. We observed that direct exposure of purified proteasome to the oxidant PN ($ONOO^-$) has a biphasic effect on proteasome activity; low levels of PN enhance enzyme activity, while high PN levels suppress it. This was confirmed *in situ* by creating differential oxidative stress levels in CYP2E1-expressing HCV^+ hepatoma cells treated with 10–100 μM *tert*-butyl hydroperoxide (t-BOOH) for 5 h and by exposure of purified 20S proteasome (containing PA28 activator) to mitochondrial and microsome fractions obtained from livers of control and EtOH-fed rodents. Low concentrations of t-BOOH as well as mitochondrial and microsome fractions from livers of control rats increased proteasome activity, but higher concentrations of t-BOOH and the same fractions from alcohol-fed animals suppressed its activity.[118] Similar bi-phasic regulation of proteasome activity was observed upon treatment of HepG2 cells with various doses of PN, which is released from mitochondria after EtOH exposure.[119]

6.8.2. Role of CYP2E1

In vivo studies reveal that EtOH-induced oxidant stress is involved in proteasome suppression.[120–122] It has become clear that EtOH-enhanced CYP2E1 expression and catalysis have important regulatory roles in proteasome activity modulation. Chlormethiazole administration, which blocks CYP2E1 activity in EtOH-fed rats, also blunts the EtOH-induced reduction in hepatic proteasome activity, to suggest that CYP2E1-generated oxidants cause proteasome suppression.[123] Further, liver proteasome activity is unaffected in alcohol-consuming *CYP2E1* knockout mice, supporting the notion that CYP2E1-generated ROS suppress proteasome activity.[124] Importantly, while CYP2E1 induction by EtOH suppresses proteasome activity, the latter suppression stabilizes hepatic CYP2E1 levels. This was demonstrated using the proteasome inhibitor PS-341 (Bortezomib), which stabilized CYP2E1 in livers of EtOH-withdrawn rats.[120] Both this and a study by Roberts *et al.*[14] demonstrated that CYP2E1 is ubiquitylated and degraded by the proteasome. As suggested by computational studies, CYP2E1 is postulated to form a complex with HSP90. EtOH inhibits such complex formation, thereby limiting exposure of microsomal CYP2E1 to the cytosolic proteasome.[125] Another recent study showed that intragastric EtOH feeding to mice enhances expression of the small ubiquitin-like modifier (SUMO)-conjugating enzyme, UBC9, which SUMOylates CYP2E1, thereby stabilizing the enzyme.[126]

6.8.3. Role of acetaldehyde

CYP2E1-catalyzed, EtOH oxidation generates acetaldehyde,[127] which reportedly suppresses proteasome activity, as demonstrated by a chronic EtOH vapor inhalation study that induced high blood alcohol levels (BAL) and high levels of acetaldehyde in Wistar

rats (and confirmed by *in vitro* experiments). These studies suggested that ethylated cytosolic proteins (*i.e.,* cytosolic proteins covalently bound to acetaldehyde) inhibit proteasome activity. Furthermore, exposure of purified proteasome to acetaldehyde inhibits its activity and reduces its ability to degrade ethylated proteins.[128] Our laboratory also reported a negative correlation between high BAL and lower proteasome activities in chronically EtOH-fed rodents.[129] An acetaldehyde-induced decline in proteasome activity was further supported by *in vitro* experiments in which HepG2 cells were exposed to an extracellular acetaldehyde-generating system (AGS). This system contains EtOH, the cofactor NAD, yeast ADH and generates physiologically-relevant amounts of acetaldehyde without causing significant cell toxicity.[130]

6.8.4. Disassembly of 26S proteasome and the role of protein aggregation

Oxidant stress usually causes disassembly of the 26S proteasome by separating the 20S and 19S particles. This results in a higher level of Ub-independent degradation of oxidized proteins by the 20S enzyme.[100] This is not a rare event, as under normal conditions about 30% of mammalian proteasomes exist as 26S particles.[131] About 20% of cellular proteins undergo 20S proteasomal cleavage under normal conditions, but during oxidant stress, the 20S enzyme form becomes the major degradation machinery because it is more resistant than the 26S enzyme to such stress conditions and degrades oxidatively modified proteins more efficiently. ATP deficiency that is associated with acute or chronic EtOH-induced impairment of hepatic mitochondria decreases 26S proteasome activity, while it increases 20S proteasome activity within 30 min after the onset of oxidant stress.[132] Furthermore, after 3–5 h of oxidant stress, suppression of 26S proteasome activity (due to its disassembly) is accompanied by accumulation of ubiquitylated and oxidized proteins, followed thereafter by 20S proteasome activation to remove the oxidized proteins. Following removal of oxidant stress, 26S proteasome reassembly occurs, and degradation of ubiquitylated substrates resumes. If the duration of oxidant stress is longer than 12 h, *de novo* synthesis of constitutive and immunoproteasome subunits resumes, followed by association of the 20S proteasome with either activators or the 19S regulatory complex. Thus, a switch from the 26S to 20S forms of the proteasome takes place during oxidant stress, with a return to the 26S form after stress is removed.[132]

One reason why EtOH exposure causes dissociation of the 19S and 20S particles is that it depletes ECM29 protein, which normally connects the two particles.[108,115,133] Because 26S proteasome disassembly prevents the degradation of Ub-protein conjugates, ubiquitylated proteins accumulate in livers of chronically EtOH-fed rats. We reported earlier that chronic EtOH administration does not affect the activities of hepatic ubiquitylation enzymes.[134] Additionally, heavy alcohol consumption by humans increases the level of Ub and Ub-protein conjugates in the sera of cirrhotic patients.[135] Furthermore, such conjugates are frequently found in liver Mallory-Denk (M-D) bodies, histological markers of alcoholic hepatitis. M-D bodies also contain the HSPs 70, 90 and 25, certain 26S proteasome subunits and cytokeratin. M-D proteins represent aggresomes left undegraded by the proteasome as a result of its inhibition in intragastrically EtOH-fed rats or after exposure to a proteasome inhibitor.[55,136] Proteasome-resistant aggregates are thought to be degraded by autophagy in lysosomes. However, alcohol-induced lysosome deficiency, as previously detailed in Section 5 would potentially prevent such degradation to exacerbate liver injury.

P62 as well as mutations in Ub also contribute to aggresome formation.[137] Thus, in liver cells, mutant Ub UBB+1, which cannot be removed from substrate proteins by the 19S particle, accumulates in Mallory bodies after exposure to various toxins, including EtOH.[138] Importantly, EtOH exposure to mice disrupts deubiquitylation of substrate proteins by decreasing expression of the 19S-associated deubiqutylating enzyme, UCHL5.[108,115,133] Chronic EtOH feeding to mice also suppresses expression of PA28α, which normally activates the 20S proteasome. The reduction in PA28α expression is associated with lower 20S ChT-L proteasome activity.[115] Because activation of the 20S proteasome by PA28α is IFN-γ-dependent, and because alcohol exposure suppresses IFN-γ signaling via the janus kinase-signal transducer and activator of transcription 1 (JAK-STAT1) pathway, EtOH-reduced PA28α expression is negatively regulated by impaired transduction of the IFN signal in hepatocytes.[139]

6.8.5. Posttranslational modifications of the proteasome

Proteasome activity is modified by co- and post-translational modifications of its subunits, including phosphorylation, sumoylation, acetylation, myristoylation, glycosylation, and ADP ribosylation.[140] EtOH oxidation-induced post-translational modifications of proteasome protein subunits affect its enzyme activity. This was shown by 2 dimensional Western blot analysis of 26S and 20S proteasomes purified from livers of chronically EtOH-fed rats. Proteasome α-subunits C9/α 3 and C8/α 7 become hyperphosphorylated, which reduces ChT-L proteasome activity. Similar proteasome suppressing effects were achieved by treatment with okadaic acid, a protein phosphatase 2A (PP2A) inhibitor.[141] Exposure to EtOH also appears to induce nitration of proteasomal proteins. We reported that nitration of purified proteasome causes a biphasic effect on its enzyme activity.[119] Such dose-dependent effects of PN were confirmed by the kinetics of protein nitration in mice exposed to molsidomine, a compound that forms PN when it is metabolized in liver cells. As shown recently, an oxidant stress-associated decrease in proteasome activity is also associated with 4-HNE adducts on the α7 subunit of the 20S enzyme.[142] It is worth noting that the proteasome likely regulates the levels of 4-HNE-adducted proteins, which are FAT10-ylated and targeted for proteasomal degradation. Using transgenic mice that express HCV proteins and fed an EtOH-containing diet, employing the acute-on-chronic (NIAAA) feeding model, we demonstrated that while IFN-α injection increased FAT10 mRNA, the expression of FAT10 protein in hepatocytes was suppressed by EtOH.[143] This impairs FAT10-ylation of oxidized proteins and leads to accumulation of 4-HNE-adducted proteins.[143]

Covalent modifications of proteasomal protein substrates make them more susceptible to degradation by the proteasome. Hence, nitration by PN of lysozyme, an often-used proteasome substrate, enhances its susceptibility to proteasomal degradation, compared with unmodified lysozyme.[144] Also, reactive aldehyde- and 4-HNE-modified proteins exhibit greater susceptibility to proteasomal degradation if they are mildly cross-linked. However, extensive modification causes their aggregation rendering them proteasome-resistant.[145]

Cellular methylation status regulates proteasome activity. We reported that a reduction in the methylation index (*i.e.*, a lower S-adenosylmethionine (SAM) to S-adenosylhomocysteine (SAH) ratio) is associated with suppressed proteasome activity.[146,147] We also reported that when control and HCV^+ mice were chronically fed 20% EtOH in water, both liver SAM:SAH ratio and proteasome activity were decreased. The latter was reflected by a decline in proteasomal protein methylation as indicated by lower methyl-lysine content in 26S proteasome purified from EtOH-fed HCV^+ mice.[115]

6.8.6. Proteasome protein substrates affected by EtOH consumption

The 26S proteasome degrades ubiquitylated, FAT10-ylated, and other post-translationally-modified proteins. The latter include

short-lived phosphorylated signal transduction factors, which are rapidly degraded after targeted gene activation. In virally-infected cells, the immunoproteasome breaks down viral proteins to generate peptides for antigen presentation. When EtOH metabolism suppresses proteasome activity, it stabilizes the aforementioned substrate proteins, thereby affecting cell metabolism and disrupting signal transduction. Here, we provide several examples:

(i) As mentioned previously, CYP2E1 and ADH are proteasome substrates.[148] This was demonstrated both in cultured hepatoma cell lines expressing CYP2E1, ADH or both and confirmed *in vivo*.[14,31,112,117,121,122,149] Stabilization of these enzymes by EtOH-oxidation-induced proteasome suppression can exacerbate oxidant stress and acetaldehyde production.

(ii) Some protein substrates are degraded by both the 20S and 26S proteasome forms. These include p21/Cip 1, c-Jun, c-Fos, p53, T-cell antigen receptor chain α, Fra-1 and Hif-1α.[150] Stabilization of these proteins by proteasome suppression likely affects cell proliferation, growth and differentiation.

(iii) Phosphorylated signal transduction factors that regulate inflammation, apoptosis and innate immunity (Iκβ, JAKs1-2 and STATs) are degraded by the proteasome.[150–152] Stabilization of these factors by EtOH metabolites may induce apoptosis and likely affect the innate immune response. Thus, undegraded IκB-α blocks survival signaling via the NFκB pathway.[153,154] EtOH-induced elevation of JAK-STAT1 signaling negative regulators, suppressor of cytokine signaling (SOCS) 1 and SOCS3, occurs in liver cells.[139] SOCS1 functions as an Ub E3-ligase for phosphorylated JAK2, (which is activated by IFN-γ upstream of STAT1), catalyzing JAK2 ubiquitylation for subsequent degradation by the proteasome.[155] Activated (phosphorylated) STAT3 is also a proteasome substrate. EtOH exposure blocks IL-6-induced STAT-3 phosphorylation survival signaling in hepatocytes. The latter event is implicated in alcoholic liver disease pathogenesis.[156] Degradation of other STAT factors (STAT4, 5 and 6) likely involve the proteasome as well, but the effect of EtOH on these types of signaling proteins has not been studied.

(iv) Proteasome degrades signal transduction factors that regulate lipid metabolism. Early growth response 1 (Egr-1) and sterol regulatory enzyme binding-protein 1c (SREBP-1c) are two transcription factors, which target genes that influence lipid biosynthesis.[157] Both are proteasome substrates and their levels rise in hepatocytes due to their enhanced synthesis.[158] In addition, their decreased catabolism by the proteasome during EtOH exposure likely sustains their levels.[158] Decreased catabolism may also apply to levels of growth factors that rise during alcohol-induced liver disease pathogenesis. These are platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and fibroblast growth factor (FGF).[129]

(v) In addition to host liver cell proteins, the UPS regulated the turnover of some viral proteins that infect liver cells.[159,160] Exposure to EtOH likely stabilizes these proteins, as we have shown for HCV proteins.[118]

(vi) Viral and other foreign proteins are degraded by the proteasome to small (8–12 aa) peptides to be presented on the cell surface in the context of major histocompatibility complex (MHC) class I. These cell surface complexes are recognized by cytotoxic T-lymphocytes that eliminate infected cells in order to maintain homeostasis. Peptide generation by the immunoproteasome usually occurs in an IFN-dependent manner.[161] We reported that EtOH metabolism suppresses MHC class I-dependent antigen presentation by hepatocytes by decreasing immunoproteasome expression, and activity as well as IFN signaling. Such suppression minimizes the chance for these cells to be recognized and eliminated by the immune system.[115,116,130,162]

7. Summary: functional consequences of ETOH-induced lysosome and proteasome dysfunction

It is clear that an adequate supply of lysosomes, complete with hydrolases, along with a well-functioning UPS, with fully active proteasome as the degradative component, are crucial for maintaining hepatic waste management and regulating cellular homeostasis. This review has described how excessive alcohol consumption undermines both degradative pathways.

7.1. Lysosome dysfunction

Lysosomes maintain both proteostasis and lipostasis. Their proteolytic function is well known, but lysosomes' importance in lipid catabolism was revealed rather recently, when Singh *et al.*[163] demonstrated that these organelles actively degrade hepatic LDs, by classical macroautophagy (Fig. 2). It is also important to note that lysosomes come into direct contact with and digest small LDs by microautophagy.[164] Larger (macrovesicular) LDs, in livers of problem drinkers are generated by fusion between smaller LDs, a process that is actually facilitated by heavy drinking.[28,165] Breakdown of macrovesicular LDs is believed to begin by their interaction with cytosolic lipases, which digest large LDs into smaller droplets that can eventually be sequestered into autophagosomes for fusion with lysosomes.[35]

Lysosomal degradation of proteins and LDs is impaired by an EtOH-elicited reduction in lysosome supply because of faulty lysosome biogenesis. The latter is caused by inadequate translocation of TFEB to the cell nucleus to activate transcription of CLEAR genes.[79] Earlier, we described evidence that, in chronically EtOH-fed mice, TFEB levels are higher in the cytosol and lower in nuclear compartment than in pair-fed control mice, suggesting that TFEB in EtOH-fed mice is phosphorylated to a greater degree and cannot enter the nucleus. This latter scenario has been elaborated by recent evidence that the intracellular localization of TFEB depends also on UPS activity, specifically the proteasome, and an E3 ligase STUB 1 to maintain TFEB proteostasis in the cytoplasm.[73] This is normally accomplished by the selective degradation, by the UPS, of the phosphorylated (inactive) form of TFEB.[73] However, because chronic EtOH consumption inhibits proteasome activity,[129] and enhances TFEB phosphorylation,[79] it is likely that these combined events disrupt the balance of phosphorylated (inactive) and unphosphorylated (active) TFEB in the cytoplasm, thereby limiting the translocation of active TFEB into the nucleus. However, the latter scenario, just described, must be tested.

7.2. Proteasome dysfunction

The evidence that chronic EtOH consumption inhibits the major catalytic (*i.e.*, the chymotrypsin-like and trypsin-like) activities of the proteasome is well substantiated.[55,108,117,119,136,162] This review has provided more detailed information than before on the UPS, particularly the different physical states of the 20S proteasome in its three forms: the constitutive enzyme, the immunoproteasome, and the intermediate proteasome, each of which can be converted to the 26S enzyme, that breaks down ubiquitylated proteins. Each form of the enzyme contains different catalytic subunits, which differentially affect its peptidase activities and its responses to oxidant stress produced by EtOH metabolism.

Adding to the physical qualities of the proteasome, itself, are its dissociable protein regulators, which alter the enzyme's specificity and activity. These include PA28 and PA200, which add to the complexity of proteasome regulation, as demonstrated in studies using hepatitis C core protein, PA28, and subcellular fractions derived from control and EtOH-fed animals.[118]

EtOH-elicited inhibition of lysosome biogenesis, autophagy and the proteasome require EtOH metabolism.[79,117,166] Circumstantial evidence implicates acetaldehyde as the offeding agent. However, there are significant challenges to confirming what is still assumed. Acetaldehyde is highly volatile (bp = 21 °C) and not easily detected *in vivo*. Detecting acetaldehyde or its secondary metabolite, MAA and their adducts on proteins, using immunochemical analysis has become an accepted procedure.[23] Thus, detecting such adducts with lysosomal proteins, TFEB, and/or the multiple subunits of the proteasome is feasible, but demonstrating whether these adducts cause diminished biological function or altered subcellular localization will likely require more refined techniques.

8. Therapeutic interventions

8.1. Abstinence

Abstaining from drinking alcohol remains the most effective action that people with alcohol use disorders can take to alleviate and/or minimize further liver injury from excessive drinking. Our recent animal studies revealed that EtOH withdrawal from chronically EtOH-fed rats, which were refed control diet for seven days, almost completely resolved their steatosis, and normalized hepatic lysosome and proteasome function, with a return of nuclear TFEB to normal (control) levels.[82]

8.2. Agents that enhance autophagy/lipophagy

In a recently-published review on alcohol and lipophagy, we describe therapeutic dietary (caffeine and zinc), herbal-derived (quercitin and salvioloic acid) and a repurposed anti-seizure medication (carbamazepine) that either enhance or are believed to enhance autophagy/lipophagy.[35] Some of the latter compounds were tested in alcohol studies with rodents and found to be effective in alleviating steatosis that results from either alcohol consumption or from obesity, which is closely associated with non-alcoholic fatty liver disease. The general mode of action of these compounds is to enhance lipophagy, but the mechanism by which each enhances the process appears unique. A larger study screened potential TFEB activators.[167] Out of 15,000 compounds tested, three compounds used for other purposes, digoxin, ikarugamycin, and alexidine dihydrochloride, activated TFEB, each by a distinct calcium-dependent mechanism. To our knowledge, none of these compounds has been tested in either human or nonhuman alcohol-related studies.

8.3. Proteasome activators

Because the activity of the hepatic proteasome declines after chronic EtOH administration, its activation by small molecules would seem advantageous in restoring proteostasis by eliminating proteopathy. Out of a screen of over 2750 compounds, Leestemaker *et al.*[168] used a proteasome activity probe fluorescence-activated cell sorting assay, which yielded 10 compounds that increased 26S proteasome activity. One of these was the p38 mitogen-activated protein kinase inhibitor, PD 169316, which could complicate findings in liver by producing side effects related to cell signaling. Cyclosporin A is another candidate compound that activates the 26S enzyme and has been used in previous liver studies as a therapeutic agent.[169,170] Those latter studies were unrelated to proteasome activation. Another investigation by Jones *et al.*[171] used derivatives of chlorpromazine, an antipsychotic, to activate the 20S proteasome in order to enhance the degradation of oxidized α synuclein, believed to form aggregates in neurons. Given recent emphasis on neuronal health and the importance of the proteasome in removing oxidized proteins, some of the same compounds, specifically directed to the liver, could well have therapeutic benefit in alleviating alcohol-induced liver injury.

Authors' contributions

All authors contributed equally to this review.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgements

This review is the result of work supported with resources and the use of the facilities at the Omaha Veterans Affairs' Medical Center and United States Department of Veterans Affairs Merit Review grant, BX004053 (to K. K. Kharbanda) and NIH grants, R01AA026723 (to K. K. Kharbanda) and R01AA027189 (to N. A. Osna). We are pleased to acknowledge the valuable assistance of Sarah Sweeney, who arranged the figures for this review.

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