Alcohol-Related Mechanisms of Acute Pancreatitis: The Roles of Mitochondrial Dysfunction and Endoplasmic Reticulum Stress

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Introduction

Excessive alcohol consumption is a major contributor to diverse pathologies with an estimated 4 in 100 deaths worldwide caused by alcohol according to the World Health Organization (65). The close association between alcohol consumption and acute pancreatitis (AP) has been recognized for a long time, with Friedrich first describing the *Drunkard's Pancreas* in 1878, although elevated intake of alcohol had been linked to pancreatic disease a century earlier (4). More recently, a Danish population-based cohort study has shown an increased risk of AP in individuals who consumed in excess of 14 drinks per week, irrespective of the type of beverage or frequency of intake (Kristiansen *et al.*, 2008). A subsequent meta-analysis indicated that an elevated risk of AP exists in those imbibing greater than 4 drinks per day (26). Despite the recognized risk of AP increasing with alcohol intake its basis remains incompletely understood and no specific therapy exists (52). Intriguingly, some individuals appear more susceptible to developing AP linked to excess alcohol consumption than others, with less than 10% of heavy drinkers developing clinical disease. However, this phenomenon has no clear explanation and clearly is an important area for investigation. Progress in elucidating the pathophysiology of alcoholic AP has been complicated by the fact that alcohol alone does not induce AP reliably in experimental animal models, with additional factors required to model alcohol-induced pancreatic inflammation and damage, including caerulein, lipopolysaccharide and ductal obstruction that may not accurately reflect the clinical situation (34). Direct sensitizing actions of ethanol are thought to contribute to damaging effects (50), including activation of NFκB in pancreatic acinar cells via the ε isoform of protein kinase C (58) and activation of cholinergic pathways (36). Recent work has focused on the way in which alcohol metabolism may be involved in mediating pancreatic toxicity.

Ethanol Metabolism

Ethanol is metabolized in the pancreas by both oxidative and non-oxidative routes (5, 16). Current evidence indicates that both pathways are likely to contribute to the detrimental effects of alcohol on the exocrine pancreas, via distinct mechanisms that ultimately compromise mitochondrial function (7, 8, 44, 60). Oxidative metabolism (OME) proceeds through several nicotinamide adenine dinucleotide (NAD^+) consuming steps performed by alcohol and aldehyde dehydrogenases (ADH and ALDH) that generate acetaldehyde and acetate, respectively. Recent findings have suggested that ethanol induces mitochondrial dysfunction via a reduction of the ratio of oxidized to reduced

nicotinamide adenine dinucleotide, a mechanism distinct from the effects of cholecystokinin hyperstimulation which are mediated by increasing cytosolic calcium ($[Ca^{2+}]_c$) (54, 60).

In contrast oxidative metabolism of ethanol (OME), non-oxidative metabolism of ethanol (NOME) promotes esterification of fatty acids to yield highly lipophilic fatty acid ethyl esters (FAEEs) via FAEE synthases including carboxylester lipase (CEL). FAEE synthase activity occurs in the human pancreas at rates of up to 54 nmol/min/g tissue, generating high localized levels of FAEEs (12). An autopsy study showed that individuals who died of acute alcohol intoxication had preferentially elevated FAEEs in the pancreas, in contrast to other organs commonly damaged by alcohol such as the heart and lungs (32), suggesting importance of NOME to pancreatic damage. *In vivo* studies in rats subsequently confirmed that saturated FAEEs induced pancreatic damage indicative of AP (63). Furthermore, administration of ethanol, under conditions of OME inhibition, generated plasma and tissue FAEEs and development of AP (64). Early (<15 mins.) redistribution of CEL into the cytosol from a predominantly apical, granular localization within the pancreatic acinar cell occurred following *in vivo* administration of fat and alcohol to mice in a new *in vivo* model of alcoholic AP (FAEE-AP) (24). Furthermore, inhibition of CEL blocked FAEE generation and ameliorated detrimental effects of fat and alcohol (24). In AP patients, elevated CEL is detectable in necrotic pancreatic lobules and in areas of fat necrosis (1), consistent with localized generation of toxic FAEEs in areas of damage. In pancreatic acinar cells FAEEs released calcium from the endoplasmic reticulum (ER) via stimulation of inositol trisphosphate (IP_3) receptors, causing a depletion of internal calcium stores that led to store-operated calcium entry (SOCE), promoting toxic, sustained elevations of cytosolic calcium ($[Ca^{2+}]_C$) that lead to necrotic cell death (7-9). Furthermore, FAEEs underwent hydrolysis to fatty acids in the mitochondria causing a localized elevation that compromised mitochondrial function (8, 25, 31). Pharmacological inhibition of hydrolase enzymes significantly reduced necrosis induced by a fat and alcohol combination, highlighting the importance of fatty acid release in the mitochondria to cellular damage (9). Diverse actions of FAEEs have been reported in pancreas including increased fragility of lysosomes (19) and inhibition of serine proteases (37) that may predispose to fibrogenesis and impaired pancreas recovery after organ damage in chronic injury (38).

Recent progress in understanding the basis of alcohol-induced damage has highlighted the importance of organellar dysfunction within the pancreatic acinar cell as central for initiation of AP. In particular, the involvement of mitochondria and the endoplasmic reticulum, two organelles that are intimately linked spatially and functionally to modulate cellular calcium homeostasis, energy production, and lipid and protein synthesis (11, 27, 55).

Mitochondrial dysfunction in alcoholic acute pancreatitis

Mitochondria perform a variety of tasks in the pancreatic acinar cell, the most important being provision of energy for cellular processes including secretion of inactive digestive enzyme precursors. In order to do this effectively mitochondria respond to oscillatory rises of $[Ca^{2+}]_C$, induced by hormonal (cholecystokinin) and neuronal (acetylcholine) stimulation (6, 45, 62), by generating NADH, via stimulation of Ca^{2+}-dependent dehydrogenases of the Krebs Cycle, that feeds into the electron transport chain to promote ATP production. Additionally, mitochondria are thought to constitute a protective perigranular buffer barrier that impedes movement of excessive Ca^{2+} released from the apical pole to the basolateral region in which the nucleus resides (53). However, when sustained rises of $[Ca^{2+}]_C$ occur in pancreatic acinar cells in response to aberrant Ca^{2+} signals induced by diverse AP precipitants, including CCK hyperstimulation, bile salts and ethanol metabolites, mitochondrial dysfunction ensues that leads to rundown of ATP production and induction of cellular necrosis (7, 10, 18, 43).

Recent evidence has shown that the trigger for mitochondrial dysfunction in AP is the opening of the mitochondrial permeability transition pore (MPTP) (44, 60), which permeabilized the inner mitochondrial membrane allowing free movement of substances up to 1.5 kDa in and out of the organelle. MPTP formation thus leads to collapse of membrane potential, dissipating the proton gradient necessary for production of ATP. Although the exact composition of the pore remains controversial, recent evidence has indicated that it may be a dimer of the F_0/F_1-ATP synthase (2, 14). In response to AP precipitants in both human and murine pancreatic acinar cells, calcium-dependent MPTP formation occurred as a consequence of IP_3 and ryanodine receptor-mediated intracellular calcium release and subsequent SOCE; diminished ATP production led to impaired calcium clearance, defective autophagy, zymogen activation, cytokine production, phosphoglycerate mutase 5 activation and necrosis (44). The crucial role played by compromised intracellular ATP levels as a result of mitochondrial dysfunction has been shown by studies in which detrimental effects of AP toxins, including non-oxidative ethanol metabolites, were preventable by intracellular ATP supplementation in isolated pancreatic acinar cells, allowing energy-dependent calcium extrusion pumps to reduce $[Ca^{2+}]_C$ and maintain homeostasis (3, 8, 44). The mitochondrial matrix protein peptidyl-prolyl cis-trans isomerase cyclophilin D (CypD) plays a pivotal role in modulating the MPTP; all biochemical, immunological and histopathological responses of AP in four experimental models,

including alcoholic (FAEE-AP), were reduced or abolished by genetic deletion or pharmacological modulation of this protein (44), suggesting the potential of CypD inhibitors for translational therapy.

Endoplasmic reticulum responses with alcohol

The endoplasmic reticulum (ER) of the pancreatic acinar cell plays a predominant role in the function of the cell as protein synthesis and transport are highly developed in this cell. It is not surprising then that the ER responds to alcohol. A previous study has shown that exposure of pancreatic acinar cells to ethanol induced a slow, gradual release of calcium from the ER (9). The ER not only translates mRNA into new proteins synthesized in its lumen but it performs several post-translational modifications including disulfide bond formation facilitated by chaperone-mediated protein folding and other post-translational modifications such as glycosylation. Correctly folded and otherwise modified proteins are directed to specific cellular organelles. As an example, the digestive enzyme proteins are segregated into the secretory pathway ending up in zymogen granules which undergo exocytosis and secretion with neurohormonal stimulation. As another example, acid hydrolases are glycosylated with mannose-6-phosphate which is necessary for their transport to the lysosome.

In general protein folding is accomplished in the ER by molecular chaperones and folding enzymes that include disulfide isomerases and oxidoreductases. In addition, there is a quality control mechanism that degrades improperly processed proteins by proteosomal degradation. This process is called ER-associated degradation (ERAD). Autophagy also participates in degradation of dysfunctional ER and damaged or misfolded proteins to prevent cellular toxicity that these proteins may cause (13, 29).

In order to adjust to changing demands encountered by the ER protein synthesis and processing machinery including ethanol and its metabolism, eukaryotic cells have developed a complex signaling system referred to as the Unfolded Protein Response (UPR). Activation of the UPR occurs with accumulated unfolded or misfolded proteins in the ER lumen, a phenomenon termed “ER stress” (56). ER stress has several sources, including a physiologic increase in the demand for protein folding; decreased chaperone function; accumulation of permanently misfolded proteins due to mutation; decreases in cellular ATP levels or calcium in the ER ($[Ca^{2+}]_{ER}$); and perturbed ER redox status that occurs with alcohol metabolism (39, 48). Interestingly, the non-oxidative ethanol metabolite palmitoleic acid ethyl ester and palmitoleic acid, which is released by hydrolysis of its parent FAEE (24), caused complete depletion of $[Ca^{2+}]_{ER}$, with concomitant falls of NADH and depletion of cellular ATP (8, 9). Also, the folding process itself generates reactive oxygen species

which, in turn, can cause aberrant disulfide bond formation (i.e. misfolding). Thus, in the case of a continuous misfolding stress as occurs with mutation or possibly with ethanol metabolism there will be a greater ER stress than would occur during a transient increase in unfolded proteins as a consequence of the need to replenish zymogen stores.

The UPR has three major response systems to ER stress. These are a global reduction in mRNA translation which attenuates the demand for protein processing; an increase in expression of chaperones and foldases as well as increased phospholipid synthesis to expand the functional ER network; and activation of the ERAD and autophagic systems to eliminate misfolded and aberrant proteins (28, 29, 42, 56, 57). These responses are accomplished by identified sensing and signaling systems (56, 57). They include Inositol-requiring protein-1α (IRE1α), activating transcription factor-6 (ATF6), and RNA-activated protein kinase (PKR)-like ER kinase (PERK).

Regarding alcohol-induced ER stress, we found a key role for IRE1α and in preventing damage to the exocrine pancreas (39, 47). Upon its activation, an endonuclease activity within IRE1α splices X-box binding protein-1 (XBP1) mRNA resulting in a shorter mRNA (spliced XBP1, sXBP1 mRNA) that encodes the active transcription factor sXBP1. sXBP1 regulates a broad spectrum of genes involved in protein folding, including chaperones, disulfide isomerases and oxidoreductases family as well as genes for protein degradation (ERAD), lipid biosynthesis for ER/Golgi biogenesis, vesicular trafficking and redox metabolism (28, 33). In the exocrine pancreas, sXBP1 is especially necessary for acinar cell homeostasis and function (33). The critical importance of sXBP1 for the function of the pancreatic acinar cell is supported by studies using *Xbp1*$^{+/-}$ mice (39, 40) and acinar cell specific *Xbp1* null mice (23, 33). XBP1 deficiency results in defective stimulated secretory response, extensive acinar cell loss and inflammation as well as severe pathology in the remaining acinar cells, as evidenced by reduced levels of ER chaperones, a poorly developed ER network and secretory system, marked reduction in zymogen granules and digestive enzymes, and accumulation of autophagic vacuoles (23, 40).

Ethanol feeding in rodents induces structural changes in the acinar cell consistent with ER stress such as ER dilation, mitochondrial swelling and some disorganization of cellular organelles (17, 30, 39). However, chronic ethanol-fed animals, as humans, do not develop pancreatitis unless challenged with other toxic factors (49, 51, 61). We found that pancreatic mRNA and protein levels of sXBP1 were significantly increased in mouse and rats fed ethanol-containing diets (39). In order to determine whether the upregulation of sXBP1 by alcohol feeding is necessary to maintain homeostasis and prevent pancreatitis, we used *Xbp1* heterozygous mice (*Xbp+/-*). Compared to ethanol-fed wild-type mice (*Xbp1+/+*), histological analysis of pancreatic tissue in ethanol-fed

Xbp1+/- mice revealed morphologic features of severe ER stress such as disorganized and dilated ER, accumulation of dense material within the ER, and a reduced number of mature zymogen granules. These features were accompanied by accumulation of autophagic vacuoles, and activation of apoptotic signals including upregulation of CHOP (see below) within patchy areas of inflammatory pancreatitis (39, 40). Moreover, recent studies indicate that cerulein-induced AP is more severe in XBP1 deficient mice compare to controls (unpublished observations). From these studies we concluded that alcohol feeding activates an adaptive and protective UPR through increased expression of sXBP1 involving activation of the endonuclease activity of IRE1α. Further, these actions of the UPR are necessary to prevent cellular toxicities of ethanol.

Whereas IRE1α/XBP1 signaling primarily mediates adaptive responses to protect ER function, this protective signal can be prematurely attenuated during severe or prolong ER stress resulting in upregulation of proapoptotic cell death mediated through the transcription factor, C/EBP homologous protein (CHOP)(35) as discussed in more detail below. Also, genetic inhibition of Xbp1 is unequivocally associated with potent upregulation of CHOP and cell death (33, 39). On the other hand, forced and sustained IRE1α/XBP1 activity enhances cell survival in conditions of severe stress (35), further supporting a protective role for sXBP1 signaling.

The PERK UPR branch has a dual role. Firstly, when activated it rapidly adjusts the cell to ER stress by mediating a general attenuation of protein synthesis (20, 21, 56, 59). On the hand, sustained activation of PERK leads to upregulation of the transcription factor, Activating transcription factor 4 (ATF4) that targets genes involved in antioxidant activities including glutathione synthesis (22) and CHOP that promotes ER stress-related cell death responses (46). CHOP also promotes inflammation by regulating cytokine production and promoting the survival of inflammatory cells (15, 41). In summary, although the PERK activation can play a transient protective role, unresolved ER stresses leads to upregulation of CHOP and promotion of inflammation and pancreatitis.

Conclusions

This chapter reviews two bodies of work related to alcohol effects on the exocrine pancreas. One addresses mitochondrial functional changes and the other endoplasmic reticulum responses, phenomena that may be interrelated in acute pancreatitis. Alcohol-induced disorders of both organelles make the pancreas susceptible to alcohol-induced injury and recent advances suggest the potential for translational therapy. Interestingly, there are some protective responses coming from the endoplasmic reticulum UPR that may be a reason why only a minority of drinkers develop pancreatitis.

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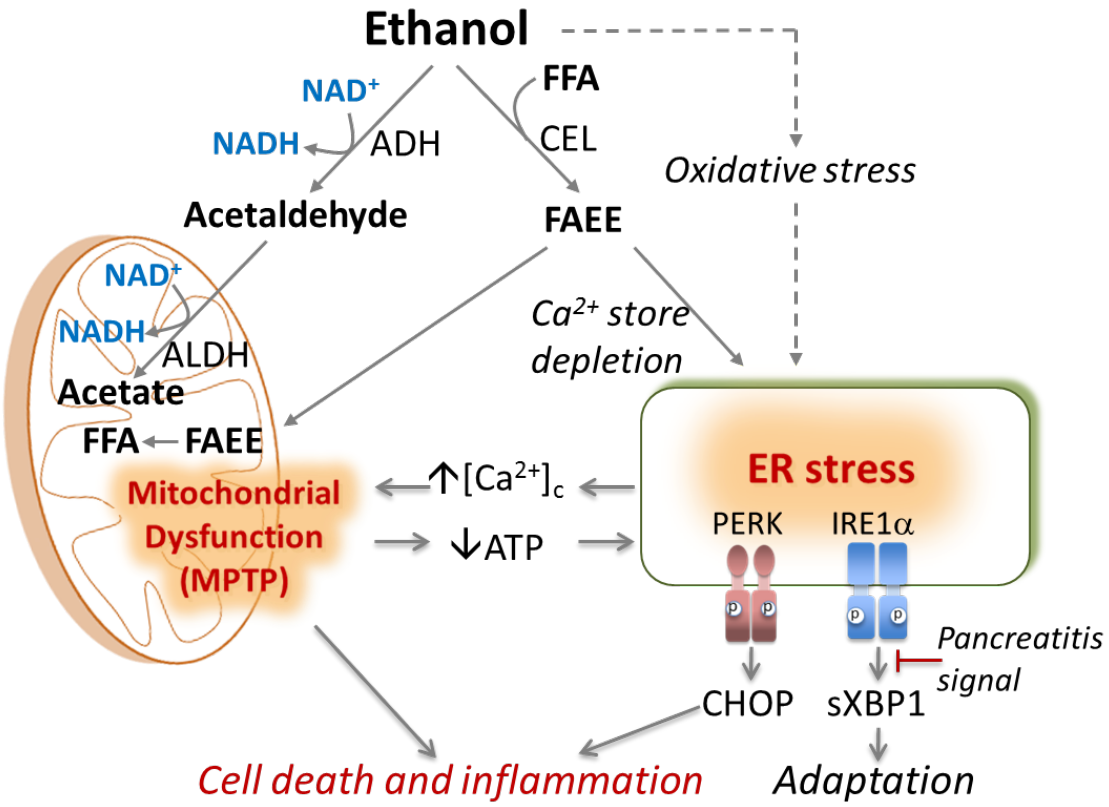
Figure 1. A schematic diagram displaying proposed mechanisms of ethanol-mediated Acute Pancreatitis (AP). In the pancreatic acinar cell, ethanol can compromise mitochondrial function via two pathways. Oxidative metabolism of ethanol to acetaldehyde, via alcohol dehydrogenase (ADH), and to acetate, via aldehyde dehydrogenase (ALDH) in the mitochondria, decreases cellular NAD^+/NADH balance. Fatty Acid Ethyl Esters (FAEEs) are esterification products of fatty acids and ethanol via FAEE synthases including carboxylester lipase (CEL). Accumulation of FAEEs elicits Ca^{2+} depletion from the endoplasmic reticulum (ER) and other cellular stores leading to sustained elevations of $[Ca^{2+}]_c$ and mitochondrial Ca^{2+} overload. Furthermore, accumulation of FAEEs in mitochondria leads to release of fatty acids, via action of hydrolases, which compromises organellar function. Both altered NAD^+/NADH ratios and $[Ca^{2+}]_c$ overload have been proposed to elicit opening of the mitochondrial permeability transition pore (MPTP), which results in mitochondrial depolarisation, ATP depletion and cellular necrosis. Besides ethanol effects on mitochondria, ethanol-induced oxidative stress alters ER redox status (not shown) and elicits chronic ER stress, an effect that can be exacerbated by FAEE-induced ER-Ca^{2+} depletion and compromised ATP

production. ER stress is manifested by activation of adaptive IRE1/XBP1 signaling that aids to preserve ER function and protein processing through the secretory pathway. However, severe ethanol-induced cellular damage or additional toxic pancreatitis signaling can compromise cellular adaptation leading to termination of protective XBP1 signaling and upregulation of cell death pathways downstream to mitochondria and PERK/CHOP signaling, and ultimately to pancreatitis.


Ethanol
NAD+
NADH
ADH
FFA
CEL
Oxidative stress
Acetaldehyde
FAEE
NAD+
NADH
ALDH
Acetate
FFA
FAEE
Ca2+ store depletion
Mitochondrial Dysfunction (MPTP)
↑[Ca2+]c
↓ATP
ER stress
PERK
IRE1α
Pancreatitis signal
CHOP
sXBP1
Cell death and inflammation
Adaptation