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Gain of function effects of Z alpha-1 antitrypsin

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Running title: Gain of function effects of ZAAT

Abstract

The serine proteinase inhibitor alpha-1 antitrypsin (AAT) is produced principally by the liver from where it is secreted into the circulation and provides an antiprotease protective screen throughout the body. Mutations leading to deficiency in AAT are associated with liver and lung disease. The most notable is the Z mutation, which encodes a misfolded variant of the AAT protein in which the glutamic acid at position 342 is replaced by a lysine. ZAAT is not secreted effectively and accumulates intracellularly in the endoplasmic reticulum (ER) of hepatocytes and other AAT-producing cells. The ER has evolved a number of elegant mechanisms to manage the accumulation of incorrectly folded proteins; ZAAT interferes with this function and promotes ER stress responses and inflammation. Until recently it was thought that gain of function was the major cause of the liver disease whilst the lung disease was entirely due to loss of anti-protease protection in the lung. This belief is now being challenged with the discovery that ER stress is also activated in bronchial epithelial cells and inflammatory cells normally resident in the lung in ZAAT deficient individuals. Here we describe the gain of function effects of ZAAT. In particular we highlight the signalling pathways that are activated during ER stress in response to accumulation of ZAAT and how these events are linked to inflammation and may contribute to disease pathogenesis.

Keywords: alpha-1 antitrypsin deficiency, apoptosis, endoplasmic reticulum stress, inflammation, NF-κB, unfolded protein response

Introduction

Alpha-1 antitrypsin (AAT) deficiency is a hereditary disorder which predominantly affects the lungs and to a lesser extent, the liver. AAT is synthesized primarily in the liver [1] but is also expressed by a variety of other cells including monocytes, neutrophils, alveolar macrophages, bronchial and intestinal epithelial cells[2-7]. AAT is the archetypal member of the serpin superfamily, whose members regulate the activity of serine proteases most notably neutrophil elastase (NE)[8]. If unchecked, NE and other serine proteases can degrade the lung matrix causing structural damage and pathologically impairing host defence. The most clinically significant mutation associated with AAT deficiency is the Z mutation, which causes the abnormal Z protein to misfold and polymerise within hepatocytes of the liver [9] and other AAT–producing cells. This intracellular retention leads to a serum deficiency of AAT, rendering the lungs vulnerable to proteolytic attack and the liver prone to cirrhosis due to the abnormal aggregation of AAT. As our understanding of ZAAT deficiency has increased over the last few years it has emerged that toxic effects associated with intracellular accumulation of ZAAT in the endoplasmic reticulum (ER) and the acquisition of new proinflammatory properties by misfolded ZAAT also contribute to the pathognesis of this disorder. Here we discuss the gain of function effects of Z alpha-1 antitrypsin.

Z AAT CAN ACT AS A CHEMOATTRACTANT

The observation by Lomas *et al.* that ZAAT can form polymers within hepatocytes[9] initially raised the possibility of polymers existing in the lung and contributing to the pathogenesis of emphysema. Although the majority of AAT is derived from the liver and enters the lung from the circulation by passive diffusion, macrophages and bronchial and alveolar epithelial cells also secrete AAT locally. Regardless of origin, ZAAT has the propensity to form polymers. The first identification of AAT polymers in lung lavage [10] and lung tissue [11] of ZZ individuals led to functional studies of AAT polymers. Initially, polymers of wild type MAAT were shown to be chemotactic for neutrophils, inducing neutrophil shape

change, enzyme release and adhesion [12]. In contrast, monomers of AAT had little effect on the migration of neutrophils *in vivo* or *in vitro*.

As the Z form of AAT is more intrinsically polymerogenic than the M form, the acquired characteristics of AAT polymers could help drive the development of emphysema in ZZ individuals. Indeed, the instillation of AAT polymers into mouse lung caused a neutrophil influx [11]. More importantly, work from this group and others demonstrated that locally produced ZAAT actually polymerises in the lung and can act as a potent neutrophil chemoattractant. ZAAT polymers have also been found co-localised with neutrophils in the alveolar wall of AAT deficiency individuals with emphysema [11, 13]. This concept of polymer-induced neutrophil influx is supported by clinical evidence of increased neutrophils in lung lavage from ZZ individuals [14]. It remains to be demonstrated whether ZAAT forms polymers *in vivo* in airway epithelial cells.

Protein Folding in the ER and ER Stress

AAT deficiency is characterised by aberrant folding of AAT protein and therefore belongs to a class of genetic conditions collectively termed conformational disorders [15], diseases caused by mutations altering the folding pathway or the final conformation of a protein. Other conformational disorders include cystic fibrosis, Parkinson's disease and Alzheimer's disease, and are all associated with intracellular accumulation of unfolded or misfolded rogue proteins. AAT deficiency can also be classified as an ER storage disease as it involves a mutation that affects the AAT polypeptide, resulting in retention of the abnormal AAT cargo in the ER [16]. This accumulation of aberrantly folded Z protein within the ER lumen has the potential to cause the phenomenon of ER stress – the three classical features of ER stress are the unfolded protein response, the ER overload response and apoptosis.

Because of the importance of accurate protein folding sophisticated cellular mechanisms have evolved to ensure correct protein folding and efficient disposal of irreversibly misfolded proteins. These mechanisms are designed to ensure that only properly

folded proteins can exit from the ER. Proteins enter the ER in an unfolded state via a heterotrimeric Sec61p channel. Various chaperones residing in the ER facilitate folding of these nascent polypeptides via modifications such as disulphide bind formation and N-linked glycosylation. Faulty polypeptides generated via this process are disposed of [17].

Polypeptides undergo quality control in the ER and are confined there by the lectin-type chaperones calnexin and calreticulin which assist in their folding until it has been properly completed. Glucose-responsive protein 78 (BIP/Grp78) can bind to exposed hydrophobic surfaces whilst oxidoreductases such as Glucose-responsive protein 58 (Grp58/ERp57) bind free thiol groups and catalyse the formation of intra- and inter-molecular disulfide bonds between paired cysteine residues. Protein disulfide isomerase, a redox-driven chaperone, is also involved in ER quality control. Immature deglucosylated glycoproteins are recognised by UDP glucose-glycoprotein glucosyltransferase (UGT1) an ER folding sensor, which retains them in the ER for reglucosylation and further calnexin-calreticulin assisted folding. Once correctly folded, proteins are transported to the Golgi apparatus for membrane insertion or secretion [18].

ER homeostasis is essential for normal cell function and survival. Environmental perturbations such as disturbances in calcium storage compromise the ER protein folding capacity, resulting in the accumulation of unfolded or misfolded protein within the ER. Any imbalance between the ER protein folding load and the ability to process the load causes ER stress, with potentially pathological consequences. ER stress can also be induced by a range of pathophysiological conditions, including ischaemia, diabetes, viral infection, and mutations which impair client protein folding [19]. Therefore, to maintain homeostasis, the ER has evolved mechanisms to sense stress and transduce signals to the cytoplasm and the nucleus, governing whether the outcome is survival or death. This protective system includes the translational attenuation of protein synthesis [20], transcriptional induction of unfolded protein response (UPR) target genes [21], and ER-associated degradation (ERAD)[22]. Collectively this tripartite management system is called the unfolded protein response (Figure 1).

UPR

(i) *Translational Attenuation - PERK Pathway.* When mammalian cells are subjected to ER stress, the first and immediate response is translational attenuation in order to reduce the load of host protein synthesis in the ER and prevent the further accumulation of unfolded proteins [23]. This is orchestrated by the type I ER transmembrane protein Protein kinase-like ER kinase (PERK), which represses global protein biosynthesis through the phosphorylation of translation initiation factor 2 (eIF2) on its alpha subunit (eIF2α). The lumenal domain of PERK is sequestered by Grp78 in unstressed cells at the ER membrane and its cytoplasmic domain is activated upon the release of Grp78 following ER stress. PERK then oligomerises and auto-phosphorylates to become active, and can *trans*-phosphorylate the translation initiation factor eIF2α [24] and the basic leucine zipper (bZIP) transcription factor NF-E2-related factor 2 (Nrf2) [25].

The phosphorylation of eIF2α at serine 51 in response to various stresses blocks the initiation of global translation in the cell, but simultaneously upregulates a subset of stress-responsive genes; eIF2α phosphorylation paradoxically promotes the translation of Activating transcription factor (ATF) 4 mRNA, another bZIP transcription factor [26]. ATF4 regulates the expression of genes essential for amino acid import, glutathione biosynthesis, resistance to oxidative stress and restoring ER homeostasis [26]. Downstream targets of ATF4 include C/EBP homologous protein (CHOP), Growth Arrest and DNA Damage-Inducible Protein (GADD34), and ATF3 [27, 28]. A key role for ATF4 in cell survival is underlined by the high expression levels found in malignant tissue from several types of cancer, including brain, breast, cervical and skin cancers, facilitating tumour growth and survival [29]. In addition, the downstream target ATF3 contributes to the expression of the pro-apoptotic protein CHOP and the phosphatase GADD34 [28], adding another layer of complexity and providing a further example of the highly coordinated and integrated approach of the cell to ER stress.

During ER stress PERK also induces phosphorylation of the transcription factor Nrf2, allowing it to dissociate from an inactive complex in the cytoplasm, and facilitating its nuclear translocation. Nrf2 transactivation appears to be independent yet parallel to eIF2α phosphorylation, and raises the possibility of two or more divergent pathways downstream of PERK. In the nucleus Nrf2 binds to antioxidant response elements (ARE), promoting a transcriptional programme of pro-survival genes, similar to ATF4 [30]. Besides genes required for coping with oxidative stress and maintaining redox homeostasis, other Nrf2-regulated genes include those involved in immune signalling, protein trafficking, protein degradation and the chaperone system [31]. Disruption of Nrf2 in mice impairs the induction of cytoprotective genes and enhances cigarette-induced emphysema caused by oxidative damage, inflammation and apoptosis [32]. Defective Nrf2 signalling has also been identified in the lungs of patients with chronic obstructive pulmonary disease, indicating that host antioxidant defences are attenuated [33]. It is known that reactive oxygen species are generated by misfolded proteins in the ER, and antioxidants have been shown to reduce UPR activation, oxidative stress and apoptosis [34].

(ii)Transcriptional Induction of UPR - ATF6 Pathway. ATF6 is an ER-resident type II transmembrane protein with an N-terminal cytoplasmic region containing bZIP and DNA transactivation domains, and a C-terminal lumenal region that senses ER stress [35, 36]. During ER stress, ATF6 translocates to the Golgi complex after the dissociation of Grp78, which masks Golgi localisation signals on the luminal region of ATF6. In the Golgi ATF6 undergoes sequential proteolytic cleavage by site-1 and site-2 proteases and the bZIP-containing N-terminal moiety becomes a soluble transcription factor that translocates to the nucleus. ATF6 binds to the ATF/CRE element [37] and to the ER stress response elements (ERSE) I and II [38, 39]. Binding of ATF6 to ERSE-I requires another transcription factor, Nuclear transcription factor Y (NF-Y) [40]. Some of the target genes regulated by ATF6 include GRP78, X-box binding protein-1 (XBP-1), CHOP/GADD153, and the membrane protein Herp [41].

(iii) *IRE1 Pathway.* The ER transmembrane protein inositol requiring kinase 1 (IRE1) regulates chaperone induction, ERAD, and expansion of the ER in response to ER stress [42]. This pathway is highly conserved in evolution and is present in all eukaryotes. Mammals have two copies of IRE1, IRE1α and IRE1β. IRE1α is ubiquitously expressed, while the expression of IRE1β is limited to the gut [43, 44]. IRE1 is an endoribonuclease, and after dissociation of Grp78 from its ER lumenal domain, IRE1 oligomerises and activates its RNase domain [45]. The target of IRE1 is the bZIP transcription factor XBP-1. The stress-induced spliced version of XBP-1 travels into the nucleus and can bind to both ERSE and UPRE, activating the transcription of ER chaperone genes, ER quality control genes and folding enzymes [46]. Degradation of unspliced XBP-1 by the proteosome is vital for efficient activation of the UPR as it occupies UPRE sites and forms less potent dimers with spliced XBP-1 [47].

Interestingly, cleavage of ATF6 occurs prior to spliced XBP-1 production, which suggests the two UPR transcription pathways are biphasic [48]. ATF6 activation is relatively rapid as it involves proteolysis of a pre-existing protein, whereas activation of XBP-1 requires mRNA transcription, splicing and translation. Furthermore, ATF6 preferentially binds to the ERSE sequence in the presence of NF-Y, while XBP-1 can bind to both ERSE and unfolded protein response elements (UPRE) sequences. This would suggest that mammalian cells activate ATF6 to induce transcription via ERSE immediately upon ER stress, and then activate XBP-1 to induce UPRE-mediated as well as ERSE-mediated transcription. The XBP-1 promoter contains a mammalian UPRE site, suggesting a positive feedback mechanism for gene transcription. This may allow for sustained UPR activation in cells undergoing chronic ER stress [46].

UPR and Inflammation

ER stress is intrinsically linked to the generation and accumulation of reactive oxygen species (ROS). The efficient folding of newly synthesised proteins into the correct

conformations in the ER is an energy-consuming process, and oxidising conditions are essential for the formation of intramolecular and intermolecular disulphide bonds [49]. It stands to reason that any increase in the protein-folding load in the ER can lead to the accumulation of ROS, with deleterious inflammatory consequences [50]. The evolutionarily conserved PERK pathway of the UPR, with an antioxidant transcriptional programme mediated by ATF4 and Nrf2, is testament to the harmful effects of ROS accumulation.

In addition to its role in preserving ER homeostasis and promoting cell survival, it is clear that ER stress-induced UPR activation influences the expression of inflammatory cytokines. The key UPR sensor IRE1 appears to coordinate ER stress-signalling with inflammatory response signalling. IRE1 has been to shown to bind the adaptor protein tumour necrosis factor-α-receptor-associated factor 2 (TRAF2) [51]. The IRE1-TRAF2 complex can recruit inhibitor of nuclear factor-κB kinase (IKK), which phosphorylates and degrades inhibitor of nuclear factor-κB (IκB), leading to the nuclear translocation of nuclear factor-κB (NF-κB) [52]. In addition, the IRE1-TRAF2 complex can also recruit the kinase Jun N-terminal Kinase (JNK), which can phosphorylate the transcription factor activator protein 1 (AP-1), a known inducer of inflammatory gene expression [53].

A major role for the UPR in the inflammatory response is now undisputed. The active spliced form of XBP-1, a key regulator of chaperone and ERAD genes, has been shown to control the production of interleukin (IL)-6 in the mouse B cell [54]. Furthermore, XBP-1 was shown to direct the production of interferon (IFN)-β in mouse macrophages subjected to ER stress [55], while both ATF6 and XBP-1 were found to control IL-6, IL-8 and monocyte chemotactic protein (MCP)-1 expression in human aortic endothelial cells [56]. Interestingly, another group demonstrated that accumulation of free cholesterol in macrophages from a mouse model of atherosclerosis induced ER stress and caused the secretion of TNF-α and IL-6 [57, 58], highlighting a mode of cytokine production induced by intracellular events, as opposed to exogenous stimuli.

However, there is a further level of complexity. Several reports have indicated that inflammatory cytokines can cause ER stress and activate the UPR. TNF-α has been shown to activate PERK, IRE1, and ATF6 in fibrosarcoma cells [59], while Tumour necrosis factor (TNF)-α, IL-1β, and IL-6 induced ER stress in hepatocytes, upregulating Grp78 and spliced XBP-1 [60]. This cytokine-induced UPR engagement in hepatocytes led to the activation of the transcription factor CREBH in the Golgi by a sequential cleavage mechanism similar to ATF6 activation, and active CREBH then mediates the acute phase response of inflammatory genes such as C-reactive protein (CRP). Furthermore, a recent study in *C. elegans* demonstrated XBP-1 is activated by *Pseudomonas* challenge, not to neutralise the invading pathogen, but to protect the host from the potentially lethal ER stress caused by mounting a massive, secretory immune response [61].

Z AAT AND THE UPR

It has been shown that all three arms of the UPR can be activated in response to over-expression of ZAAT in Chinese hamster ovary (CHO), human embryonic kidney (HEK) 293, liver HepG2 and human bronchial epithelial 16HBE14o- cell lines *in vitro* [7, 62-64] and basally in human peripheral blood monocytes isolated from ZZ individuals [6]. However, there is an absence of UPR activation in inducible models of ZAAT deficiency liver disease and in liver cells *in vivo*. Several studies have failed to discover UPR activation in cell culture and animal liver models of ZAAT deficiency [65, 66]. This has led to the hypothesis that an absence of UPR engagement allows the survival of cells with high intracellular levels of ZAAT, for example globule-containing hepatocytes.

Recently it has emerged that ZAAT expression is also associated with abnormal immune cell function [6]. Peripheral blood monocytes express AAT and Carroll *et al.* showed evidence of UPR activation *in vivo* in monocytes from ZZ individuals and linked this phenomenon to an exaggerated immune response. Monocytes isolated from healthy ZZ individuals were hyper-responsive when stimulated with *Pseudomonas* lipopolysaccharide (LPS), and the expression of ATF4, XBP-1 and a subset of genes involved in the UPR were

all increased compared to MM individuals. Furthermore, increased cytokine production and the same UPR genes could be induced in MM monocytes by treatment with the ER stress inducer thapsigargin, linking the observed ZZ monocyte changes to ER stress. Confocal microscopy demonstrated Z AAT is retained in the ER of ZZ monocytes but that Grp78 is also increased even in resting cells. These altered gene expression patterns contribute to enhanced basal and agonist-induced cytokine production by ZZ monocytes and activation of the NF-κB pathway.

These findings broaden our current understanding of the mechanisms underlying the lung and liver disease associated with ZAAT deficiency and shed light on the emerging systemic gain of function effect of the Z mutation. The traditional protease/antiprotease paradigm for the lung disease associated with AAT deficiency has to evolve to accommodate the inflammatory gain of function from accumulated ZAAT protein, including neutrophil chemoattraction, ER stress and concomitant inflammation.

Several groups have confirmed that the inflammatory mediators LPS, IL-6, IL-1β, oncostatin-M (OSM), neutrophil elastase (NE), and TNF-α can upregulate AAT production in monocytes, macrophages and alveolar epithelial cells [67-71]. This is an important mechanism of locally regulating proteolytic activity. However, if inflammatory stimuli upregulate this local AAT production from alveolar macrophages and epithelial cells in the lungs of AAT deficient individuals, the subsequent increase in ZAAT production could cause activation of the UPR. Therefore, in cells producing polymerogenic ZAAT, cytokine production induced by the UPR could represent a self-perpetuating cycle of enhanced inflammatory gene expression and immune cell recruitment.

Removal of misfolded ZAAT from the ER

At least two pathways exist for the degradation of ZAAT that accumulates in the ER, these are known as the ER-associated degradation (ERAD) and autophagic pathways. The current consensus purports that soluble ZAAT is degraded in the cytosol by the proteosome via ERAD, whilst polymerized ZAAT is degraded by autophagy [72].

(i) ER associated degradation (ERAD). This is the process by which the production of mutant glycosylated secretory proteins are monitored, detected and diverted to the ubiquitin-proteosome system for degradation [73, 74]. Most ERAD components exist at the ER membrane however the proteosome reside in the cytosol, therefore ERAD substrates are required to undergo retrograde transport into the cytosol for proteolysis. In order to prevent congestion in the ER with terminally misfolded proteins, those that cannot be refolded are extracted from calnexin and calreticulin. ER degradation-enhancing alpha-mannosidase-like (EDEM) proteins and ER mannosidase I (ERManI) regulate this process. In the context of ZAAT folding in the ER, the ER luminal and transmembrane proteins EDEM1, EDEM2 and EDEM 3 can extract misfolded ZAAT from ER chaperones and enable enzymatic trimming of mannose from its carbohydrate side-chain by ERManI [72]. It is likely that ZAAT is then 'retro-translocated' or 'dislocated' to the cytosol, possibly via Sec61, Derlin and p97/Valosin-containing Protein (VCP), modified with polyubiquitin and ultimately degraded by the proteosome [74]. In addition to ERAD, ZAAT can also be degraded by autophagy, a cellular mechanism for the degradation of cytoplasmic constituents within lysosomes.

(ii) Autophagy. The macroautophagic process (herein referred to as autophagy) is the cellular process managing the disposal and recycling of excess or defective organelles and cytoplasmic and membrane constituents [75-77]. It involves *de novo* vesicle formation. Initially a flat membrane cistern – most likely derived from the ER membrane - termed the isolation membrane or phagophore, elongates and wraps itself around a misfolded protein or organelle that is destined for disposal. These components then become enveloped in a double-membraned vesicle termed the 'autophagosome'. The outer membrane of this vesicle fuses with a lysosome to generate an 'autophagolysosome' which contain lysosomal membrane proteins and proton pumps. The generation of an autophagolysosme facilitates access of hydrolytic enzymes to the inner content of the vesicle and it is within these vesicles that constituent misfolded or long-lived proteins and excess or aberrant organelles (e.g. peroxisomes and mitochondria) are catabolised by resident acidic hydrolases. The

degradation products are transported back to the cytoplasm where they are reused for energy and biosynthesis of new molecules and organelles.

Autophagy is crucial for diverse physiological processes including starvation responses, lipid metabolism, erythropoiesis and immune function. It is a key modulator of diseases characterised by aggregrate-prone proteins and has been much studied in neurological conformational disorders. In addition to being a nutrient starvation and misfolded protein response, autophagy has now also been implicated in the processes of infection, repair and apoptosis and a variety of cellular stresses are known to induce autophagy including viral infection or the UPR [75, 77, 78]. Much of what we know regarding autophagy has been gleaned from studies in yeast. This work has demonstrated a key role for autophagy-related gene (ATG) 6 (called Beclin-1 in mammals) in autophagy. It can interact with a complex of proteins including the phophoinositol-3-kinase vesicle-mediated vacuolar sorting protein (Vsp) 34 and Ambra 1 to promote Vsp15, UV-irradiation resistance-associated gene (UVRAG) and BAX-interacting factor-1(Bif-1)-mediated autophagy [79-83].

Autophagy is also an important mechanism involved in ZAAT deficiency. There are three autophagy gene products ATG5, ATG6 and ATG16 that are particularly important for the autophagic digestion of aggregated ZAAT [84]. Expansion and dilatation of the ER and increased numbers of autophagosomes, two morphological changes characteristic of autophagy, are evident in fibroblasts overexpressing ZAAT, in ZAAT mouse liver cells and liver cells from ZAAT individuals [85-88]. In addition to macroautophagy, other autophagic processes exist including microautophagy and chaperone-mediated autophagy, each with specific functions. Another form of autophagy that involves removal of damaged or exhausted mitochondria is termed ‘mitophagy’ [89]. In ZAAT deficiency mitophagy is likely to be an important process in the liver given that mitochondrial dysfunction has been observed in patient liver samples and mouse models [88].

ER Overload response (EOR)

The proinflammatory transcription factor NF-κB can be activated by a range of diverse stimuli including UV light, microbial infection or proinflammatory cytokines. Classically these agonists engage cell surface receptors and initiate intracellular signal transduction pathways converging at IKK, an enzyme complex composed of IKKα,-β and -γ subunits. Importantly however for individuals with ZAAT deficiency, IKK can also be activated in response to accumulation of misfolded proteins in the ER. The IKK complex phosphorylates the NF-κB inhibitory proteins IκB-α, -β and -γ on key serine residues, leading to their tagging with ubiquitin and ultimate recognition and degradation by the proteosome. Following removal of IκB proteins from NF-κB dimers, nuclear localisation sequences are exposed on NF-κB freeing it to translocate to the nucleus where it can binds to NF-κB recognition elements in the promoter of target genes and promote their transcription.

ZAAT AND THE EOR

We and others have investigated NF-κB activation in the context of ER accumulation of misfolded ZAAT in a variety of cell types including CHO cells, human bronchial epithelial cells (16HBE14o-), liver cell lines and animal and human biopsies, and also in human peripheral blood monocytes [7, 62]. In all cell types studied expression of ZAAT is associated with activation of NF-κB. Electrophoretic mobility shift assays of nuclear extracts from CHO cells transfected with an empty vector, normal M or mutant Z AAT cDNAs showed that expression of ZAAT enhances NF-κB nuclear localisation and ability to bind to DNA compared to control cells. We also performed studies overexpressing wild type or mutant AAT transgenes in 16HBE14o- human bronchial epithelial cells and found significantly increased IL-8 and IL-6 production in ZAAT-expressing cells. IL-6 and IL-8 are proinflammatory cytokines; their expression is positively regulated by NF-κB. This study provided the first evidence that misfolded ZAAT is associated with a gain of function that may have the potential to impact on the lung disease associated with AAT deficiency.

In liver cells in cell culture, in animal models of ZAAT deficiency and also *in vivo* in liver biopsies from ZZ homozygous individuals NF-κB activation has been detected [64, 65]. In liver cells it is possible that activation of NF-κB in response to accumulation of ZAAT may mediate inflammation and neutrophil infiltration via up regulation of IL-8. Given that NF-κB is involved in inflammation-associated carcinogenesis, EOR may have a role in the pathogenesis of hepatocellular carcinoma in ZAAT-deficient individuals [90].

Whilst UPR and EOR are often depicted as discrete signals activated during the ER stress response it now becoming evident that UPR signalling can directly impact on and regulate proinflammatory gene expression (Figure 2). Indeed various forms of cross-talk between different ER stress-induced pathways can occur.

ER stress-induced apoptosis

Although NF-κB is largely cytoprotective, when the stress induced by the presence of a misfolded protein in the ER is prolonged cell death may occur via apoptosis. ER stress-induced apoptosis can occur by a number of different mechanisms. The best characterised pathways involve key UPR proteins and other ER membrane-resident factors (Figure 3) [91].
(i) *PERK-CHOP-GADD34.* Activation of PERK can promote cell survival by inducing the expression of specific UPR survival genes, however under conditions of prolonged ER stress, PERK-mediated activation of ATF4 leads to the expression of CHOP [92] (also called GADD153) and the pro-apoptotic genes NOXA and Bim [91, 93]. Cell cycle arrest occurs following CHOP overexpression due to enhanced expression of the pro-apoptotic genes Tribbles homologue 3 (TRB3), death receptor 5 (DR5) and carbonic anhydrase IV (CAVI) [94-96]. CHOP also promotes apoptosis by activating the ER oxidase ERO1α which dimerises with cAMP-responsive element binding protein (CREB) and suppresses expression of the anti-apoptotic protein Bcl2 [97, 98]. A further apoptotic strategy facilitated by CHOP includes the upregulation of GADD34.

(ii) *IRE1-TRAF2-JNK.* Following its activation IRE1α not only splices XPB-1 leading to the transcriptional induction of CHOP, but also interacts with TRAF2 and apoptosis signal regulating kinase 1 (ASK1) initiating a signalling cascade leading to activation of the mitogen-activated protein kinase JNK [99-102]. Phosphorylation of Bcl2 and Bim concomitantly inhibits and activates these two proteins, respectively, further promoting apoptosis [53].

(iii) *Bax/Bak-calpain-caspases.* Although not directly related to UPR signalling the third apoptotic pathway induced by ER stress does also emanate from the ER membrane. In response to ER stress the pro-apoptotic proteins Bak and Bax can undergo a conformational change enabling Ca2+ efflux from the ER into the cytoplasm [103]. This event leads to activation of the Ca2+-dependent cysteine protease calpain which can cleave caspase-12 [104]. In turn procaspase-9 is cleaved, activated and forms an apoptosome with cytochrome c (also released from mitochondria by the cytoplasmic Ca2+ influx) and apoptotic protease activating factor 1 (Apaf-1). Finally the executioner caspase, caspase-3, becomes activated. As caspase-12 is not expressed in humans, caspase-4 has been proposed to represent the functional homologue of murine caspase-12, however its exact role in ER stress-induced apoptosis remains unclear.

ZAAT AND ER STRESS-INDUCED APOPTOSIS

We have evaluated the effect of ER accumulation of ZAAT on apoptosis in HEK293 cells using the ER stress agonist thapsigargin and MAAT and ZAAT transgenes [63]. We found that, similar to treatment with thapsigargin, expression of ZAAT, but not MAAT, induced cleavage of procaspase-4 and downstream executioner caspases. Given the poorly defined role of caspase-4 in ER stress-induced apoptosis which tends to be stimulus and cell type specific we decided to investigate its role in ZAAT-induced cell death. To evaluate this we knocked-down caspase-4 protein expression in HEK293 cells. Interestingly this did not promote cell survival in ZAAT expressing cells, nor did it inhibit ZAAT-induced caspase-7 activation. These data led us to conclude that caspase-4 is not at the apex of the ZAAT/ER-

induced apoptotic signalling pathway and is not essential for ER-mediated apoptosis in HEK293 cells.

Next we used the bile acid tauroursodeoxycholic acid (TUDCA) to further investigate the mechanism of ZAAT-induced apoptosis. We focussed on the effect of TUDCA on the pro-apoptotic Bcl family member Bad, an inhibitor of Bcl2. TUDCA treatment of ZAAT-expressing HEK293 cells led to phosphorylation and inactivation of Bad, an event that can release and activate Bcl2 and promote cell survival. These studies suggest that ZAAT-induced apoptosis in HEK293 cells is likely to involve Bad-mediated inhibition of Bcl2 and supports other models that describe Bcl2 as an important inhibitory target during ER stress-induced apoptosis

We performed similar studies in airway epithelial cells. When we assayed 16HBE14o- cells expressing ZAAT for indices of ER stress-induced apoptosis we found that ZAAT had no apparent affect on apoptosis, notwithstanding its ability to activate other ER stress cascades [7]. Surprisingly we actually observed an inhibition of basal caspase-3 activity in 16HBE14o- cells expressing either MAAT or ZAAT. Concurrently it was reported that MAAT can directly inhibit caspase-3 activity and apoptosis in lung endothelial cells via inhibition of caspase-3 substrate binding [105].

In order to address the apparent dichotomy regarding the pro- and anti-apoptotic effects of ZAAT when expressed in HEK293 versus 16HBE14o- cells we investigated the effects of ZAAT on the expression of a range of pro- and anti-apoptotic genes in both cell types. Our experiments showed no involvement of Bax, cellular inhibitor of apoptosis (cIAP) 2 or x-linked inhibitor of apoptosis protein (XIAP), nor did we see a down regulation of the expression of a range of pro-apoptotic factors by ZAAT. We did however observe an increase in the expression of cIAP1 in 16HBE14o- cells expressing ZAAT and also detected increased expression of cIAP1 in ZAAT-expressing bronchial epithelial cells *in vivo*. cIAP1 is a member of the inhibitor of apoptosis family that enhances cell survival in response to diverse stimuli [106, 107]. It also acts as an upstream regulator of NF-κB [108]. Interestingly expression of ZAAT failed to induce cIAP1 expression in the HEK293 cells, providing an

explanation for the different apoptotic responses displayed by both cell lines in response to over expression of ZAAT.

Using terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining we could detect terminal apoptosis in HEK293 cells expressing ZAAT [63]. However in contrast to these *in vitro* studies, increased apoptosis has not been detected histologically or by TUNEL staining in the livers of ZAAT mice. One possible explanation for this may be the robust regenerative and cell survival properties of hepatocytes. Alternatively it may be a consequence of the highly specific stimulus- and cell type-specific nature of ER stress responses. The mechanism by which the caspase pathway is inhibited in ZAAT-containing hepatocytes remains unknown but could possibly be related to MAAT and ZAAT's known anti-apoptotic effects [7, 105]. However, the mechanism by which ZAAT might gain access to caspase-3 in the cytosol of liver cells is not known.

In summary expression of ZAAT can induce apoptosis mediated via inhibition of Bcl2 in HEK293 cells however, to date, ZAAT expression *in vivo* in hepatocytes or bronchial epithelial cells does not appear to have the same effect. It remains to be demonstrated whether endogenous expression of ZAAT in human monocytes or alveolar macrophages promotes cell death. Taken together these data imply that ZAAT-induced apoptosis or survival is an inherent feature of the cell in which ZAAT is expressed.

Conclusion

In ZAAT deficiency the misfolded protein results in both loss of function and gain. In addition to the chemotactic and proinflammatory properties of misfolded ZAAT, its accumulation in the ER has the potential to induce multiple signalling events related to ER stress. With respect to this toxic gain it is now emerging that these effects are not limited solely to hepatocytes, as previously thought. Interestingly, although perturbation of the ER can be induced by ZAAT expression in most cells, important differences exists in the responses induced in different cell types. The next steps in enhancing our understanding of these mechanisms will be to identify these stimulus- and cell-specific responses. Over the

past few years research has increased our awareness of the effects of misfolded ZAAT not only in the liver but throughout the body and particularly in the lung. This has importance for future therapies aimed at ZAAT deficiency. New treatments for both the liver disease and the chronic inflammation in the lungs are likely to be aimed at suppressing the pro-inflammatory effects of misfolded proteins and selectively modulating ER stress responses to enhance folding and/or disposal. Harnessing control of individual pathways may become possible by specifically targeting key ER stress proteins positioned at the apex of signalling cascades. For example promoting autophagy [109, 110] or ERAD could decrease the load of misfolded ZAAT within the ER and enhance cellular function. This knowledge we will advance our progress in the development of specific therapeutics for ZAAT deficient individuals.

Abbreviations

Activating transcription factor (ATF)

Activator protein 1 (AP-1)

Alpha-1 antitrypsin (AAT)

Antioxidant response elements (ARE)

Apoptosis signal regulating kinase 1 (ASK1)

Apoptotic protease activating factor 1 (Apaf-1).

Autophagy-related gene

Basic leucine zipper (bZIP)

BAX-interacting factor-1(Bif-1)

Carbonic anhydrase IV (CAVI)

C/EBP homologous protein (CHOP)

Cellular inhibitor of apoptosis (cIAP)

Chinese hamster ovary (CHO)

cAMP-responsive element binding protein (CREB)

Death receptor 5 (DR5)

Endoplasmic reticulum (ER)

ER-associated degradation (ERAD)

ER degradation-enhancing alpha-mannosidase-like (EDEM)

ER mannosidase I (ERManI)

ER overload response (EOR)

ER stress response elements (ERSE)

Growth Arrest and DNA Damage-Inducible Protein (GADD34)

Glucose-responsive protein 58 (Grp58/ERp57)

Glucose-responsive protein 78 (BIP/Grp78)

Human embryonic kidney (HEK)

Inhibitor of nuclear factor-κB (IκB)

Inhibitor of nuclear factor-κB kinase (IKK)

Inositol requiring kinase 1 (IRE1)

Interferon (IFN)

Interleukin (IL)

Jun N-terminal Kinase (JNK)

Lipopolysaccharide (LPS),

Monocyte chemotactic protein (MCP)

NF-E2-related factor 2 (Nrf2)

Nuclear factor-κB (NF-κB)

Nuclear transcription factor Y (NF-Y)

Neutrophil elastase (NE)

Oncostatin-M (OSM)

Protein kinase-like ER kinase (PERK)

Reactive oxygen species (ROS)

Tauroursodeoxycholic acid (TUDCA)

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

Translation initiation factor 2 alpha subunit (eIF2α)

Tribbles homologue 3 (TRB3)

Tumour necrosis factor (TNF)

Tumour necrosis factor-α-receptor-associated factor 2 (TRAF2)

UDP glucose-glycoprotein glucosyltransferase (UGT1)

Unfolded protein response (UPR)

Unfolded protein response elements (UPRE)

UV-irradiation resistance-associated gene (UVRAG)

Valosin-containing Protein (VCP)

Vesicle-mediated vacuolar sorting protein (Vsp)

X-box binding protein-1 (XBP-1)

X-linked inhibitor of apoptosis protein (XIAP),

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Figure 1. Conceptual schematic of the unfolded protein response.

Accumulation of a misfolded protein e.g. ZAAT in the ER lumen titrates Bip/Grp78 away from PERK, ATF6 and IRE1 leading to their activation. PERK dimerises, becomes phosphorylated and inactivates eIF2α - this blocks translation via eIF2B. ATF4 gene and protein expression are induced leading to ATF4-regulated gene expression, including GADD34 which stimulates protein phophatase-1 (PP1) and triggers dephosphorylation of eIF2α. Nrf2 is activated by PERK, translocates to the nucleus and induces antioxidant gene expression. The transcription factor ATF6 is transported to the Golgi and cleaved by the proteases S1P and S2P to generate the active ATF6p50 which upregulates expression of XBP1 and chaperone genes. IRE1 becomes phosphorylated, assembles into multimers and splices XBP1 (uXBP1) to generate XBP1s mRNA which encodes a transcription factor that regulates expression of EDEM1 and chaperones.

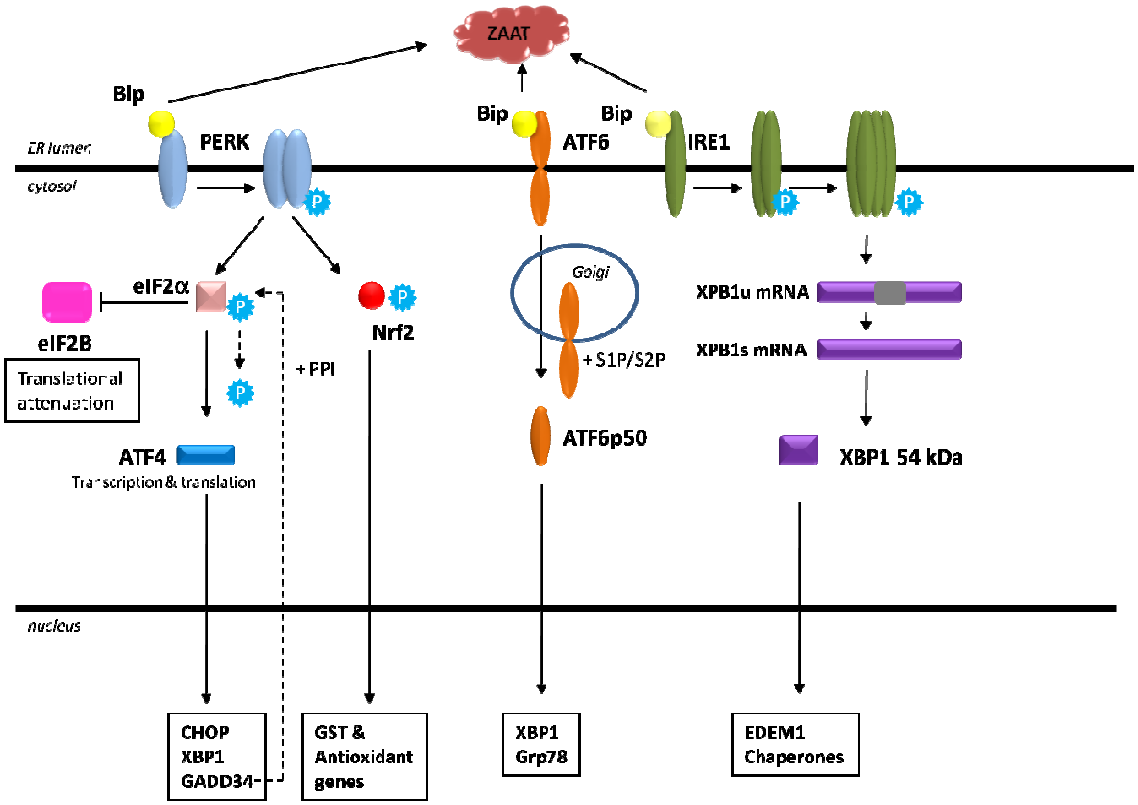
Figure 2. Potential cross-talk between different ER stress pathways.

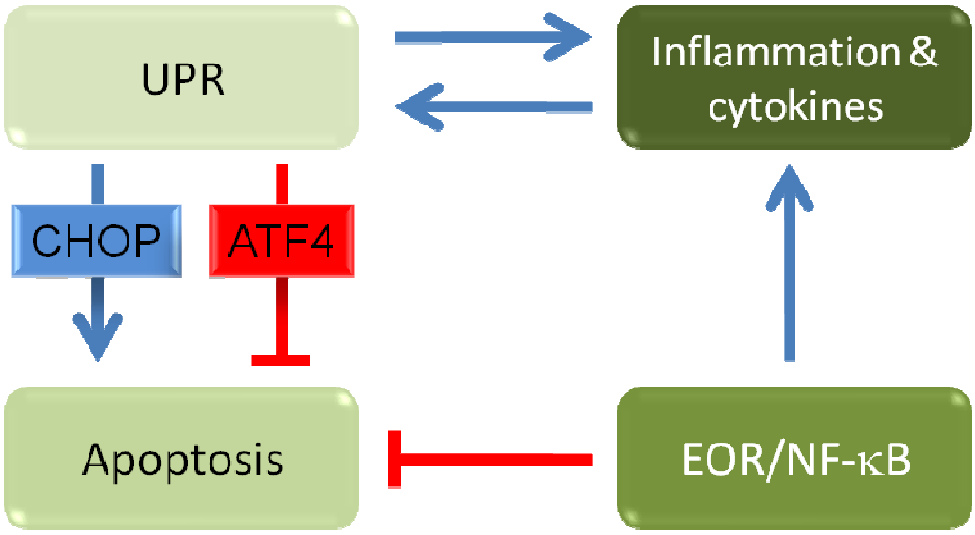
UPR can induce and inhibit apoptosis via CHOP and ATF4, respectively, whilst EOR has a largely negative effect on apoptosis by the activation of NFκB. UPR can activate NFκB/EOR and promote proinflammatory cytokine expression, with these in turn leading to further UPR activation.

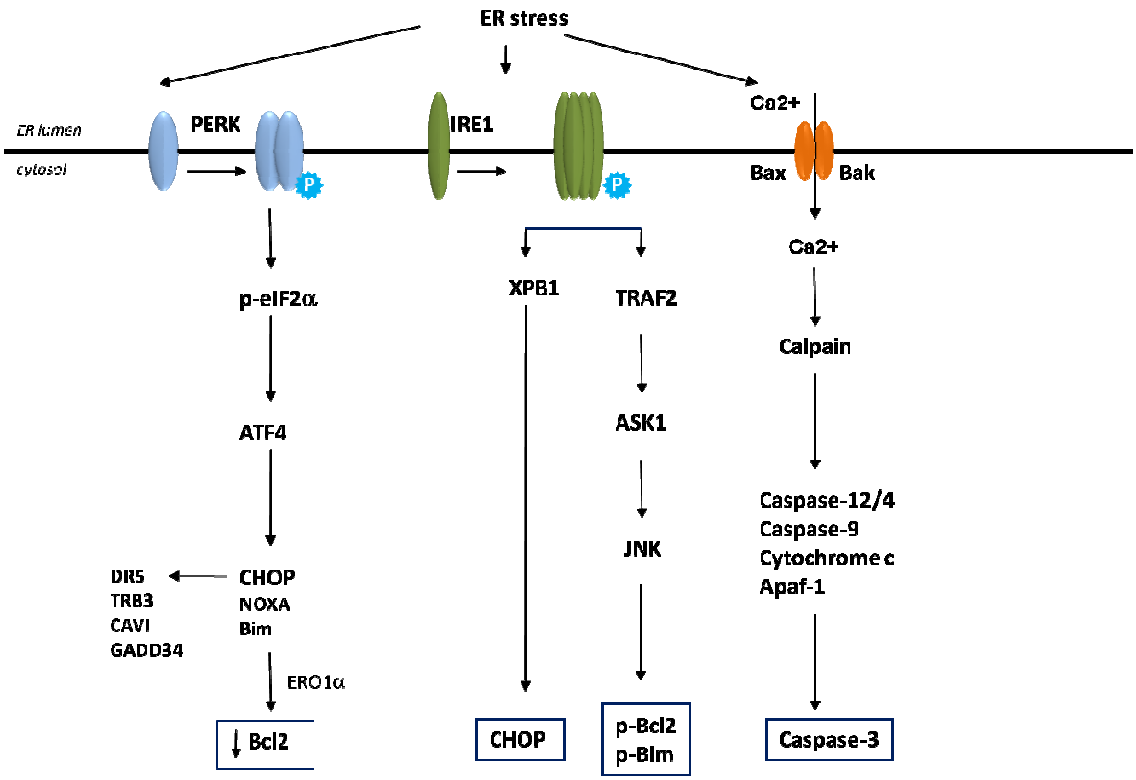
Figure 3. Pathways of ER stress-induced apoptosis.

PERK-mediated activation of ATF4 leads to the expression of pro-apoptotic CHOP, NOXA and Bim. CHOP also enhances expression of DR5, TRB3, CAVI and GADD34 and activates ERO1α leading to suppression of Bcl2. IRE1α splices XPB-1 leading to upregulation of CHOP expression. IRE1α signals via TRAF2 and ASK1 to activate JNK which then phosphorylates of Bcl2 and Bim. The proapoptotic proteins Bak and Bax undergo a conformational change, Ca2+ effluxes from the ER and activates calpain which cleave

caspase-12 (or possibly caspase-4). Signalling via caspase-9 cytochrome c and Apaf-1 activates caspase-3.




UPR
Inflammation & cytokines
CHOP
ATF4
Apoptosis
EOR/NF-κB


ER stress
ER lumen
cytosol
PERK
IRE1
Ca2+
P
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Bax
Bak
p-eIF2α
ATF4
CHOP
NOXA
Bim
DR5
TRB3
CAVI
GADD34
ERO1α
Bcl2
XPB1
CHOP
TRAF2
ASK1
JNK
p-Bcl2
p-Bim
Ca2+
Calpain
Caspase-12/4
Caspase-9
Cytochrome c
Apaf-1
Caspase-3