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Citation

Wout, E. F. A. van 't. (2014, June 4). *Endoplasmic reticulum stress in the lung : lessons from α 1-antitrypsin deficiency*. Retrieved from <https://hdl.handle.net/1887/25869>

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Title: Endoplasmic reticulum stress in the lung : lessons from α 1-antitrypsin deficiency

Issue Date: 2014-06-04

Chapter 2

The integrated stress response in lung disease

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American Journal of Respiratory Cell and Molecular Biology, in press

Abstract

Lungs are repeatedly exposed to inhaled toxic insults, such as smoke, diesel exhaust and microbes, which elicit cellular stress responses. The phosphorylation of eIF2 α by one of four stress-sensing kinases triggers a pathway called the integrated stress response (ISR) that helps protect cellular reserves of nutrients and prevents that accumulation of toxic proteins. In this review, we will discuss how activation of the ISR has been shown to play an important role in pulmonary pathology and how its study may help in the development of novel therapies for diverse conditions from hypoxia to cancer.

Introduction

Because of its direct contact with the outside world, the lung is continuously challenged by inhaled insults, including smoke, diesel exhaust and a multitude of microbes, all of which trigger various cellular stress pathways. Recent studies have underlined the critical role played by one of these stress pathways, which involves phosphorylating the alpha subunit of eukaryotic translation initiation factor 2 (eIF2 α). This single molecular event serves to integrate signalling from multiple stress-sensors and so has been termed the “integrated stress response” (ISR) (Figure 1). In this review, we will discuss the ISR with particular regard to its evolving role in pulmonary medicine and we highlight how this knowledge is enabling the development of novel therapies.

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The integrated stress response (ISR)

Activation of the ISR occurs when one of four homologous stress-sensing kinases is triggered. This family comprises General Control Nonrepressible-2 (GCN2), Heme-Regulated Inhibitor (HRI), Protein Kinase R (PKR) and PKR-like Endoplasmic Reticulum Kinase (PERK) (1). GCN2 evolved first and allows all eukaryotes, including yeast, to respond to amino acid starvation. As multicellular animals developed, this kinase gave rise to a family of proteins that could respond to other stresses. HRI was identified as a kinase responsive to iron-deficiency; however, over time, it has been shown to respond also to other stressful stimuli including damaging oxidation. PKR responds to the appearance of double-stranded RNA within the cell, which occurs most commonly during viral infections; and finally, PERK senses the efficiency of protein folding within the endoplasmic reticulum (ER), thus enabling cells to trigger the ISR during ER stress. This pathway induced by PERK activation is also one of the three arms of the unfolded protein response (UPR) to ER stress, which has been reviewed elsewhere (2).

When eIF2 α is phosphorylated, protein synthesis is blocked, which serves a number of cytoprotective roles. During ER stress, it lowers the rate at which new proteins enter the ER thereby off-loading its overburdened chaperones; in conditions of amino acid starvation or iron limitation, it reduces the rate at which these nutrients are consumed

to make new proteins; and during viral infection, blocking protein synthesis can impede viral replication. Furthermore, the phosphorylation of eIF2 α paradoxically promotes the translation of a subset of mRNAs, most notably that encoding activating transcription factor 4 (ATF4). Indeed, most canonical ISR target genes are transactivated by this transcription factor and act to adapt the cell to these stresses. For example, up-regulation of numerous transporters increases the rate of amino acid import, thus overcoming nutrient limitation, while also providing the substrates needed for the synthesis of the antioxidant glutathione (Figure 1). In addition, ATF4 induces expression of another transcription factor, C/EBP homologous protein (CHOP), which cooperates with ATF4 to induce the eIF2 α phosphatase growth arrest and DNA damage-inducible protein 34 (GADD34; also known as PPP1R15A) (3). GADD34 completes a negative feedback loop and allows protein synthesis to recover by dephosphorylating eIF2 α (4).

It is often claimed that CHOP is a pro-death transcription factor, but this is inaccurate. In some situations, the expression of CHOP promotes cell survival (5); however, in conditions of very prolonged stress, for example in models of chronic disease, the loss of CHOP is clearly protective. This protective effect of CHOP deletion reflects the toxic consequence of recovering protein synthesis (via GADD34) if the original stress has not adequately been ameliorated by the ISR. In addition, another target of CHOP is the ER oxidase ERO1 α , which is up-regulated during the ISR to promote oxidative protein folding, but can itself contribute to cellular oxidative stress (Figure 1). Finally, CHOP contributes to the induction of pro-inflammatory genes, such as interleukin (IL)-8 (6).

Activation of the ISR in lung pathology

Inhaled toxins

Of the pulmonary insults recognised to trigger the ISR, perhaps the best known is oxidative stress, frequently caused by exposure to cigarette smoke. *In vitro* studies have shown that cigarette smoke extract induces apoptosis in a CHOP-dependent manner and this can be antagonised by antioxidants (7, 8). ER stress appears to be a major mediator of

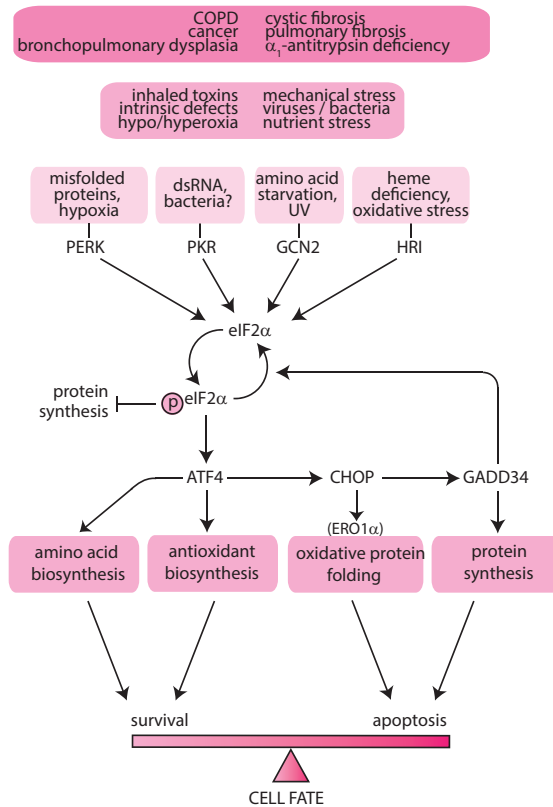


Figure 1. The integrated stress response (ISR) in the lung.

Phosphorylation of eIF2α by the stress-sensing kinases GCN2, PKR, PERK or HRI, leads to activation of the ISR by a wide range of insults including amino acid starvation, viral infection, protein misfolding and iron deficiency. This attenuates global protein synthesis via the inhibition of eIF2B, which normally functions to maintain the translation initiation factor eIF2 in its active GTP-bound state. Simultaneously, limiting levels of active (GTP-bound) eIF2 lead to the translation of the transcription factor ATF4. Targets of ATF4 include amino acid transporter and synthesases, which serve a cytoprotective role. Via induction of the transcription factor CHOP and the eIF2α phosphatase GADD34, ATF4 also promotes the recovery of protein synthesis and oxidative protein folding (through induction of the oxidase ERO1α). During severe or prolonged stress, the recovery of protein translation and protein oxidation can contribute to the failure of homeostasis and ultimately to cell death. COPD, chronic obstructive pulmonary disease.

the response to cigarette smoke extract because inhibition of the kinase PERK can block this induction of CHOP (7). Consistent with this, heightened levels of ER stress have been observed within the lungs of current smokers (9) and patients with COPD (10). It has recently been demonstrated that exposure even to the smoke of a single cigarette can impair oxidative protein folding in lungs of mice, probably through impaired function of the ER enzyme Protein Disulphide Isomerase (PDI) (11).

Recently, an airway gene expression signature associated with COPD was described and included 98 genes, many of which are targets of ATF4 (12). When ATF4 is overexpressed in epithelial cells *in vitro*, this COPD gene signature is recapitulated. The observation that many of the COPD-associated changes in gene expression in bronchial biopsies can be reversed by inhaled corticosteroid therapy suggests that treatments might be targeted to this mechanism in future (12).

When mice are exposed to inhaled environmental particulate pollution, they also show evidence of ISR activation due to ER stress (13), as do cultured human cells treated with diesel exhaust particles (14). Since the effects of particulate matter can be antagonised by antioxidants, these too most likely reflect oxidative stress impairing the function of the ER (13). In fact, the toxicity of particulates may be mediated by the ISR, since CHOP deficient cells are protected from particulate-induced apoptosis.

It would be wrong to suggest that the ISR is purely toxic during pulmonary oxidative stress or that PERK is the sole kinase involved. For example, when mice are exposed to hyperoxia (95% O₂), phosphorylation of eIF2 α does not involve ER-stress (5). Instead, PKR is activated by unknown mechanisms to induce ATF4 and CHOP. In this instance, CHOP is protective since *Chop*^{-/-} mice reared in 95% O₂ develop more severe lung injury, and even modest hyperoxia (80% O₂) causes higher mortality in CHOP deficient animals (5). The mechanism for this protection is unclear but CHOP appears to prevent increased epithelial permeability. But the complex role of CHOP in hyperoxic lung injury requires more study because in newborn mice with hyperoxia-induced bronchopulmonary dysplasia CHOP appears to mediate pathological apoptosis (15).

Intrinsic defects

Point mutations of secreted proteins can cause ER stress and activate PERK. Surfactant protein C (SFTPC), which is normally secreted by type II pneumocytes, is mutated in some rare cases of familial interstitial lung disease (16). A splice-site mutation (c.460+1A>G) that causes deletion of *SFTPC* exon4 results in protein misfolding and familial interstitial pneumonia. The *Sftpc* null mouse has a non-lethal phenotype suggesting that the disease-associated mutations in man may be caused by toxic gain-of-function. Accordingly, when the Δ exon4 mutant is expressed in cells it causes ER stress and phosphorylation of eIF2 α (17). It remains to be tested, however, whether *CHOP* or *GADD34* genotype can alter the toxicity of the SFTPC mutants. Although currently only a correlation, it is intriguing that elevated levels of ATF4 and CHOP protein have been detected in lung homogenates and in type II cells from patients with idiopathic pulmonary fibrosis compared with controls (18). It is therefore tempting to speculate that ISR activation may be a final common pathway in many cases of interstitial lung disease.

Mutations of the *SERPINA1* gene, which encodes α_1 -antitrypsin, can lead to its accumulation within the ER, but surprisingly most of these mutants fail to trigger the UPR, although they do enhance the cell's susceptibility to ER stress (19). The most common of these, the so-called Z allele, results from a missense mutation (E342K) that destabilises the protein such that most is degraded while some polymerises within the ER resulting in serum deficiency and early onset emphysema in most homozygous individuals. Approximately 10% of homozygotes also develop clinically significant liver disease but the mechanism for this is not entirely clear. The increased sensitivity of hepatocytes to ER stress and therefore activation of the ISR by PERK may be involved, since mice made to express the Z variant are far more susceptible to developing hepatic fibrosis following bile duct ligation, which causes ER stress, than are animals expressing wild type α_1 -antitrypsin (20). In this model, Z expressing mice show an exaggerated ISR as evidenced by significantly higher induction of CHOP (20) and it is known that CHOP expression is essential for cholestasis-induced hepatic fibrosis (21). However, these effects are critically dependent on the level of Z α_1 -antitrypsin expressed by a cell. Primary bronchial epithelial cells,

which secrete detectable levels of Z α_1 -antitrypsin, fail to achieve concentrations of the mutant protein within their ER to allow protein polymerisation (22). As a result, in contrast to hepatocytes, airway epithelial cells show no augmentation of CHOP or GADD34 expression upon a second hit of mild ER stress (22). Each tissue should therefore be considered individually, since monocytes, another α_1 -antitrypsin-expressing cell, have been reported to display enhanced expression of ATF4 when purified from Z homozygous individuals (23). Whether these cells, unlike hepatocytes, experience constitutive activation of the ISR in Z individuals is not clear. But it is plausible that isolation of the cells may be sufficiently stressful to trigger the ISR, which in itself represents an interesting question for future research.

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Infection

The lung is exposed daily to large numbers of inhaled infectious micro-organisms. These represent the third class of stimuli causing activation of the ISR. The canonical stimulus for the eIF2 α kinase PKR is dsRNA, which reduces the rate of protein translation and so impedes viral replication (as reviewed in (24)). Because of the need to recover translation in order to synthesise inflammatory cytokines, there is also a requirement for GADD34 induction and translational recovery as demonstrated by the increased susceptibility of GADD34 deficient cells and mice to viral infection (25). But there is redundancy within the system: for example the coronavirus IBV (infectious bronchitis virus) can activate both PKR and PERK, leading to expression of ATF4 and CHOP (26). Surprisingly, both kinases appear to mediate the cytotoxicity of this infection via CHOP-dependent apoptosis. Curiously, although PKR serves an antiviral role in most instances, in IBV infection, eIF2 α phosphorylation contributes to enhance viral replication.

Activation of Toll-like receptors (TLRs), specifically TLR3 by dsRNA and TLR4 by lipopolysaccharide (LPS), can also trigger phosphorylation of eIF2 α (24). The mechanism for this is not entirely clear, but may plausibly reflect increased ER activity due to the need to secrete cytokines and antimicrobial peptides upon TLR activation. Interestingly, the ISR shows specific modulation by TLR signalling that goes beyond simple activation.

In macrophages, when phosphorylation of eIF2 α is induced by other stimuli, for example ER stress, activation of TLR4 by LPS blocks induction of ATF4, CHOP and GADD34 (27). It is now becoming clear how this can be explained. Normally, when eIF2 α is phosphorylated it binds to and inhibits eIF2B, an enzyme responsible for maintaining eIF2 α in its active GTP-bound state. Indeed, binding of phospho-eIF2 α to eIF2B is responsible both for the inhibition of translation seen during the ISR and for the up-regulation of ATF4 and CHOP. However, activated TLR4 stimulates eIF2B thus overcoming the effects of eIF2 α phosphorylation (28). It has been proposed that this mechanism may enable cells selectively to inhibit the ISR during chronic ER stress caused by infection and thus avoid the toxic effects of inducing CHOP and its target GADD34.

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But the phosphorylation of eIF2 α and subsequent activation of the ISR can have negative consequences during chronic infection and so may represent a therapeutic target. In cystic fibrosis, most causative mutations of CFTR do not cause robust ER stress directly because these mutations lie within its cytosolic portion (29, 30). But loss of CFTR function leads to the generation of thickened airway mucus with reduced clearance, which is prone to chronic colonisation by bacteria such as *Pseudomonas aeruginosa*. This induces local ER stress and can be recapitulated in normal airway epithelia by application of CF mucus directly (30), and explains why primary CF epithelia recover from ER stress if allowed to grow *in vitro* in the absence of colonised mucus (29). It has been noted, however, that the ER stress seen in models of CF in response to *P. aeruginosa* is deficient in PERK-eIF2 α signalling (31). This may contribute to the chronic inflammation seen in CF, since pharmacological induction of eIF2 α phosphorylation with the drug salubrinal was found to lessen the inflammatory response of CF cells. The mechanism by which ER stress fails to activate the ISR in this context has not been studied in detail, but may involve the novel TLR-eIF2B pathway introduced above.

Where on-going translation is essential for efficient immune responses, for example in PKR-activated dendritic cells, a modified ISR is activated in which robust induction of GADD34 prevents significant translational attenuation (32). This and other observations, including those of the TLR-eIF2B pathway, have given rise to the concept of a

specific “microbial stress response”, which shares many features of the canonical ISR, but with modifications such as less robust induction of CHOP (reviewed in (24)). However, the induction of components of the ISR, including CHOP, can differ substantially between models, perhaps because of cell type or the stimuli used, and thus many ‘flavours’ of the ISR may exist. For example, when dendritic cells are challenged either with ER stress or TLR agonists, efficient induction of CHOP is required for secretion of interleukin (IL-) 23 (33), which appears to contrast with the response elicited by PKR activation. It should also be noted that viruses and bacteria have evolved numerous mechanisms to escape the antimicrobial effects of the ISR. For example, respiratory syncytial virus (RSV) can sequester activated PKR and prevent phosphorylation of eIF2 α (34).

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Nutrient stress

Being the oldest in evolutionary terms, GCN2 is ubiquitous in eukaryotes. In terms of pulmonary pathology, it has been studied most in the context of tumour biology as it functions to match amino acid supplies with demand, which can be a limiting factor in cancer growth. The induction of ATF4 during nutrient stress promotes the induction of many amino acid transporters and synthetic enzymes in tumour cells, such as asparagine synthetase (35). As a result, inhibition of the GCN2-ATF4 pathway can reduce proliferation and cell survival (35). This accounts for the up-regulation of GCN2 (both activated and total) in lung cancer samples compared to healthy controls or the surrounding non-tumorous cells (35).

In addition to limiting the levels of nutrients, such as amino acids required for macromolecule biosynthesis, nutrient deprivation also profoundly affects the metabolism of a tumour. A consequence of this is a heightened level of ER stress that is seen in many hypoxic tumours (36, 37). It is therefore unsurprising that ER stress and the UPR have been implicated in the pathophysiology of solid tumours including human lung cancer (8). Evidence of ER stress is associated with more aggressive tumours and resistance to chemotherapy (36). As the mediator of eIF2 α phosphorylation during ER stress, PERK has proved necessary for the growth of larger solid tumours (37) and contributes to resistance

to therapy (36). It ensures that protein synthesis remains at a level consistent with the supply of nutrients and energy, and through ISR-mediated activation of autophagy it also promotes the recycling of nutrients from old and damaged organelles (38). Under such conditions, the recovery of translation mediated by CHOP and GADD34 would be expected to be toxic. Indeed, loss of GADD34 in tumours may prevent apoptosis and promote cell survival in hypoxic conditions (39). Accordingly, GADD34 expression correlates with the degree of differentiation of malignant mesothelioma, with lower levels of expression being seen in the more aggressive sarcomatoid subtype (40). It is therefore tempting to speculate that GADD34 may be a tumour suppressor in ER stressed environments, but this has yet to be tested formally.

Mechanical stress

Finally, the pulmonary epithelium is not a static culture of cells as it is frequently treated in the laboratory. Instead, it is a rhythmically flexing and stretching structure. The forces placed upon it profoundly alter its biology, as the challenges of invasive ventilation in acute lung injury have shown. Marked mechanical stress has been shown to induce apoptosis in a PERK dependent manner (41). Surprisingly, other limbs of the UPR do not appear to be activated, even during prolonged mechanical stress. Moreover, the mechanism of apoptosis is independent of CHOP-GADD34. This is likely to be a fruitful area for research and there will be much to learn from other areas of biology, for example the bone where cyclical loading, rather than static loads, have been shown to modulate protein synthesis via activation of PKR (42).

Therapeutic options

It is clear that the ISR plays a crucial role in many aspects of pulmonary pathophysiology and the basic science of ISR signalling is sufficiently well understood that it could plausibly be manipulated for therapeutic gain. In many cases, the transient activation of the ISR plays a cytoprotective role. For that reason, agents that promote phosphorylation of eIF2 α have been shown to be protective. The small molecule salubrinal

was identified as a compound that prevented the dephosphorylation of eIF2 α during ER stress and during infection with herpes simplex virus (HSV) (43). Although it has been claimed to inhibit the eIF2 α phosphatases directly, convincing evidence for this is lacking. Nevertheless, it can increase cellular levels of phosphorylated eIF2 α with cytoprotective results. Amelioration by this drug of inflammatory signalling in *Pseudomonas*-exposed CF cells has already been mentioned (31), but salubrinal has also been shown to be protective in the monocrotaline model of pulmonary hypertension (44) and the toxicity of cigarette smoke extract (45). More recently, the drug guanabenz, used previously as an anti-hypertensive, has been found to inhibit GADD34 thus increasing phosphorylation of eIF2 α and promoting cell survival during chronic ER stress (46). The hypotensive effects of this drug, which are unrelated to its effects on the ISR, limit its use in animals, but it is likely that future GADD34 inhibitors will be useful in situations where activation of the ISR would be beneficial.

In contrast, blockade of the cytoprotective effects of the ISR will be useful in chemotherapy. Potent small molecules have been developed, such as GSK2656157, that show high selectivity for PERK, which is especially important in malignancy, and in animal models have proved effective at inhibiting tumour growth through alterations in amino acid metabolism and angiogenesis (47).

Conclusion and future directions

The progress from understanding the basic biology of the ISR to its role in pulmonary pathology has been dramatic and rapid. The breadth of noxious stimuli to which it responds may hamper efforts to identify which stress or stresses were the original insult in some diseases; however, the very fact that multiple kinases converge on a single substrate to regulate translation during disease offers many advantages for research and treatment. If selectivity is required, identification of the upstream kinase should offer this, while modulation of the downstream phosphatase(s) can generate a more broad effect. But many unknowns remain, which will make the ISR an exciting and fruitful area for respiratory research for many years to come.

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