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Pathological consequences of the unfolded protein response and downstream protein disulphide isomerases in pulmonary viral infection and disease

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Nicolas Chamberlain and Vikas Anathy*

Department of Pathology and Laboratory Medicine, University of Vermont Larner College of Medicine, 149 Beaumont Ave, Burlington, VT 05405, USA

*Vikas Anathy, Department of Pathology and Laboratory Medicine, University of Vermont Larner College of Medicine, 149 Beaumont Ave, HSRF Rm 218, Burlington, VT 05405, USA. Tel.: +1-802-656-0395, Fax: 802-656-8892, email: vikas.anathy@med.uvm.edu

Protein folding within the endoplasmic reticulum (ER) exists in a delicate balance; perturbations of this balance can overload the folding capacity of the ER and disruptions of ER homoeostasis is implicated in numerous diseases. The unfolded protein response (UPR), a complex adaptive stress response, attempts to restore normal proteostasis, in part, through the up-regulation of various foldases and chaperone proteins including redox-active protein disulphide isomerases (PDIs). There are currently over 20 members of the PDI family each consisting of varying numbers of thioredoxin-like domains which, generally, assist in oxidative folding and disulphide bond rearrangement of peptides. While there is a large amount of redundancy in client proteins of the various PDIs, the size of the family would indicate more nuanced roles for the individual PDIs. However, the role of individual PDIs in disease pathogenesis remains uncertain. The following review briefly discusses recent findings of ER stress, the UPR and the role of individual PDIs in various respiratory disease states.

Keywords: disulphide bond; ER stress; PDI; pulmonary disease; UPR.

Abbreviations: ATF4, activating transcription factor 4; ATF6, activating transcription factor-6; CF, cystic fibrosis; CHOP, C/EBP homologous protein; CoV, coronaviruses; eIF2a, eukaryotic initiation factor 2a; ER, endoplasmic reticulum; ERAD, ER-associated degradation; ERSEs, ER stress response elements; HTBE, human tracheobronchial epithelial; IAV, influenza A virus; IRE1, inositol-requiring protein 1; PDIs, protein disulphide isomerases; PERK, (PKR)-like ER kinase; PKR, protein kinase RNA; RIDD, regulated IRE1 dependent degradation of mRNA; RSV, respiratory syncytial virus; RV, rhinovirus; TRAF2, tumour necrosis factor associated factor 2; UPR, unfolded protein response; XBP1, X-box binding protein 1.

The endoplasmic reticulum (ER) is a highly specialized organelle that plays numerous roles in the cell. It is the primary site of the synthesis of membrane-bound and secreted proteins, and as such maintains an oxidizing redox environment to facilitate the formation of disulphide bonds required for the stabilization of peptide structure (*1*). Additionally, numerous posttranslational modifications such as N-inked glycosylation occur solely within the ER (*2*). Approximately one-third of all proteins that traffic through the ER contain disulphide bonds and the ER contains a vast array of chaperones and foldases to assist in the folding of newly synthesized peptides (*1*, *3*). Properly folded proteins are essential for the normal function of the cell, and potentially misfolded proteins are rapidly degraded by the ER-associated degradation (ERAD) (*4*). Under basal conditions, ~30% of newly synthesized peptides are targeted for degradation (*5*).

Protein folding within the ER exists in a delicate balance, and the physiological states of increased protein synthesis can quickly overload the folding capacity of the ER leading to a buildup of unfolded or misfolded peptides in the ER lumen, termed ER stress. In an effort to combat this stress the cell activates the unfolded protein response (UPR), a highly conserved, multifaceted stress response aimed at restoring normal ER homoeostasis (*6*). Collectively the UPR attenuates normal protein synthesis, up-regulates ERAD machinery, increases the size of the ER and up-regulates various chaperones, including protein disulphide isomerases (PDIs) a large family of proteins that assists in the oxidative folding of nascent peptides (*6*). Failure of the UPR to restore normal ER homoeostasis leads to cell death through the activation of apoptotic pathways (*6*).

ER stress and activation of the UPR are common in the progression of numerous diseases including various cancers (*7*), neurodegenerative disorders (*8*) and viral infections (*9*). The individual aetiology of these disorders is as diverse as the cell's response to each. And the exact intricacies of the molecular mechanisms underlying activation of the UPR and subsequent up-regulation of distinct PDIs remains poorly understood. A greater understanding of host pathways involved could potentially aid in the development of future treatments. The following review briefly discusses recent findings of ER stress, the UPR and the role of individual PDIs in various respiratory disease states.

The Unfolded Protein Response

The UPR is a highly conserved collection of pathways responsible for monitoring the status of the ER. In mammals, the UPR consists of three pathways each

controlled by a particular sensor, inositol-requiring protein 1 (IRE1), protein kinase RNA (PKR)-like ER kinase (PERK) and activating transcription factor-6 (ATF6) (Fig. 1). IRE1 exists in two isoforms α and β, this review focuses on solely IRE1 α, as the role the β isoform in activation of the UPR and induction of PDIs during pulmonary disease is less well characterized. These three pathways work in concert to decrease the protein load of the ER while simultaneously increasing its folding capacity. If the ER stress is too pronounced, or prolonged, the UPR directs the cell towards apoptosis (*10–12*).

Generally, the PERK pathway limits protein synthesis, the IRE1 pathway increases mRNA and protein degradation and the size of the ER, while the ATF6 pathway up-regulates chaperone proteins and protein degradation (*10*). Each of these transducers are integral membrane proteins residing in the ER membrane. Under normal conditions are held in inactive conformations by GRP78, an ER resident chaperone. Under conditions of ER stress unfolded or misfolded protein builds up within the ER lumen, GRP78 dissociates from the sensors owing to higher affinity to exposed hydrophobic residues on the unfolded proteins (*11*). While GRP78 is considered a master regulator of the UPR, the ER chaperone HSP47 has recently been shown as a selective regulator of the IRE1 arm of the UPR, displacing GRP78 and facilitating IRE1 oligomerization (*13*). Additionally, there is evidence that the individual transducers can bind unfolded protein directly (*14*).

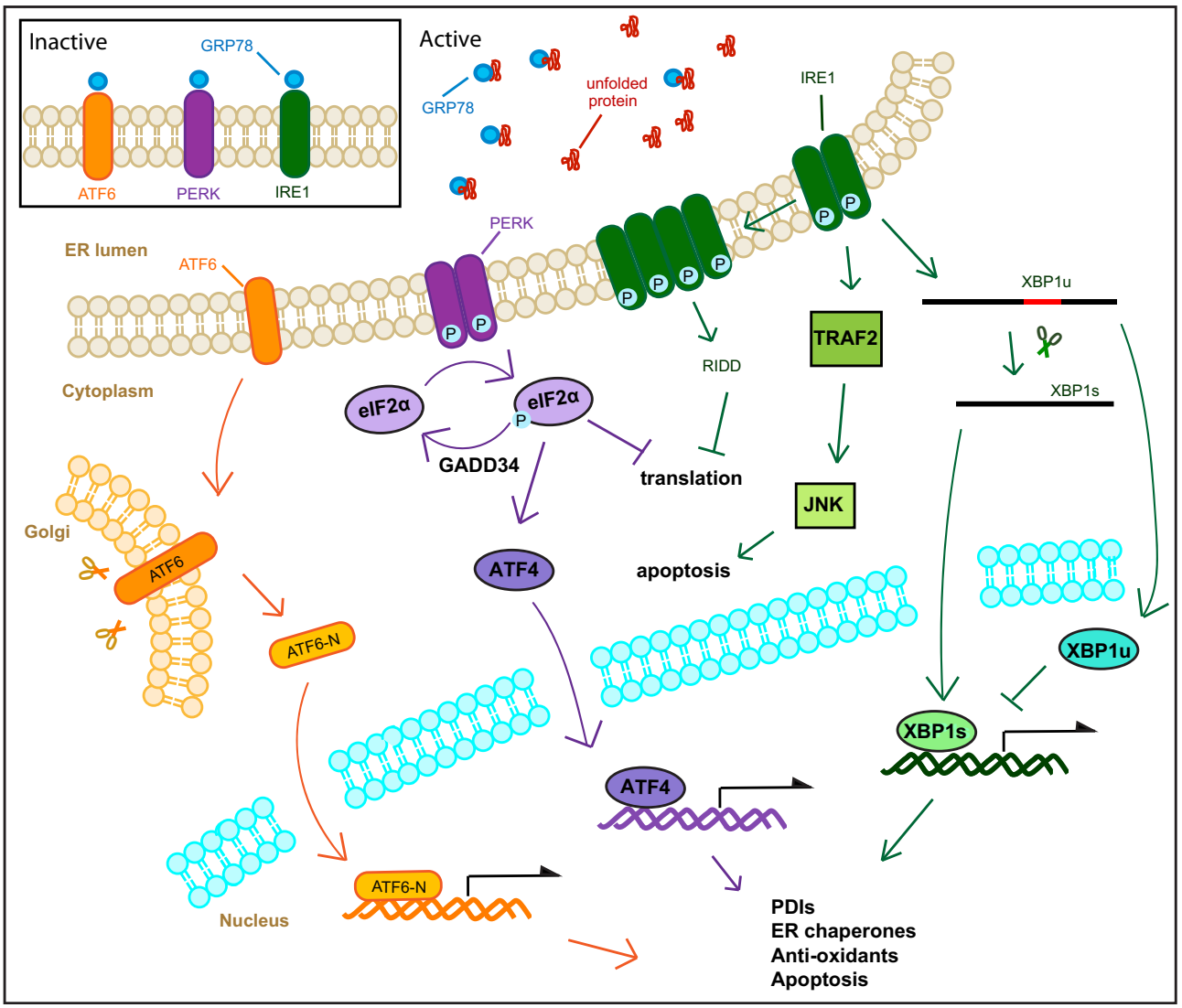

Fig. 1. Representation of canonical UPR signalling pathways. The UPR is activated by a buildup of unfolded protein within the ER lumen. GRP78 dissociates from the three ER stress sensors IRE1, ATF6 and PERK. Dimerization of IRE1 leads to autophosphorylation activating ribonuclease activity specific to XBP1 mRNA. This splicing generates XBP1s which is transported to the nuclease and induces the expression of UPR target genes. IRE1 phosphorylation also activates TRAF2 which directs the cell towards apoptosis through JNK signalling. IRE1 is also capable of associating into higher order structures which allow for non-specific degradation of ER associated mRNAs (RIDD). PERK dimerization leads to autophosphorylation activating kinase activity specific to eIF2a, halting protein translation. This loss of translation drives expression of ATF4 which acts as a transcription factor and induces the expression of UPR target genes. eIF2a is regenerated by GADD34. Upon dissociation of GRP78 from ATF6, ATF6 is transported to the Golgi where it is cleaved by cellular proteases to produce a transcription factor which induces the expression of UPR target genes.

Following GRP78 disassociation IRE1 dimerizes and undergoes trans-autophosphorylation of cytosolic kinase domains (*11*). Upon phosphorylation IRE1 displays endonucleolytic activity specifically targeting X-box binding protein 1 (XBP1) mRNA, this activity removes an intron and ultimately induces a frame shift by removing a stop codon. The spliced XBP1 (XBP1s) is translated and acts as a transcription factor driving the expression of ER chaperones, ERAD proteins and lipid synthesis (*15*). In mammals both spliced and unspliced (XBP1u) are translated, interestingly while XBP1s acts a potent transcriptional activator of UPR effector genes, XBP1u acts as a repressor of the UPR (*16*) (Fig. 1).

Tumour necrosis factor associated factor 2 (TRAF2) is known to interact with IRE1 under conditions of extended ER stress leading to activation of downstream inflammatory and apoptotic signalling (*10*).

IRE1 is also capable of forming higher order structures that utilize their endonucleolytic activity to degrade ER-localized mRNAs in a process called regulated IRE1 dependent degradation of mRNA (RIDD) (*17*).

Like IRE1, PERK also undergoes dimerization and transphosphorylation following release from GRP78. PERK then phosphorylates eukaryotic initiation factor 2a (eIF2a) attenuating cap-dependent protein translation. This decrease in global translation leads to the cap-independent translation of activating transcription factor 4 (ATF4). ATF4 leads to the expression of amino-acid transporters, genes important in protecting the cell against oxidative stress and XBP1 (*10*). ATF4 also leads to the expression of C/EBP homologous protein (CHOP), another transcription factor that drives the cell towards apoptosis (*10*).

Unlike both IRE1 and PERK, ATF6 can exist as either a monomer or an oligomer, stabilized by disulphide bonds, while still bound to GRP78. ATF6 contains a Golgi localization signal that is masked by GRP78, upon disassociation ATF6 is translocated to the Golgi body where it is consecutively cleaved by two proteases SP1 and SP2 (*18*). Interactions with PDIs ensure that only reduced monomeric ATF6 is moved to the Golgi. The proteases liberate the N-terminal cytosolic domain of ATF6 (ATF6-N). ATF6-N is a transcription factor that moves to the nucleus and induces expression of various UPR target genes. Chaperone proteins are the primary targets of ATF6-N, including GRP78, GRP94 and PDIs (*10*).

While it is useful to separate UPR signalling pathways into discrete units, there exists a large amount of crosstalk between them. Genes under the control of the UPR often contain ER stress response elements (ERSEs) in their promoter regions that are responsible for transcriptional induction. Both XBP1s and ATF6-N can bind to these elements, though ATF6-N binding requires additional transcription factors (*19*). Interestingly, XBP1s and ATF6-N can form heterodimers, which further complicates signalling (*20*). Moreover, all three UPR pathways often involve the same proteins (*10*). The PERK and ATF6 pathways lead to XBP1 expression, which is then processed by IRE1. And all three pathways converge on NF-κB activation, though each uses a distinct mechanism.

However, this does not mean the UPR exists in a binary state of either active or inactive. The individual pathways of the UPR can be activated independently of one another. For instance, numerous groups have shown differential pathway activation following influenza infection, suggesting distinct triggers for each signalling pathway (*21*, *22*).

Furthermore, while UPR activation is classically thought to involve the accumulation of unfolded protein within the ER lumen, there are numerous studies demonstrating activation of the UPR in the absence of unfolded protein. Toll-like receptor (TLR)2 and 4 have been shown to activate IRE1 and subsequent XBP1 maturation in macrophages (*23*). Notably, this XBP1 activation did not induce expression of canonical ER stress genes but was required for the continued production of proinflammatory cytokines (*23*). Similarly, it has been reported dendritic cells constitutively activate the IRE1 arm of the UPR in the absence of ER stress, and this activation was required for homoeostasis of CD8α^+ dendritic cells (*24*, *25*). Additionally, mitochondrial reactive oxygen species have been shown to exacerbate TLR induced activation of the UPR (*26*). These findings are particularly interesting as the utilization of TLRs as an alternative activation pathway would suggest that pathogens themselves or pattern-associated molecular patterns (PAMPs) (*27*) and damage-associated molecular patterns (DAMPs) are directly capable of activating the UPR (*28*).

Protein Disulphide Isomerases

The UPR up-regulates a wide variety of chaperone proteins in an effort to restore normal proteostasis, among these are PDIs a large family of redox-active chaperones that play important roles in the formation, reduction and isomerization of disulphide bonds (*29*). Currently, there are over 20 members of the PDI family, each differentiated from one another by the number and organization of TRX domains (*29*, *30*). Individual TRX domains are classified as catalytically active (a) or protein binding (b) by the presence or absence of a largely conserved CXXC sequence (Table I). The CXXC motif allows for the oxidoreductase activity of PDIs by alternating between an oxidized form, where both cysteines are linked through a disulphide bond, and a reduced form containing two free sulphydryl groups. This effectively transfers a disulphide to the client protein. While the individual catalytic sequences vary the overall mechanism remains the same, with the intervening residues modulating the pKa of the reactive residues. The N terminal Cys exists as a thiolate (-S^-) anion due to its lower pKa, which mediates nucleophilic attack forming a mixed disulphide. The C terminal Cys is partially buried within the protein elevating its pKa relative to other thiols and preventing the reverse reaction from occurring. The pKa of this C terminal Cys is rapidly decreased by a conformational change in the protein itself, which brings the side chain of a highly

Table I. Characteristics of the human PDI gene family

Protein name	Gene name	Accession number	Gene location	TRX domain organization	Catalytic sequence	ER locatlization	ER retention sequence	Molecular weight (Da)
PDIA1	*P4HB*	P07237	17q25.3	a-b-b′-a′	CGHC, CGHC	ER lumen	Yes	57,116
PDIA2	*PDIA2*	Q13087	16p13.3	a-b-b′-a′	CGHC, CTHC	ER lumen	Yes	58,206
PDIA3	*PDIA3*	P30101	15q15.3	a-b-b′-a′	CGHC, CGHC	ER lumen	Yes	56,782
PDIA4	*PDIA4*	P13667	7q36.1	a′-a-b-b′-a′	CGHC, CGHC, CGHC	ER lumen	Yes	72,932
PDIA5	*PDIA5*	Q14554	3q21.1	b-a′-a-a′	CSMC, CGHC, CPHC	ER lumen	Yes	59,594
PDIA6	*PDIA6*	Q15084	2p25.1	a′-a-b	CGHC, CGHC	ER lumen	Yes	48,121
PDIA7	*PDILT*	Q8N807	16p12.1	a-b-b′-a′	SKQS, SKKC	ER lumen	Yes	66,657
PDIA8	*ERP27*	Q96DN0	12p12.3	b-b′	No catalytic site	ER lumen	Yes	30,480
PDIA9	*EPR29*	P30040	12q24.13	b	No catalytic site	ER lumen	Yes	28,993
PDIA10	*ERP44*	Q9BS26	9q31.1	a-b-b′	CRFS	ER lumen	Yes	46,971
PDIA11	*TMX1*	Q9H3N1	14q22.1	a	CPAC	Membrane bound	No	31,791
PDIA12	*TMX2*	Q9Y320	11q12.1	a	SNDC	Membrane bound	Yes	34,038
PDIA13	*TMX3*	Q96JJ7	18q22.1	a-b-b′	CGHC	Membrane bound	Yes	51,872
PDIA14	*TMX4*	Q9H1E5	20p12.3	a	CPSC	Membrane bound	Yes	38,952
PDIA15	*TXNDC5*	Q8NBS9	6p24.3	a′-a-a′	CGHC, CGHC, CGHC	ER lumen	Yes	47,629
PDIA16	*TXNDC12*	O95881	1p32.3	a	CGAC	ER lumen	Yes	19,206
PDIA17	*AGR2*	O95994	7p21.1	a	CPHS	ER lumen	Yes	19,979
PDIA18	*AGR3*	Q8TD06	7p21.1	a	CQYS	ER lumen	Yes	19,171
PDIA19	*DNAJC10*	Q8IXB1	2q32.1	a′-b-a′-a-a′	CSHC, CPPC, CHPC, CGPC	ER lumen	Yes	91,080
PDIB1	*CASQ1*	P31415	1q23.2	b-b-b′	No catalytic site	ER lumen	No	45,160
PDIB2	*CASQ2*	O14958	1p13.1	b-b-b′	No catalytic site	ER lumen	No	46,436

conserved arginine in close proximity to the active site. This shift in pKa changes the C terminal Cys from a thiol to a thiolate anion, which acts to resolve the mixed disulphide through a subsequent nucleophilic attack. Additionally, salt bridges located beneath the active site also serve to modulate the pKa of the Cys residues. PDIs in the reduced dithiol state participate in isomerization reactions, shuffling disulphide bonds between Cys residues, whereas oxidized (-S-S-) PDIs introduce disulphide bonds into associated peptides. The introduction of a disulphide bond into the client protein leaves the oxidized PDI in the reduced state where it can be rapidly re-oxidized by an intricate network of enzymes (Fig. 2). The isomerization of disulphide bonds does not involve a net change in disulphides, so the enzyme remains in the reduced state following the reaction. The above process is extensively reviewed in the following references (*31*, *32*).

The re-oxidation of PDIs falls primarily to ERO1, which using FAD as a cofactor, transfers electrons from PDIs to molecular oxygen reducing it to hydrogen peroxide (*33*). Additional enzymes can oxidize PDIs: such as GPx7 and GPx8, two peroxidases that directly oxidize PDIs while reducing hydrogen peroxide (*34*). Glutathione is the primary redox buffer in the ER, and reduced glutathione is known to oxidize PDI *in vitro* (*35*). Interestingly oxidized glutathione has been shown to reduce PDIA3 *in vivo*, demonstrating its buffering role. The ability to reduce PDIA3 suggests glutathione possesses the ability to reduce other PDIs *in vivo* as well (*36*). While the non-catalytic b domains lack an active site, they nonetheless assist in the chaperone activity of PDIs by assisting in protein binding.

PDIs were originally characterized as ER resident proteins; most members of the family contain either a canonical KDEL sequence or a non-canonical retention sequence. Despite the near total presence of an ER retention sequence PDIs are commonly found throughout the cell, at the cell surface or even preferentially secreted from the cell (*37*). The dispersal throughout the cell despite the presence of a retention sequence may suggest unexplored roles for non-canonical retention sequences.

As one might expect, owing to the high degree of homology in the PDI family there exists a large amount of redundancy in terms of both functionality and client proteins. However, certain proteins appear to be clients of specific PDIs (*38*). PDIA3 has enhanced specificity towards glycoproteins owing to its association with both calreticulin and calnexin, two lectin-based chaperones within the ER lumen (*39*).

UPR and PDIs in asthma and pulmonary fibrosis

The UPR is initiated to manage the ER stress, but intense ER stress can result in apoptosis. Excessive ER stress and unhindered UPR can lead to apoptosis, proinflammatory signalling and epithelial-mesenchymal transition, features that have all been linked to lung fibrosis (*40–43*) and asthma (*39*, *44–46*).

Although evidence is emerging, that downstream of UPR, PDIs are up-regulated in both asthma and pulmonary fibrosis, their function in the pathophysiology of lung diseases is not well understood. We have identified that various PDIs are up-regulated in allergic asthma (*39*, *45*), and their increases correlated with the higher bronchodilator response or blood eosinophilic counts in allergic asthmatics (*39*, *45*). Intriguingly our in-depth analysis of lung epithelial-specific knockouts of PDIA3 demonstrated that PDIA3 specifically regulate, eosinophilic and pro-fibrotic responses in lung epithelial cells by oxidizing cysteine sulphydryl (-SH) groups in eotaxin, periostin and epidermal growth factor (EGF) (*45*). Furthermore, we also demonstrated that PDIA3 facilitates -S-S- mediated oligomerization of pro-apoptotic BAK to induce intrinsic apoptosis in allergic airway disease models (*45*, *46*). Ablation of *Pdia3* specifically in lung epithelial cells attenuated,

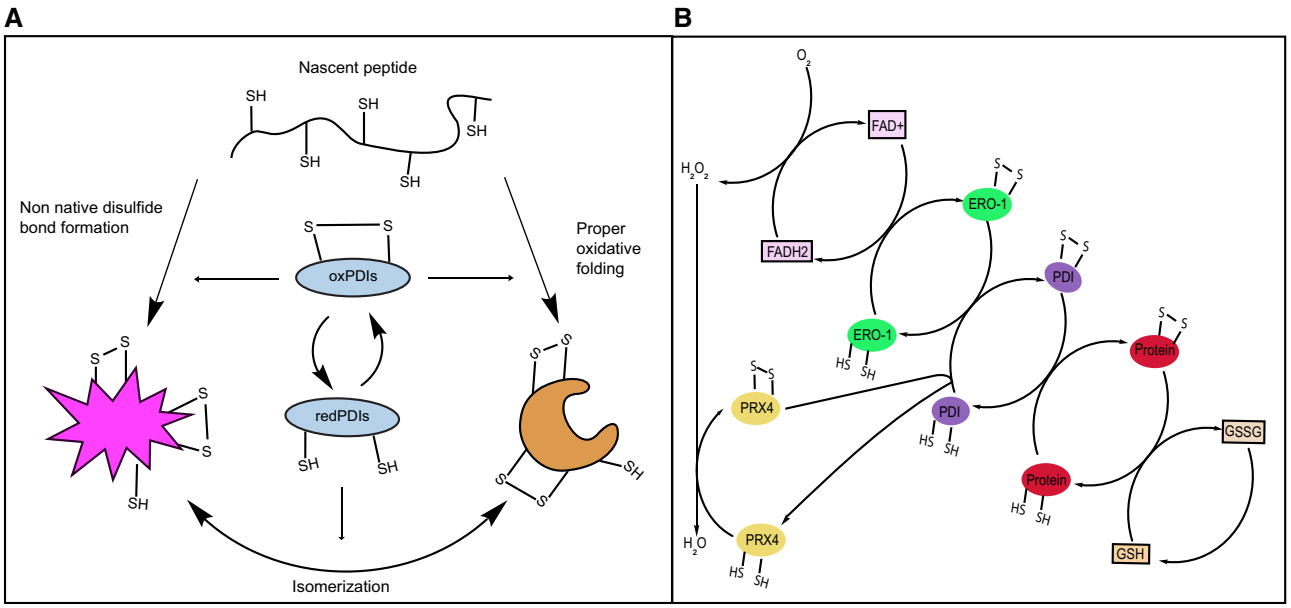

Fig. 2. Functions of PDIs in oxidative folding. (**A**) Oxidized PDIs catalyse disulphide bond formation of nascent peptides in the ER. Leading to proper oxidative folding or non-native disulphide bond formation. Reduced PDIs facilitate isomerization of disulphide bonds. (**B**) PDIs are oxidized via interactions with ERO1. ERO1 uses FAD to transfer electrons to molecular oxygen generating hydrogen peroxide. PRX4 can also directly oxidize PDIs. PDIs transfer disulphides to client proteins. Glutathione contributes to disulphide bond reduction.

apoptotic, inflammatory and fibrotic responses in a model of allergic airway disease (*45*). These and other literature have led to the hypothesis that heterogeneous severe asthma could potentially be classified as an endotype of asthma (*47*).

Although, there is very little known about the impact of PDIs in pulmonary fibrosis recent literature has highlighted that PDIs potentially regulate disulphide bonds in many pro-apoptotic and pro-fibrotic proteins including collagen crosslinking enzyme lysyl oxidase like 2 (LOXL2) (*45*, *46*, *48*). Literature has also indicated that PDIA3 drives the trans-differentiation of murine alveolar epithelial cells and it is regulated by pro-fibrotic injury in mice (*49*). We have also identified that PDIA3 as a regulator of -S-S- bonds in death receptor CD95 (FAS) and inhibition or down-regulation of PDIA3 decreases -S-S- bonds in FAS, lung epithelial apoptosis and ultimately attenuation of pulmonary fibrosis in murine models of pulmonary fibrosis (*46*).

So far there are no proven therapeutics available to inhibit PDIs in the clinic, however, decades of research from various laboratories have identified many inhibitors that have shown *in vivo* and *in vitro* efficacy in inhibiting PDIs. Interestingly, rutinosides (plant flavonoids) that are known to inhibit PDIs are now being used in different clinical studies (*50*), also it is interesting to note that Dr Stockwell's group have identified LOC14 as a specific inhibitor of PDIA1 and –A3 (*51*, *52*). This literature suggests that UPR and subsequent induction of PDIs regulate pathology of various diseases and inhibiting PDIs may be a potential therapeutic approach that would benefit patients with chronic diseases.

UPR and PDIs in respiratory viral infection

Approximately 40 viruses are known to interact with the UPR, with many of these ultimately causing the induction of ER chaperone proteins (*53*). In this section, we highlight a few common respiratory viruses that display significant morbidity and mortality while also being known to cause exacerbations of lung diseases (*54*).

Influenza A virus (IAV) is known to activate different arms of the UPR depending on the model (*21*, *22*, *55*). Hassan *et al.* (*21*) demonstrated in isolated primary human tracheobronchial epithelial (HTBE) cells that IAV infection activated the IRE1 branch of the UPR but not the PERK or ATF6 branches. That same year Roberson *et al.* (*22*) showed in isolated primary mouse tracheal epithelial cells IAV infection strongly activated the ATF6 branch, but not PERK or IRE1. As indicated the models for these two studies are different which may account for the differences in UPR activation. However, one less explored difference between the two studies is the time post-viral infection in which the activation status of the UPR was explored. Hassan *et al.* examined UPR activation shortly after infection while Roberson *et al.* explored UPR activation at 24 and 48 h post-infection. This distinction may help explain the contradictory results as the two studies are examining the UPR at different points of the viral replication cycle where reproductive needs and thus utilization of host proteins may be quite different. A recent third study by Landeras-Bueno *et al.* (*55*) using chemical genomics showed IAV infection leads to the attenuation of the PERK branch of the UPR in A549 cells. Interestingly, in contrast to the previous studies, they showed no down-regulation of IRE1 or ATF6. Again, this may be due to differences in the time points examined.

The relationship between IAV and PDIs is more straightforward, in 2006, Solda *et al.* (*38*) clearly demonstrated PDIA3 was required for efficient folding of IAV HA *in vitro*. They also showed while other PDIs

were able to act as surrogate chaperones for various proteins, efficient HA disulphide bond formation required PDIA3. Loss of IAV replication in cells treated with siRNA against PDIA3 supported this finding, though direct results on viral proteins were not examined (22). More recently expanded siRNA screens identified PDIA1 and four in addition to PDIA3 as having a role in IAV replication (56). The authors found substantial decreases in both IAV NP and M1 protein levels as well as significant decreases in viral transcript levels following siRNA treatment. Both M1 and NP lack disulphide bonds as part of their functional conformations (57), thus it is unclear whether the examined PDIs are interacting directly with the NP or M1.

Building on those results, we have shown PDIA3 plays an important role in IAV replication *in vivo*. Utilizing conditional PDIA3 knockout mice, we demonstrate PDIA3 deletion in lung epithelial cells significantly decreases levels of viral transcripts and proteins, as well as corresponding decreases in inflammatory cytokines and inflammatory and immune cells in the bronchoalveolar lavage fluid (BALF) (39). Moreover, we show ablation of *Pdia3* in the lung epithelium diminishes IAV mediated methacholine induced AHR, providing a physiological readout illustrating the importance of PDIA3 in IAV replication (39).

Rhinovirus (RV) has recently been shown to cause impaired UPR activation in primary cystic fibrosis (CF) bronchial cells (58). However, this impairment of UPR activation does not appear to be cell intrinsic as activation with known chemical inducers of the UPR produces a robust UPR response as indicated by increased GRP78 and CHOP expression (58). Moreover, chemical activation of the UPR in RV infected primary cells significantly impeded viral replication and subsequent release of the virus. Suggesting that like IAV, RV is capable of directly activating the UPR. This is confirmed by a recent study from Song *et al.* (59) showing the human RV16 infection, specifically the non-structural protein 2B activates both PERK and ATF6 UPR pathways in H1-HeLa cells. Interestingly, this protein also simultaneously inactivates the IRE1 pathway by blocking phosphorylation and subsequent XBP1 splicing (59).

Like the above viruses, respiratory syncytial virus (RSV) infection activates the UPR. In both A549 cells and primary HTBE cells, UPR activation is characterized by increased GRP78 levels, and activation of both the IRE1 and ATF6 pathways, though no PERK activation was detected (60). However, a study involving a mouse model exploring RSV's role in lung fibrosis found PERK activation 7 days post-infection along with elevated ATF6, GRP78 (61). XBP1 levels were not elevated and IRE1 activation was not explored. Intriguingly, while RSV activates the IRE1 branch of the UPR in both cell lines and isolated primary cells, IRE1 inhibits RSV replication (60). In both $Ire1^{-/-}$ mouse, embryonic fibroblasts and A549 cells treated with an IRE1 inhibitor viral transcript and protein levels were significantly higher than in RSV infected control cells (60). Though the exact mechanism for this is unclear, it may be related to IRE1 RIDD endonuclease activity.

Coronaviruses (CoV) are another virus family capable of causing respiratory exacerbation as well as significant illness on their own (62). SARS-CoV and MERS-CoV, in particular, have high pandemic potential and are associated with significant mortality (62). It is well-established CoV can induce the UPR in culture, this UPR activation has been linked to the spike (S) protein and for certain CoV, the exact amino acid domain responsible is known (63, 64). The S protein of SARS-CoV and CoV-HKU1, both betacoronaviruses, induce the transcription of GRP78, GRP94 and CHOP through activation of the PERK pathway *in vitro* (63). Cells infected with MHV-A59, a model murine CoV in the same family as SARS and MERS, show activation of all three UPR branches, though ATF6 activation is limited later in infection (62). Relative activation of UPR branches varies between individual CoV as cells infected with SARS-CoV show limited XBP1 slicing and ATF6 cleavage (65, 66). Interestingly, an *in vitro* study using a selective PERK inhibitor found alleviating translational repression increased the levels of viral proteins but decreased viral titers (62). This was hypothesized to be due to increased translation of host anti-viral proteins.

There is currently little information on the relationship between RV, RSV and CoV and host PDIs. Thus, any potential interactions remain to be characterized, and any prospective anti-viral pharmacological influence need to be explored.

While we have limited ourselves to a discussion of respiratory viruses known to interact with the UPR, there are numerous other non-respiratory viruses that do so (53). Herpes Simplex Virus (67), Dengue Virus (68), Hepatitis B (69) and HIV (70) all interact with the UPR or PDIs despite all infecting drastically different cell types. These host-viral interactions may provide promising targets for the basis of the development of future antivirals.

UPR and PDIs in immune signalling

Both the UPR and PDIs play important roles in immune signalling and activation. Increased expression of proinflammatory cytokines during ER stress has been firmly established (71). And because it allows for ER expansion, increased protein production, and subsequent protein secretion, activation of the UPR is needed for the development and function of both secretory and immune cells (15, 72, 73). Additionally, GRP94 has been shown to be involved in the maturation of Toll-like receptors (74).

PDIA3 is a core component of the peptide loading complex, required for antigen presentation through MHC Class I (75). In addition, PDIs play important roles in cytokine folding and maturation. siRNA knockdown of PDIA3 in the lung epithelium has been shown to alter the oxidative folding of eotaxin and periostin in mice (45). PDIA1 is found at high levels in the secretory granules of eosinophils, suggesting a direct role in cytokine secretion (76).

Table II. Characteristics of various PDI inhibitors

Compound name	Mode of action	Clinical trial[a]	References
Bacitracin	Competitive inhibitor binds to free thiols in substrate binding region. Cell impermeable	Yes (*57*)	*85*
16F16	Irreversibly binds to cysteine residues in active site. Cell permeable	No	*52*
LOC14	Allosteric inhibitor. Binds adjacent to active site, forces protein to maintain oxidized conformation. Reversible. Cell permeable	No	*39*, *51*
PACMA31	Irreversibly binds to cysteine residues in active site. Cell permeable	No	*86*
CCF642	Allosteric inhibitor. Irreversibly binds to conserved lysine directly adjacent to the active site. Cell permeable	No	*7*
P1	Irreversibly binds to cysteine residues in active site. Cell permeable	No	*87*
E64FC26	Pan-PDI inhibitor mechanism unknown. cell permeable	No	*93*
KSC-34	PDIA1 inhibitor selective for C53 in a domain active site. Cell permeable	No	*92*
ML359	PDIA1 inhibitor mechanism unknown. Reversible. Cell permeable	No	*88*
RB-11-ca	PDIA1 inhibitor selective for C53 in a domain active site. Cell permeable	No	*89*
Juniferdin	PDIA1 inhibitor mechanism unknown. Cell permeable	No	*88*
Eupatorin	Flavonoid compound binds to tryptophan residues near the active site of PDIA3	No	*94*, *95*
Eupatorin-5-methyl ether	Flavonoid compound binds to tryptophan residues near the active site of PDIA3.	No	*94*, *95*
Quercetin-3-rutinoside	PDIA1 inhibitor binds to b' domain. Reversible. Cell impermeable	No	*50*
T8	Allosteric inhibitor binds near active site. Reversible. Cell permeable	No	*90*
RL90	Anti-PDIA1 antibody	No	*83*
17β-estradiol	Binds to bb' domain	Yes (2,054)	*84*
35G8	Believed to bind with cysteine residues in active site	No	*91*

[a]Clinicaltrial.gov.

UPR and PDIs in feedback regulation

There are an increasing number of papers exploring the redox regulation of the UPR sensors through thiol-disulphide exchange (*77*). In other words, modulation of the UPR itself through its downstream PDI effectors. A recent study determined PDIA1 and A3 are important in the regulation of PERK, utilizing lentiviral PDIA3 depletion in combination with small molecule-based PDI inhibition the authors concluded oxidized PDIA1 was an important activator of PERK, while PDIA3 was critical to regulating the oxidation state of PDIA1 (*77*).

One group found PDIA6 is important in modulating IRE1 signalling through interaction with Cys148 of the activated protein, facilitating its decay, thus acting as an attenuator of IRE1 signalling (*78*). Another study by the same group exploring the UPR and glucose-stimulated insulin secretion found PDIA6 regulates the RIDD activity of IRE1 (*79*). Utilizing shRNA against PDIA6, Eletto *et al.* (*79*) found that during UPR activation through chemical stressors, PDIA6 modulates the kinase activity of PERK as well as the XBP1 splicing ability of IRE1. However, upon activation due to glucose concentration, only RIDD activity of IRE1 is triggered, PDIA6 regulates this activity, and that this regulation was dependent of the enzymatic activity of PDIA6, rather than expression of PDIA6.

Another different study found PDIA5 is important for ATF6 activation and export during ER stress (*80*). Higa *et al.* (*80*) determined this activation was redox dependent and PDIA5 was involved in disulphide bond rearrangement of ATF6 which facilitated its transport to the Golgi. A very recent study utilizing trap mutants of various PDIs found PDIA16 modulates trafficking of ATF6 to the Golgi and assists in proteolytic cleavage (*81*). Another study using shRNA depletion of PDIs found PDIA4 regulates ATF6 activity, as increased GRP78 expression was detected specifically following PDIA4 deletion as opposed to other PDIs (*82*). Though, the exact nature of this control was not explored.

This avenue of research is promising as it demonstrates the possibility of controlling aberrant or exuberant UPR activation, or modulating specific arms of the UPR, through inhibition of individual PDIs.

Small molecule inhibition of PDIs

Given the scope of PDIs, it is not surprising there is plentiful investigation into modulating their activity. Numerous chemicals have been shown to inhibit PDI activity, from antibodies (*83*) and hormones (*84*) to antibiotics (*85*). In the past few years, there has been an influx of inhibitors identified from small-molecule screen libraries with increasing specificity towards PDIs (*86–91*). Most of these molecules are identified as potential chemotherapeutic agents and have been characterized against PDIA1, the prototypical member of the PDI family (*92*). However, given the high degree of homology between PDI family members some inhibitory activity against other PDIs is often predicted. A full description of currently available small molecule PDI inhibitors can be found in Table II, the following section will highlight a few key inhibitors.

16F16 was the first of this new wave of inhibitors, it acts by covalently binding to reactive Cys residues in the active site of PDIs (*52*). Hoffstrom *et al.* (*52*) utilized it to explore mechanisms linking PDIs to apoptotic cell death. E64FC26 is a novel PDI inhibitor-based off of an indene moiety that acts as a pan inhibitor of numerous PDIs though the inhibitory

mechanism in unclear (*93*). The authors found E64FC26 improved survival in a mouse model of multiple myeloma, with little adverse effects. KSC-34 is a particularly interesting inhibitor, it displays enhanced specificity towards PDIA1, which is not unusual, but KSC-34 is selective towards the N-terminal CGHC active site of the protein (*92*). This means KSC-34 can be used to explore specific functionalities and protein interactions of each individual active site. Additionally, the specificity of this inhibitor is encouraging in that is suggests the possibility of identifying novel inhibitors with the same level of specificity targeted towards other PDIs.

LOC14 is unique, it is a reversible PDI inhibitor that binds adjacent to the CGHC active site and locks the enzyme in an oxidized conformation, though the exact residues remain to be elucidated (*51*). LOC14 has been found to be neuroprotective in both cell culture and animal models and displays high stability (*51*). We have recently demonstrated LOC14 is also capable of inhibiting PDIA3 as well as PDIA1, and that treatment with LOC14 significantly decreased Influenza replication and the maturation of viral proteins (*39*). Another group has demonstrated the redox modulating effects of flavonoid compounds on PDIA3 (*94*). Rather than directly interacting with the active site these compounds bind to residues on the protein binding b and b' domain absent on other PDIs (*94*, *95*). These flavonoids are interesting as they may provide a structural basis for the design of specific PDIA3 inhibitors.

The above compounds represent an expansive range of mechanisms and targets within the PDI family, from E64FC26 acting as a pan PDI inhibitor (*93*) to KSC-34 acting towards a specific active site on PDIA1 (*92*). PDIs are being found to play increasingly important roles in a wide variety of conditions and cellular activities, though current techniques to elucidate their exact role remain limited. Conventional RNA interference techniques take time to ensure sufficient knockdown of a target gene and targeting multiple genes simultaneously can pose additional problems. Employing targeted inhibitors allows for rapid decreases in protein activity and could theoretically be titrated to achieve a desired activity level. We have already demonstrated the efficacy of these inhibitors by utilizing them to determine the role of PDIs in oxidative folding of influenza proteins (*39*), while Cole *et al.* (*92*) explored the specific role of the PDIA1 A site on protein folding and secretion. Current compounds are able to target some of the PDI family, though inhibitory action against all PDIs has yet to be explored, and highly specific inhibitors towards PDIs other than PDIA1 remain to be developed. Nonetheless, the development of increasingly specific inhibitors specifically targeted towards individual PDIs would provide an invaluable tool to determine the distinct role of unique PDIs during disease pathogenesis.

Conclusion

The UPR and PDIs play critical roles in numerous cellular processes. Much remains unknown regarding their role in normal responses and their impact on the development of disease. Investigation into the branches of the UPR and their downstream effectors remains challenging. However, small molecule PDI inhibitors provide an exciting opportunity to tease apart the molecular mechanisms of ER stress and provide potential platforms for the development of future therapeutics.

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Conflict of Interest

V.A. hold patents: U.S. Patent No. 8,679,811, 'Treatments Involving Glutaredoxins and Similar Agents' and U.S. Patent, 9,907,828, 'Treatments of oxidative stress conditions'. V.A. have received consulting fees and research funds (contracts) from Celdara Medical LLC, N.H. for his contributions with the commercialization of glutaredoxin for the treatment of pulmonary fibrosis.

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