The P5 disulfide switch: taming the aging unfolded protein response

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Abbreviations

ATF	Activating transcription factor
BiP	Binding immunoglobulin protein
CHOP	CCAAT-enhancer-binding protein homologous protein
ER	Endoplasmic reticulum
ERAD	ER-associated degradation
GADD	Growth arrest and DNA-damage-inducible
GRP	Glucose-regulated protein
IRE-1	Inositol-requiring enzyme-1
NADPH	Nicotinamide adenine dinucleotide phosphate
NOX	NADPH oxidases
PDI	Protein disulfide isomerase
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
QC	Quality control
ROS	Reactive oxygen species
UPR	Unfolded protein response
XBP-1	X-box binding protein-1

Introduction

Aging is a process that involves a complex interplay between different factors that affect the overall integrity of an organism (Lopez-Otin et al. 2013). The phenomenon of aging can be

summarized as a cumulative effect of the breakdown of various cellular functions resulting in altered intracellular communication (Dickey et al. 2009; Fulop et al. 2014), an increase in genomic instability (Moskalev et al. 2013), telomere attrition (Harley et al. 1990), epigenetic alterations (Chambers et al. 2007), loss of proteostasis (Ben-Zvi et al. 2009), deregulated nutrient sensing (Powers et al. 2006), mitochondrial dysfunction (Cadenas and Davies 2000), and loss of stem cell proliferation (Oreffo et al. 1998).

One of the more comprehensive theories regarding the underlying cause of the phenomenon of aging is the aptly named quality control theory or the QC theory of aging. This theory postulates that aging is a concomitant breakdown of several QC systems. Briefly, this theory states that reduction in the endoplasmic reticulum (ER)-protein processing capacity (Brown and Naidoo 2012; Naidoo 2009), the nutrient sensing capability, the histone modifying capacity (genomic quality control), and cell signaling, along with emotional and environmental stress (Miller et al. 2011) all contribute to the overall phenotype of aging (Ladiges 2014). The protein processing capability, as well as the protein quality control mechanisms that reside in the ER have received a lot of attention in recent years (Naidoo 2009).

One of the most important aspects of cell survival is linked to the ability of a cell to sense and respond to abnormal and antagonistic conditions, or stress. To a cell, stress can take many forms, ranging from alterations in cellular communication resulting in the loss of hormone regulation that control important functions such as cellular proliferation and apoptosis, to a breakdown in intracellular signaling systems resulting in disruptions in the cellular maintenance mechanisms such as protein quality control and DNA damage response elements. With regard to the former, alterations in the mineralocorticoid (MR) and the glucocorticoid receptors (GR) observed in aging mice (Van Eekelen et al. 1992) have been shown to have serious consequences with regard to stress responsiveness in the neuroendocrine system (Pedersen et al. 2001). In the latter case, a

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breakdown in the cellular protein processing systems (Ben-Zvi et al. 2009; Koga et al. 2011; Schipanski et al. 2013; Taylor and Dillin 2011) and intracellular DNA-linked stress sensors and response elements (Choi et al. 1995) have been linked to many of the defects observed in aging cells.

The unfolded protein response or UPR is an important part of protein quality control, and functions by sensing misfolded proteins or excessive oxidative stress within the ER. The UPR signalling cascade triggers those cellular functions that serve to contain and alleviate the hostile conditions that led to the activation of the UPR.

The UPR system provides a paradox of its own; experiments studying the effects of ER stress on the longevity of organisms as well as those employing *vitro* cell cultures have shown that a low level of ER stress prolongs the life of the cell whereas, sustained high levels of ER stress can trigger the activation of pro-apoptotic pathways that result in cell death (Szegezdi et al. 2003; Armstrong et al. 2010; Watanabe and Lam 2008). The double-edged effect of environmental stress on an organism is called hormesis (Salminen and Kaamiranta 2010).

Recent findings regarding regulation of cellular UPR by the P5 disulfide isomerase (Eletto et al. 2014; Groenendyk et al. 2014) and its discovery in various age-related pathologies have provided new potential targets with regard to regulation of the UPR. This review discusses these findings and attempts to bridge these discoveries and highlight the importance of P5 as an important target for aging research.

The double-edged UPR

The cellular UPR comprises three primary signaling cascades that together serve to reduce the protein load within the ER and selectively upregulate chaperones and other proteins that play an active role in alleviating or containing the effects of stress (Wu et al. 2014). These pathways are regulated by three ER sensors, namely, inositol requiring enzyme-1 (IRE-1), activating transcription factor (ATF-6), and protein kinase RNA-like endoplasmic reticulum kinase (PERK). All these sensors react to disruptions in the molecular environment of the ER and trigger various signaling cascades that drastically alter the ER environment (Shoulders et al. 2013; Fels and Koumenis 2006). The major signaling events and some of the processes they affect are illustrated in Fig. 1. These effects initially exert cytoprotective functions; however, constitutive UPR activation results in the activation of a number of proapoptotic pathways. Thus, proper regulation of the UPR is central to the maintenance of optimal cellular function.

ATF-6 signaling comprises the processing and transport of the cleaved ATF-6 protein to the nucleus via the golgi where it regulates the expression of various ER stress response elements such as BiP and GRP94 (Haze et al. 1999). ATF-6 has also been shown to upregulate the expression of the

Xbp-1 mRNA (Yoshida et al. 2001), which plays a central role in the IRE-1-mediated signaling pathway.

IRE-1 responds to the presence of unfolded proteins by transautophosphorylation followed by oligomerization leading to the splicing and subsequent activation of Xbp-1 pre-mRNA, which in turn results in the upregulation of various chaperones such as certain PDI family members, BiP, and some ERAD-specific proteins (Shoulders et al. 2013).

Prolonged IRE-1 activation leads to the activation of the c-Jun amino terminal kinase (JNK) (Yoshida et al. 2001), which in turn phosphorylates members of the B cell lymphoma-2 (Bcl2) family (Urano et al. 2000) that ultimately signal the activation of a caspase signaling cascade (Lei and Davis 2003) which triggers apoptosis.

Initially, PERK activation leads to translational attenuation for most proteins. This effect is not universal as PERK signaling also results in the activation of ATF4, which enhances expression of various UPR genes. This is achieved through autophosphorylation of PERK and the subsequent phosphorylation and activation of eIF2- α (Shoulders et al. 2013), which in turn activates ATF4.

Constitutive PERK activation results in an apoptotic response via the expression of the transcription factor ATF-4, which in turn upregulates the expression of the CHOP/GADD153 protein. CHOP/GADD153 is known to be involved in the Bcl-2 proapoptotic pathway (McCullough et al. 2001; Bi et al. 2005). Furthermore, PERK plays an important role in establishing communication between the mitochondria and the ER during excessive oxidative stress and is central to initiating oxidative stress-related apoptotic pathways within the mitochondria (Verfaillie et al. 2012). However, studies have shown that the PERK pathway may also exert an antiapoptotic function (Fels and Koumenis 2006).

Thus far, three distinct pathways have been identified with regard to UPR-linked apoptosis. To summarize, these comprise the expression and activation of proapoptotic caspases within the mitochondria (Zhang et al. 2001; Naidoo et al. 2008; Lee et al. 2010), upregulation of the proapoptotic CHOP (Marciniak et al. 2004; Wu et al. 2014; Honjo et al. 2014), and disruption of the mitochondrial membrane in the presence of excessive oxidative stress via UPR-related upregulation of protein disulfide isomerase (PDI) (Jaronen et al. 2014). Thus, a goldilocks-like, “just right” level of activation is necessary to ensure that the cellular UPR serves to relieve ER stress rather than trigger cell death (Hetz and Mollereau 2014).

Although the UPR is an important process in maintaining cellular fitness, defects in UPR regulation, as postulated to occur in aging cells (Naidoo 2009; Brown and Naidoo 2012), can result in premature cell death. These studies have employed several model organisms such as mice, *C. elegans*, and yeast to show an age-related decrease in chaperone activity during ER stress and a corresponding increase in proapoptotic signaling (Brown and Naidoo 2012).

Fig. 1 A simple schematic of the cellular UPR. This diagram represents a simple description of the major signaling events that are associated with the UPR and the effects of unfolded proteins on the three major arms of the UPR. Phosphorylation of a protein has been represented by the addition of an encircled “P” to the phosphorylated proteins.

Upregulation of a protein has been indicated by a “↑.” The names of all the proteins involved in this have been clearly labeled. ER lumen refers to the region above the line and indicates that all the events above this line occur within the ER

Another important component of the UPR is the endoplasmic reticulum-associated degradation (ERAD) pathway. The ERAD system works to identify and eliminate misfolded proteins, via a complex mechanism involving the action of ER-specific ligases and members of the heat shock protein family (Meusser et al. 2005; Sommer and Jentsch 1993). ERAD protein expression is upregulated during ER stress via IRE1 and PERK activation and disruption of ERAD has been shown to prolong the UPR (Travers et al. 2000). Recent studies have shown that protein degradation is attenuated in aging cells resulting in a reduction in the capacity of the cells to process misfolded proteins produced within the ER secretory pathway (Schipanski et al. 2013; Martinez-Vicente et al. 2005) and thus promoting prolonged activation of the UPR in these cells.

Protein disulfide isomerase in the UPR

The ER resident protein disulfide isomerase (PDI) has been shown to be an important marker of cellular stress. PDI family members are so named due to their ability to isomerize

disulfide bonds and promote proper folding of proteins containing these bonds (Fig. 2b). In addition to its catalytic activity, it has been reported that PDI can exert a level of chaperone-like activity and assist in thiol-independent folding of a number of protein substrates (Quan et al. 1995; Puig and Gilbert 1994; Wang 1998). Indeed, the role of PDI in protein folding has been explored in depth and has been shown to promote the folding of proteins in vitro (Freedman et al. 1994).

The chaperone-like and isomerase (Tu and Weissman 2004; Rutkevich et al. 2010; Lu and Holmgren 2014) activity of PDI makes it an indispensable part of the protein QC system in cells. However, in vitro experiments have shown that one of the major by-products of the isomerase activity of PDI is reactive oxygen species (ROS) (Gross et al. 2006; Tu and Weissman 2002) that effect cellular health by induction of DNA and protein damage. However, there have been no reports thus far linking the ROS generated in the course of PDI activity and the activation of any ER-specific stress pathways.

Studies have shown the mitochondria to be another site for PDI localization and activity (Kimura et al. 2008; Rigobello

Fig. 2 **a** Domain organization of P5. This image illustrates the domain organization of canonical human P5 relative to another well-studied PDI family member; P4HB, to highlight the differences in the domain organization and content of P5 in relation to other PDI family members. The domains are represented by *solid rectangles*, where “a” and “a’” represent catalytically active thioredoxin domains containing a CXXC motif, “b” and “b’” represent domains containing a thioredoxin like fold but lacking the active CXXC domains, the domain labeled “c” represents the acidic C-terminal region present in PDI, while the “KDEL” sequence is the ER retention sequence. The active site cysteines along with their

amino acid positions have been labeled as well. The domains of both proteins were identified using the interpro tool nMitchell et al. 2015) available at <http://www.ebi.ac.uk/interpro/>. **b** The isomerase activity of PDI. This diagram represents the mode of action of the isomerase function of PDI. The cysteines in the misfolded protein substrate have been labeled as S1, S2, and S3, while the presence of a line between these residues indicates a disulfide bond. The figure represents the isomerization of the incorrect S1-S2 disulfide bond to the correct S2-S3 conformation via PD activity. The cysteine residues in PDI before oxidation have been labeled as “SH” and as “S” after oxidation

et al. 2001). Within the mitochondria, it has been reported that PDI plays an important role in UPR-associated NADPH oxidase (NOX) activation (Jaronen et al. 2013). Furthermore, it has been postulated that excessive PDI import into the mitochondria, as seen during UPR, may trigger disruption of the mitochondrial membrane potential (Jaronen et al. 2014).

Among the members of the PDI family, recent studies have shown that the isomerase P5 plays an important role in several pathologies associated with protein aggregation, including Alzheimer’s (Honjo et al. 2014), ALS (Honjo et al. 2011), and ischemia/reperfusion-mediated cell death (Vekich et al. 2012).

Thus far, these findings have been postulated to be a consequence of the loss of the oxidative folding and chaperone-like functions proffered by P5. However, recent discoveries regarding the role of this protein in regulating the PERK and IRE-1 (Eletto et al. 2014; Groenendyk et al. 2014) UPR arms provide a more comprehensive and convincing explanation to the perceived effects of this protein in the aforementioned pathologies. This interaction may hold the key to better

understanding the observations linking P5 to UPR-associated cell death.

The P5 disulfide isomerase

The P5 isomerase was first isolated from rat liver microsomes in 1989 by Nguyen et al. at the University of Göttingen (Van et al. 1989). A detailed functional study of this protein revealed distinct similarities to other PDI family members but also highlighted certain variations in the content and organization of the domains of P5 relative to other well-known PDI family members such as P4HB (Ferrari and Soling 1999; Kikuchi et al. 2002). These differences have been illustrated in Fig. 2a.

Like most other members of the PDI family, P5 contains two enzymatically active thioredoxin fold-containing domains (CGHC), which confer thiol-disulfide oxidoreductase activity. However, unlike other PDI family members, which contain two additional inactive thioredoxin domains, P5 only has one

(Ferrari and Soling 1999). Figure 2a illustrates the domain organization of P5 in relation to the usual domains present in PDI family members and highlights the key residues that are involved in its catalytic activity. The protein sequences used for the construction of this figure were obtained from the UniProt database (UniProt Consortium 2015) available at www.uniprot.org. The P5 and P4HB proteins that have been represented here are indexed under Q15084 and P07237, respectively. As observed in Fig. 2a, the P5 isomerase has a single non-functional thioredoxin domain unlike P4HB, which contains two such domains. This non-functional domain is known to confer thiol-independent peptide binding activity to PDI family members; the absence of a second such domain in the case of P5 may be responsible for the reduced chaperone-like activity attributed to P5 relative to other PDI family members (Kikuchi et al. 2002; Freedman et al. 1994).

The role of P5 in oxidative folding in vivo is controversial, as studies have yet to identify any P5-specific clients with regard to oxidative protein folding in cells (Eletto et al. 2014; Jessop et al. 2009). Furthermore, P5 depletion has been shown to have a negligible effect on the folding and secretion of insulin in β cells (Eletto et al. 2014). Despite a lack of consensus regarding the activity of P5 at a molecular level, there have been several reports linking P5 to various pathological conditions.

Vekich et al. reported in 2012 that ATF-6 up-regulates the P5 coding gene, *PDI46*, which in turn exerts a protective influence on the mouse heart when subject to ER stress related to ischemia. Additionally, knockdown of this gene in neuroblastoma cells was reported to cause an increased susceptibility to ER stress-related death (Honjo et al. 2014). These findings provide important evidence linking P5 activity and ER stress in pathological conditions.

P5 and the UPR

In 2014, Eletto et al. from the University of Pennsylvania reported two key discoveries that are central to the regulation of the IRE-1 and PERK signalling pathways of the UPR. First, cysteine 148 in the luminal domain of IRE-1 was found to be oxidized during IRE-1 activation; furthermore, it was found that P5 formed a covalent linkage with this residue and directed the decay of IRE-1 signaling through this interaction (Fig. 3). A P5 trapping mutant, in which the CGHC domains of P5 were mutated to CGHS domains, thereby blocking the isomerase activity of P5 (Fig. 2b) and trapping the bound substrate to P5, was shown to co-precipitate with the IRE-1 and PERK receptors, respectively. Furthermore, P5 ablation resulted in a hyperactive UPR resulting in embryonic lethality in *C. elegans*. Due to the homology of the luminal domains of IRE-1 and PERK, a similar mechanism was postulated to control PERK signal decay.

Another recent publication exploring the ER restructuring caused via Xbp-1 expression and ATF-6 activation reported that Xbp-1 expression leads to a considerable increase in the expression of *PDI46* (>75 %) (Shoulders et al. 2013). Furthermore, ATF-6 was shown to induce a significant increase in *PDI46* levels, independent of Xbp-1 activation as well (Vekich et al. 2012). These findings, when taken together, provide a possible mechanism by which the IRE-1 UPR pathway may be able to self-regulate. This feedback mechanism may serve to curb the apoptotic effects of prolonged IRE-1 and PERK responses.

However, in June 2014, Groenendyk et al. from the University of Alberta reported that *PDI46* silencing led to an attenuation of the IRE-1 response, indicating that the role of P5 in UPR regulation may be more complex than previously thought.

A possible explanation for these conflicting findings may lie in the localization of P5 in the mitochondria (Kimura et al. 2008). As a consequence of increased P5 expression triggered by the UPR, localization of increasing concentrations of P5 over time within the mitochondria may trigger NOX activation (Jaronen et al. 2013), which in turn could result in the constitutive activation of the UPR via excessive cellular stress brought upon by the ROS generated by NOX activity within the mitochondria, in combination with a disruption of the mitochondrial membrane caused due to excessive P5 import.

Furthermore, it was shown that the initial IRE-1 response observed in cells overexpressing P5 was incomplete relative to wild-type cells (Groenendyk et al. 2014), suggesting that P5 may be playing both sides by deactivating the IRE-1 and PERK responses via a thiol-based interaction on one hand, while promoting the activation of the UPR by stimulating NOX-associated ROS generation on the other. Further study regarding the localization of P5 during ER stress, and the effect of NOX-associated UPR activation may help further elucidate the effect of P5 on the UPR and resolve these findings. Furthermore, studying this effect will lead to a greater understanding of the relationship between mitochondrial stress and alterations within the ER environment.

All these reports serve to showcase the importance of studying the P5 protein in the context of the UPR. Investigating this effect will not only serve to resolve the role of P5 in the various pathologies it has been implicated in, but will also lead to a deeper understanding of the regulation of the UPR and its relationship with mitochondrial oxidative stress.

P5 in aging

There have been a few studies that have reported changes in P5 at the protein as well as the mRNA level in aging cells. Expression of P5 has been shown to be attenuated in aging mesenchymal cells (Yoo et al. 2013). With regard to age-

Fig. 3 Proposed mechanism of IRE1-associated apoptosis and regulation of the same by P5. This figure illustrates the basic scheme of P5 control of IRE1 signaling, and also represents the key signaling events that are associated with constitutive IRE1 signaling in the presence of prolonged

ER stress, or on depletion of P5. The mechanism of the P5/IRE1 interaction has been derived from the work of Eletto et al., published in 2014. All of the molecules have been labeled in the figure; the “P” represents phosphorylation of a given molecule within the pathway

related pathologies, studies employing SH-SY5Y cells showing an AD-like phenotype (Honjo et al. 2014) found that knock-down of *PDI6* in these cells resulted in loss of cellular viability. Furthermore, this study also reported a decrease in the level of P5 expression in the brains of patients suffering from Alzheimer's, as well as high levels of P5 S-nitrosylation (Lu and Holmgren 2014).

S-nitrosylation of PDI has been implicated in several molecular mechanisms that are linked with the promotion of protein aggregation and abrogation of PDI activity (Mattson 2006; Nakamura and Lipton 2011). A report in 2012 by Chen et al. from the University of Manitoba showed a positive correlation between S-nitrosylated PDI and levels of ubiquitinated protein aggregates in ischemia/reperfusion injury (Chen et al. 2012). Furthermore, there have been reports that have linked increased levels of protein S-nitrosylation with aging (Nakamura et al. 2007; Santhanam et al. 2007; Wu et al. 2009). However, further study is required in order to understand the extent of S-nitrosylation-linked loss of P5 activity in aging cells. Lu et al. postulated that the loss of cell viability observed in Alzheimer's may be a result of the accumulation of misfolded proteins within the ER, brought about by a combination S-nitrosylation-linked deactivation of P5 and the decreased expression of the same.

The conclusions of Lu et al. are logical; however, these results can be better explained when taken in the context of the role of P5 in regulating IRE-1 and PERK activity. It is fair to hypothesize that the loss in cell viability may be attributed in some part to the loss of IRE-1/PERK regulation caused by the attenuation of P5, leading to prolonged UPR activation and eventual cell death.

Another important feature reported in aging cells is the accumulation of oxidative damage to the proteins involved in redox processes (Berlett and Stadtman 1997). Reports have shown an age-related decrease in PDI activity that has been attributed to accumulative oxidative damage (Nuss et al. 2008;

Rabek et al. 2003). This loss in the enzymatic activity of PDI may contribute toward altering the P5-dependent regulation of the PERK and IRE-1 UPR arms.

The discovery of the P5 disulfide switch in the IRE-1 and PERK UPR arms and the mounting evidence with regard to the central role of P5 in various UPR-associated pathological conditions give the findings regarding P5 in aging cells a new significance. Further study of P5 in aging cells and age-related pathologies in the context of an aging unfolded protein response will provide important information regarding the alterations in the regulation of the UPR observed in these conditions.

Summary

Aging cells are characterized by a loss of proteostasis and a decreased ability to survive under environmental stress (Lopez-Otin et al. 2013; Brown and Naidoo 2012; Martinez-Vicente et al. 2005). Regulation of the UPR in aging cells has been under much scrutiny, and studies have shown that the UPR in these cells differs considerably from younger cells with regard to the induction of apoptosis and chaperone activity (Brown and Naidoo 2012). The role of IRE-1 and PERK in UPR-associated apoptosis (Shoulders et al. 2013) makes the regulation of these signaling cascades an important target of study.

The seemingly contradictory findings regarding the role of P5 in activating and deactivating these responses (Eletto et al. 2014; Groenendyk et al. 2014) warrant further investigation and may hold the key to unlocking the role of this protein in various pathological conditions. Another important target for study with regard to P5 is the effects of the localization of this protein in the mitochondria and the consequences, if any, of these effects on the activation of the UPR.

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