Glutathione- and non-glutathione-based oxidant control in the endoplasmic reticulum

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Summary

The redox-active tripeptide glutathione is an endogenous reducing agent that is found in abundance and throughout the cell. In the endoplasmic reticulum (ER), the ratio of glutathione to glutathione disulfide is lower compared with non-secretory organelles. This relatively oxidizing thiol-disulfide milieu is essential for the oxidative folding of nascent proteins in the ER and, at least in part, maintained by the activity of ER-resident endoplasmic oxidoreductin 1 (Ero1) enzymes that oxidize cysteine side chains at the expense of molecular oxygen. Glutathione disulfide and hydrogen peroxide formed as a consequence of Ero1 activity are widely considered as being inoperative and potentially dangerous by-products of oxidative protein folding in the ER. In contrast to this common view, this Commentary highlights the importance of glutathione- and non glutathione-based homeostatic redox control mechanisms in the ER. Stability in the thiol–disulfide system that prominently includes the protein disulfide isomerases is ensured by the contribution of tightly regulated Ero1 activity, ER-resident peroxidases and the glutathione–glutathione-disulfide redox pair that acts as a potent housekeeper of redox balance. Accordingly, the widely held concept that Ero1-mediated over-oxidation in the ER constitutes a common cause of cellular demise is critically re-evaluated.

Key words: Endoplasmic reticulum, Disulfide-bond formation, Glutathione, Ero1, Reactive oxygen species, Apoptosis

Introduction

Glutathione is an ubiquitous low molecular weight compound synthesized in the cytosol. It consists of the three amino acids glutamate, cysteine and glycine that are linearly linked by a γ-peptide and a conventional α-peptide bond, respectively. Owing to the sulfhydryl (–SH) group of the cysteine residue, reduced glutathione (GSH) is a redox-active molecule that can participate in a variety of antioxidant reactions. It can either act as an electron donor in the reduction of peroxides, which is catalyzed by glutathione peroxidases, or that of disulfides, a reaction catalyzed by glutaredoxins. Alternatively, but with a relatively slow kinetics (Winterbourn and Metodiewa, 1999), GSH can also directly react with various oxidants in a nonenzymatic manner. The product of most types of these scavenging reaction that involve GSH is its dimeric oxidized form glutathione disulfide (GSSG). The intracellular glutathione concentration is in the millimolar range (Hwang et al., 1992), and the GSH–GSSG redox pair is considered a biological redox buffer. Apart from constituting the GSH–GSSG redox pair, a small percentage of cellular glutathione (~0.1% at steady state) is disulfide-linked to protein (Hansen et al., 2009).

The maintenance of the glutathione redox state, which is defined by the squared GSH concentration divided by the GSSG concentration $[GSH]^2 \div [GSSG]$ (see Box 1), is highly compartmentalized within the cell (Go and Jones, 2008). In the unstressed cytosol, for instance, the value of $[GSH]^2 \div [GSSG]$ is kept constantly high by the NADPH-dependent activity of glutathione reductase. Thus, in line with the value measured in yeast (Ostergaard et al., 2004), the cytosolic glutathione reduction potential in cultured human cells ranges between –280 and –320 mV depending on the growth condition (Gutscher et al., 2008). When a cytosolic concentration of 8 mM glutathione is assumed (Hwang et al., 1992), this translates into a 2800- to 63,000-fold increase of GSH in the GSH:GSSG ratio[1]. Cytosolic GSH and, during oxidative stress, GSSG are also exported to the extracellular space by plasma membrane transporters (Ballatori et al., 2005; Hirrlinger et al., 2001). In exocytic cell organelles, however, where energy-driven reduction or extrusion mechanisms for glutathione in analogy to the cytosol have not been identified to date, the GSH–GSSG redox pair is in a more-oxidized state (Fig. 1). This is catalyzed by the oxidative protein folding machinery residing in the endoplasmic reticulum (ER), which converts the oxidizing power of molecular oxygen into disulfide bonds in proteins and GSSG (as detailed further below). Accordingly, the ER potentially constitutes an oxidative sink for cellular GSH, and a model has been put forward, in which increased oxidative protein folding in the ER can lead to the detrimental accumulation of GSSG and hydrogen peroxide (H_2O_2; the product of the reduction of oxygen) (Cenci and Sitia, 2007; Chakravarthi et al., 2006; Shimizu and Hendershot, 2009; Thorpe and Kodali, 2010; Tu and Weissman, 2004).

In this Commentary, I discuss the regulation of thiol oxidation in the ER in the light of potent homeostatic control mechanisms that have been uncovered only recently. These include negative-feedback regulation of oxidases, catalysis of the reduction of H_2O_2 to water and GSSG-driven protein oxidation. Apart from sustaining secretory activity, the stable maintenance of ER redox conditions has implications on cellular redox homeostasis as well as on current models of ER-generated cell death signals. The available evidence

[1]It is important to stress that the frequently cited GSH:GSSG ratio of 30–100 to 1 (Hwang et al., 1992) is not representative of the cytosol, but rather the integrated ratio of the entire cell.

Box 1. The reduction potential of glutathione depends on the molar ratio between GSH and GSSG, and the total glutathione concentration

Redox reactions are composed of two corresponding half-cell reactions. For instance, thiol-disulfide exchange between PDI^{red} (in the equations, only one of the di-cysteine active sites is outlined by two –SH groups) and GSSG that involves two reaction steps (Eqn 1) can be split into the two half-cell reactions shown in eqns 2 and 3. Because reduction of GSSG produces two molecules of GSH, [GSH] enters as a squared term into the reaction quotient Q (Eqn 4). The two half-cell reduction potentials (calculated by the Nernst equation, Eqns 5 and 6) express the affinity of the conjugated redox pairs for electrons, which by definition is higher the more positive the value. Thus, the driving force for thiol-disulfide exchange between the GSH–GSSG and PDI^{red}–PDI^{ox} redox pairs is given by the difference in reduction potential $\Delta E'$ (Eqn 7).

$$PDI_{SH}^{SH} \xrightleftharpoons{GSSG\ GSH} PDI_{SH}^{SSG} \xrightleftharpoons{GSH} PDI_{S}^{S}, \quad (1)$$

$$PDI_{SH}^{SH} \rightleftarrows PDI_{S}^{S} + 2e^{-} + 2H^{+}, \quad (2)$$

$$GSSG + 2e^{-} + 2H^{+} \rightleftarrows 2GSH, \quad (3)$$

$$Q = \frac{[PDI_{S}^{S}] \times [GSH]^2}{[PDI_{SH}^{SH}] \times [GSSG]}, \quad (4)$$

$$E'_{PDI_{SH}^{SH}/PDI_{S}^{S}} = E^{o'}_{PDI_{SH}^{SH}/PDI_{S}^{S}} - \frac{RT}{2F} \ln \frac{[PDI_{SH}^{SH}]}{[PDI_{S}^{S}]}, \quad (5)$$

$$E'_{GSH/GSSG} = E^{o'}_{GSH/GSSG} - \frac{RT}{2F} \ln \frac{[GSH]^2}{[GSSG]}, \quad (6)$$

$$\Delta E' = E'_{GSH/GSSG} - E'_{PDI_{SH}^{SH}/PDI_{S}^{S}} = E^{o'}_{GSH/GSSG} - E^{o'}_{PDI_{SH}^{SH}/PDI_{S}^{S}} - \frac{RT}{2F} \ln Q. \quad (7)$$

Since the reaction quotient Q that contains $[GSH]^2$ (Eqn 4) enters the calculation of ΔE (Eqn 7), the oxidative (or reductive) power of glutathione cannot be adequately defined by the linear [GSH]:[GSSG] ratio. It also depends on the absolute concentrations of GSH and GSSG.

E' is the reduction potential at pH 7, $E^{o\prime}$ is the standard reduction potential at 1 atm, 298 K, pH 7 [–240 mV for glutathione (Rost and Rapoport, 1964), –163 mV and –169 mV for PDI (**a** and **a′** domain, respectively) (Chambers et al., 2010)], R is the gas constant (8.315 J K^{-1} mol^{-1}), T is the temperature (K) and F is the Faraday constant (96485 C mol^{-1}).

is supporting a model, in which pro-apoptotic oxidative stress ensuing from unfolded protein accumulation in the ER is triggered by mitochondrion- rather than ER-derived metabolites.

Reducing and oxidizing inputs on glutathione in the ER

The ER is the founding organelle of the secretory pathway, in which secretory and membrane proteins are synthesized in a process that most often involves the introduction of disulfide bonds. Almost 20 years ago, the global GSH:GSSG ratio in the ER, the Golgi and the endosomal–lysosomal system was measured by rapid acidification-quenching of a glutathione-reactive peptide probe targeted to the exocytic compartment in mammalian cells (Hwang et al., 1992) (Fig. 1). The reported range in the ratio of GSH to GSSG (1:1–3:1) has demonstrated an in situ redox environment in the secretory pathway that is well-suited for disulfide-bond formation, and has remained a benchmark for the research in

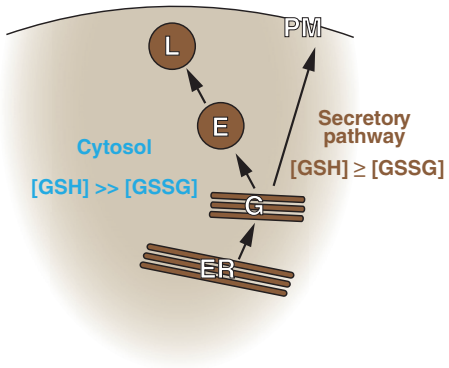

Fig. 1. Oxidized redox state of glutathione in the secretory pathway. Schematic overview of the organelles of the secretory pathway that mediate the exocytic export from the ER through the Golgi complex (G) to the plasma membrane (PM) or, alternatively, to endosomes (E) and lysosomes (L). The redox environment in the secretory pathway is supportive of disulfide-bond formation with approximately equal concentrations of GSH and GSSG. By contrast, the concentration of GSH is substantially higher than that of GSSG in the cytosol, so that the cytosolic glutathione reduction potential is reducing.

oxidative protein folding ever since. The effective reduction potential of glutathione in the ER, however, has not yet been determined and will either require the knowledge of absolute in situ concentrations of GSH and GSSG (Box 1) or the development of a glutathione-specific, ER-adapted redox biosensor (Gutscher et al., 2008; Lohman and Remington, 2008).

The maintenance of the relatively low GSH:GSSG ratio in the ER has long been thought to rely on privileged cytosol-to-ER transport of GSSG compared with that of GSH, as indicated by accumulation of [^{35}S]GSSG rather than [^{35}S]GSH within microsomes[2] in the form of mixed protein–[^{35}S]glutathione disulfides (Hwang et al., 1992). This view has, however, been abandoned following the discovery of the conserved endoplasmic oxidoreductin 1 (Ero1) sulfhydryl oxidases, of which there are two in humans (ERO1A and ERO1B, also known as and hereafter referred to as Ero1α and Ero1β) (Sevier and Kaiser, 2008). These ER-resident flavoenzymes oxidize active-site cysteine SH-groups in protein disulfide isomerase (PDI) by reducing molecular oxygen (Fig. 2). Ero1-dependent GSH-to-GSSG conversion (through PDI) in the yeast ER (Cuozzo and Kaiser, 1999) and bi-directional passage of GSH, but not of GSSG through rat liver microsomal membranes (Banhegyi et al., 1999), have been demonstrated almost at the same time. The observed virtual inability of GSSG to cross the microsomal membrane (Banhegyi et al., 1999) is in clear contrast to the earlier results that have been obtained by Hwang et al. (Hwang et al., 1992) (see above). It is possible that these earlier results regarding the import of GSSG have been affected by the leakiness of the microsomal preparation (as evidenced by the considerable loss of a luminal glycopeptide) and by the hypo-oxidation of microsomal proteins due to the reducing storage conditions (Hwang et al., 1992). Moreover, the combined data obtained from experiments that used living or semi-permeabilized mammalian cells and with microsomes unequivocally support the notion that cytosolic GSH, rather than cytosolic GSSG, directly

[2]Microsomes are vesicle-like, predominantly ER-derived structures that can be isolated by cell homogenization and differential centrifugation. They serve as an in vitro model system for diverse processes associated with the ER.

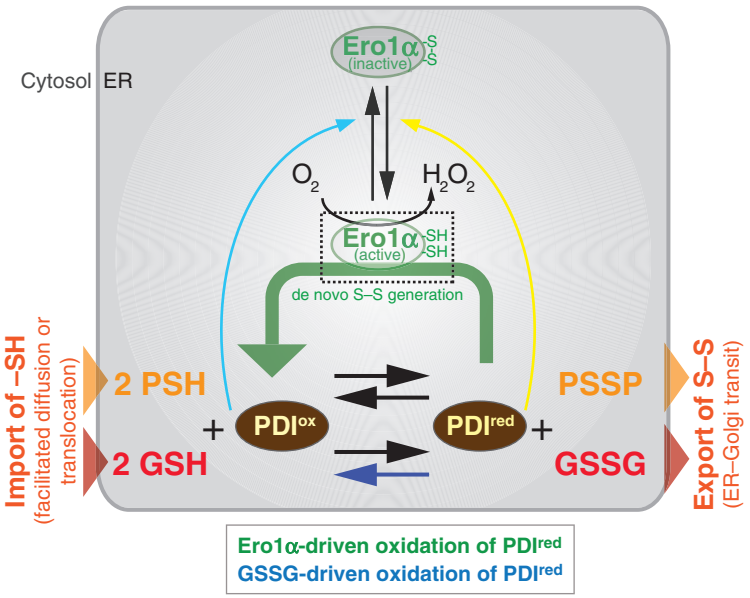

Fig. 2. Pathways of thiol-disulfide exchange that involve PDI, Ero1, nascent proteins and glutathione. Thiol groups (carbon-bonded SH-groups) in nascent secretory or membrane proteins (PSH) and glutathione (GSH) enter the ER from the cytosol and react with PDI^{ox}, yielding the disulfide-bonded proteins (PSSP), GSSG, and PDI^{red}. Correctly folded PSSP and GSSG are exported to the Golgi complex by vesicular transport. PSSP with non-native disulfide pairings (which are, therefore, misfolded) can be reduced by PDI^{red} (or by other reduced PDI family members or by GSH directly; not depicted), yielding PSH and PDI^{ox}. Alternatively, PDI^{red} is recycled to PDI^{ox} by reacting with the active form of Ero1α that passes the electrons on to molecular oxygen (O_2) [referred to as Ero1α-driven oxidation of PDI^{red} or de novo disulfide (S–S) generation; green arrow]. Ero1α is positively regulated by a high concentration of PDI^{red} (yellow arrow). H_2O_2, which is generated by Ero1α for every PDI^{red} molecule that is oxidized, is also converted to water by oxidizing PDI^{red} (see text for details). When PDI^{ox}, GSSG and PSSP become abundant, the high PDI^{ox} : PDI^{red} ratio promotes the formation of regulatory disulfides in Ero1α (light-blue arrow) that render Ero1α inactive. In addition, a high concentration of GSSG facilitates GSSG-driven oxidation of PDI^{red} (dark-blue arrow). In some tissues, Ero1α is complemented by Ero1β, which is also redox regulated (Wang et al., 2011).

contributes to ER redox regulation (Jessop and Bulleid, 2004; Molteni et al., 2004).

Thus, the model originally proposed by Cuozzo and Kaiser, by which cytosol-derived GSH delivers reducing equivalents to the ER and balances luminal thiol oxidation by Ero1 (Fig. 2), is firmly established and undisputed (Cuozzo and Kaiser, 1999). During oxidative protein folding, GSH is required for the corrective reduction (or isomerization) of non-native disulfide pairings (Chakravarthi et al., 2006). Given the features of facilitated diffusion for energy-independent GSH entry into microsomes (Banhegyi et al., 1999), the inherent – presumably protein-dependent – permeability of the ER membrane for small molecules (Le Gall et al., 2004) rather than active transporters is likely to determine the preferential permeation of GSH. The selective entry of GSH also implies that the prominent GSSG pool in the secretory pathway (Hwang et al., 1992) (Fig. 1) is predominantly formed through the oxidation of GSH within the ER.

GSSG pools are tightly regulated and help to control ER redox homeostasis

If incoming GSH can be continuously oxidized to GSSG in the ER by the machinery responsible for oxidative protein folding, what then happens to the amounting GSSG? Does it accumulate in the ER? Indeed, once GSSG is generated within microsomes, it can no longer diffuse out and remains trapped (Banhegyi et al., 1999). Extrapolation from this finding to the situation in the ER, together with the observation that the vesicular export of GSSG from the ER does not effectively contribute to ER redox homeostasis (Appenzeller-Herzog et al., 2010), indicate considerable intraluminal consumption of GSH and substantial accumulation of GSSG. Accordingly, the generation of ER-luminal disulfide bonds will – if unregulated – over-oxidize the ER, deplete cellular reductants and compromise cell homeostasis (Thorpe and Kodali, 2010; Tu and Weissman, 2004).

However, the process of thiol oxidation in the ER is tightly regulated. This has, for instance, been illustrated in dithiothreitol (DTT) washout experiments. Thus, the restoration of the steady-state redox distributions of ER oxidoreductases and glutathione, upon complete reduction by and subsequent removal of DTT that is accompanied by the induction of disulfide-bond formation in folding proteins (Braakman et al., 1992), is exquisitely rapid and well controlled (Appenzeller-Herzog et al., 2010) (Fig. 3B, left panel). As this process involves the oxidation of reduced PDI (PDI^{red}) by the ubiquitous Ero1 isoform Ero1α (Appenzeller-Herzog et al., 2010) (Fig. 3A), one level of regulation is provided by non-catalytic disulfide bonds in Ero1α that negatively regulate its activity (Appenzeller-Herzog et al., 2008; Baker et al., 2008) (Fig. 2). At least one of these disulfide switches is feedback-controlled through the concentration of PDI^{red} (Appenzeller-Herzog et al., 2008). Accordingly, the repression of Ero1α through this regulatory disulfide bond is relieved when the substrate PDI^{red} is present in abundance – as is the case, for instance, immediately following the washout of DTT (Fig. 3B). Conversely, Ero1α converts into an enzymatically inactive form under oxidizing conditions in the ER (i.e. when the disulfide pool is full and the PDI^{red} : PDI^{ox} ratio is low) (Fig. 2 and Fig. 3A).

A complementary mechanism of ER redox regulation – the hallmark of which is the oxidation of PDI^{red} by GSSG – was discovered in cells transfected to overexpress Ero1α (Appenzeller-Herzog et al., 2010). When these cells are subjected to DTT washout, the concentration of GSSG in the ER transiently exceeds steady-state levels several-fold (Fig. 3B, right panel)[3]. Importantly, however, this overshoot quickly reverses to basal GSSG levels. This behavior can best be explained with the ability of GSSG to serve as an alternative disulfide-donor in the ER thereby being reduced to GSH – a process that is favored at a high GSSG concentration or, in thermodynamic terms, at a more-positive ER–glutathione reduction potential (Box 1). Assuming that ER-luminal GSSG-to-GSH conversion also takes place in cells with endogenous Ero1α levels, a model was proposed in which a dynamic interplay between Ero1α- and GSSG-driven oxidation of PDI^{red} determines glutathione redox homeostasis in the ER (Appenzeller-Herzog et al., 2010) (Fig. 3A,B, vertical arrows). It is important to add that

[3]By simplification, the measured overall cellular GSSG:GSH ratio (for original data see Appenzeller-Herzog et al., 2010) can be interpreted as a (non-absolute) readout of the GSSG concentration in the ER, because (1) the vast majority of cellular GSSG resides in the ER and (2) the cellular GSH content is ~100-fold higher than that of GSSG (Appenzeller-Herzog et al., 2010; Hwang et al., 1992).

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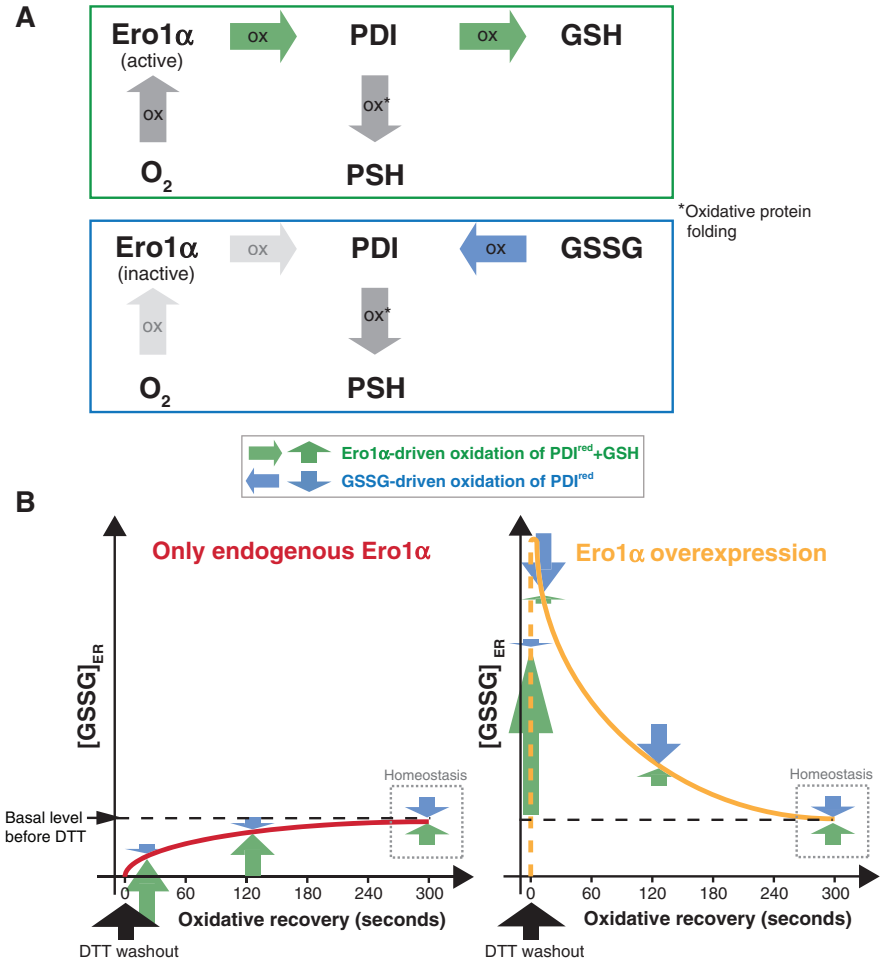

Fig. 3. Interplay between Ero1α- and GSSG-driven oxidative protein folding determines the glutathione redox homeostasis in the ER. (**A**) Representation of two disulfide relay pathways that can fuel oxidative protein (PSH) folding. Ero1α-driven oxidation of PDI^{red} and GSH (green box and arrows) and GSSG-driven oxidation of PDI^{red} (blue box and arrow). Arrows denote the flow of oxidizing equivalents (ox) in, e.g. the form of disulfide bonds. Under conditions that favor the GSSG-driven oxidation pathway (blue box), Ero1α is inactive because of the presence of inhibitory intramolecular disulphide bonds. ox*, oxidative protein folding. (**B**) Course of the concentration of GSSG in the ER ($[GSSG]_{ER}$) upon DTT washout in mammalian cells in the presence of endogenous (left graph, red line) or overexpressed Ero1α (right graph, orange line), adapted from Appenzeller-Herzog et al., 2010 (Appenzeller-Herzog et al., 2010). In cells with only endogenously expressed Ero1α (left graph), $[GSSG]_{ER}$ rises rapidly when DTT is removed and returns to the exact steady-state concentration (i.e. basal level before DTT, dashed black line). By contrast, overexpression of Ero1α leads to a very sudden, transient overshoot in the formation of GSSG that cannot be experimentally resolved because it happens so quickly (depicted by the dashed orange line). The reason for this behavior is that Ero1α is maximally activated by treatment with DTT through the reduction of regulatory disulfide bonds so that its overexpression becomes clearly manifested. After a recovery phase of 300 seconds, the basal steady-state value of $[GSSG]_{ER}$ is reached, irrespectively whether exogenous Ero1α is present or not, indicating that the regulatory disulfide bonds in the majority of Ero1α molecules have reformed and any excess GSSG has been converted to GSH. The vertical green and blue arrows denote Ero1α-driven oxidation of PDI^{red} and GSH, and GSSG-driven oxidation of PDI^{red}, respectively (see also panel A with the same color coding), the extent of which is represented at different time points during oxidative recovery by the length of the arrow. Whereas the former pathway causes an increase in GSSG (and a decrease in GSH), the latter consumes GSSG (and produces GSH). Thus, interplay between Ero1α- and GSSG-driven oxidative folding can effectively balance the reduction potential of glutathione in the ER. Note that the Ero1α- and GSSG-driven oxidation pathways are inversely regulated by $[GSSG]_{ER}$. Homeostasis is achieved when the two oxidation pathways balance each other in a dynamic equilibrium (dashed box).

the catalyzed reduction of H_2O_2 in the ER can also drive disulfide-bond formation in the presence of PDI – and even substitute for Ero1-driven oxidation (Tavender et al., 2010; Zito et al., 2010b) (see below).

As both, Ero1-mediated GSH oxidation (Appenzeller-Herzog et al., 2010; Tu et al., 2000) and GSSG-mediated protein oxidation (Karala et al., 2009), are only efficient when catalyzed by the intermediate disulfide-carrier PDI, the redox poise of ER glutathione appears to be primarily maintained through thiol-disulfide exchange with PDI (Fig. 2 and Fig. 3A). Accordingly, the recovery curve of GSSG levels in the ER after DTT washout (Fig. 3B, left panel) is the result of the rapid reaction kinetics with which both GSH and GSSG react with PDI (Darby and Creighton, 1995). To what extent PDI homologs (Appenzeller-Herzog and Ellgaard, 2008) participate in this process is less clear, but there is evidence that other PDIs, such as the thiol-disulfide oxidoreductases ERp57 and ERp46, can also act as physiological reductants of Ero1α and/or GSSG (Appenzeller-Herzog et al., 2010; Inaba et al., 2010; Jessop et al., 2009; Schulman et al., 2010). Taken together, the ability of Ero1α to shut down under oxidizing conditions in the

Box 2. ER-stress-induced cell death signaling

ER stress activates a homeostatic program termed the unfolded protein response (UPR), which augments the protein-folding capacity in the ER, lowers de novo protein synthesis and eliminates terminally misfolded proteins from the ER. If homeostasis fails to be restored, however, the UPR can switch to signaling a complex network of intertwined apoptotic messages. Important branches in this network include expression of the pro-apoptotic transcription factor CHOP, activation of the MAP3K5–JNK signaling cascade and of ER-localized initiator caspases (Heath-Engel et al., 2008; Malhotra and Kaufman, 2007). Decimation of the ER protein folding machinery by local degradation of chaperone-encoding mRNAs at the ER membrane also contributes to the induction of apoptosis under sustained ER stress (Han et al., 2009). In addition, ER stress can activate the mitochondria-dependent intrinsic apoptosis pathway by IP_3R-mediated funneling of Ca^{2+} from ER to mitochondria through specialized, synapse-like contact sites termed mitochondria-associated membranes (MAMs) (Deniaud et al., 2008; Timmins et al., 2009). Several mechanisms that contribute to this death-inducing crosstalk between ER and mitochondria have been described: (1) a UPR-induced, truncated isoform of sarco endoplasmic reticulum calcium ATPase 1 that localizes to MAMs stimulates both resting and activated Ca^{2+} transfer to mitochondria and promotes apoptosis (Chami et al., 2008); (2) CHOP-dependent upregulation of Ero1α potentiates ER Ca^{2+} release through IP_3R1 and ER-stress-induced cell death (Li et al., 2009); (3) Ca^{2+} homeostasis at the ER membrane is modulated by UPR-dependent transcriptional and post-translational activation or repression of distinct Bcl2-family proteins (Heath-Engel et al., 2008; Hetz and Glimcher, 2008). In the last decade, ER-stress-induced cell death has gained a lot of attention and has been implicated in a wide array of human diseases (Kim et al., 2008).

ER, together with the presumably millimolar pool of disulfide bonds in the form of GSSG that can buffer this shutdown and transiently drive oxidative protein folding, provides a robust system of homeostatic redox housekeeping in the ER.

ER stress and reactive oxygen species

Another possible mechanism of ER over-oxidation and GSH depletion is represented by the broad and mostly unspecific oxidative activities of peroxides and oxygen radicals, collectively termed reactive oxygen species (ROS). Indeed, Ero1 oxidases produce stoichiometric amounts of H_2O_2 for every disulfide bond formed in vitro (Gross et al., 2006; Wang et al., 2009; Wang et al., 2011), which, presumably, also occurs in the cellular context (Enyedi et al., 2010) (Fig. 2). Ero1-derived ROS have been proposed to broadly affect cell viability, a situation that might manifest itself most clearly in cell types with particularly high secretory activity, such as B lymphocytes or pancreatic β-cells (Cenci and Sitia, 2007; Chakravarthi et al., 2006; Shimizu and Hendershot, 2009; Tu and Weissman, 2004).

In line with this understanding, intracellular ROS have been detected under different conditions that interfere with ER homeostasis and lead to persistent ER stress (Back et al., 2009; Cullinan and Diehl, 2004; Harding et al., 2003; Haynes et al., 2004; Kitiphongspattana et al., 2007; Malhotra et al., 2008; Santos et al., 2009; Tan et al., 2009) – a physiological imbalance that can ultimately trigger cell death signals (Box 2). Some of these reports have also described a connection to Ero1. For instance, when nematodes are treated with the ER-stress-inducing agent tunicamycin ROS accumulate in the entire animal, but accumulation is suppressed upon knockdown of Ero1 (Harding et al., 2003). RNA interference of the gene encoding Ero1 also increases the lifespan in nematodes (Curran and Ruvkun, 2007; Harding et al., 2003; Marciniak et al., 2004), which is adversely affected by ROS. Similarly, in a yeast strain that is genetically predisposed to sustained ER and oxidative stress – by deletion of ER housekeeping genes and overexpression of a mutated and folding-incompetent variant (CPY*) of the ER client protein carboxypeptidase Y (CPY) – increased levels of intracellular ROS are detected upon transformation with a plasmid carrying the gene encoding Ero1 (Haynes et al., 2004). This finding suggests that ROS that are generated by Ero1 activity can directly contribute to oxidative stress in the cell. Unlike the situation in nematodes, and for reasons as yet unknown, however, lowering Ero1 activity in this yeast strain did not promote cell survival, as observed following treatment with antioxidants but, instead, killed the cells (Haynes et al., 2004). In mammalian model systems, conflicting results have been reported with regard to the function of attenuated Ero1 expression in improving the tolerance towards severe ER stress (Blais et al., 2010; Zito et al., 2010a). However, as shown in macrophages, expression and transcriptional induction of Ero1α by the DNA damage-inducible transcript 3 protein (DDIT3; also known as and hereafter referred to as C/EBP homologous protein, CHOP), is required for ER-stress-induced apoptosis that involves Ca^{2+} flow from the ER to the mitochondria through the inositol 1,4,5-trisphosphate receptor type 1 (IP_3R1) (Li et al., 2009) (Box 2). As this Ca^{2+} channel appears to be positively regulated by the oxidation of an ER-luminal cysteine pair (Higo et al., 2005), it has been proposed that CHOP-mediated upregulation of Ero1α triggers ER over-oxidation and, thereby, increases the probability of the channel being open (Li et al., 2009). Along the same line, induction of CHOP following prolonged ER stress promotes the formation of disulfide-linked homo-oligomers of the ER protein Hsp47 (Stefan J. Marciniak, School of Clinical Medicine, University of Cambridge, UK, personal communication), which potentially serves as an indicator of ER hyper-oxidation (Marciniak et al., 2004).

Despite these data, there is also strong evidence arguing against the idea that ER-stress-induced ROS are actually formed in the ER. Overexpression of CPY* in a slightly different yeast strain background than that used by Haynes et al. (Haynes et al., 2004) leads to the detection of intracellular superoxide (O_2^-) radicals, a fact that does not correlate with the level of Ero1 activity, which indicates a ROS source other than Ero1 as the trigger (Tan et al., 2009). Likewise, the growth of another yeast strain that is hypersensitive to exogenous H_2O_2 is not affected by overexpression of Ero1 (Tan et al., 2009) and expression of CPY* or tunicamycin treatment in yeast has been demonstrated to unexpectedly effectuate a reductive rather than an oxidative shift in the ER-luminal redox state (Merksamer et al., 2008). These data challenged the widely held model that cycles of aberrant disulfide-bond formation and their corrective breakage by the ER-folding machinery cause oxidative stress in the ER and beyond (Fig. 4A). The most convincing argument that ROS induced by ER stress do not derive from the ER, however, comes from the finding that both the ROS response to ER stress and apoptosis are alleviated in mammalian (Cullinan and Diehl, 2004; Harding et al., 2003) and yeast cells (Haynes et al., 2004) that lack a mitochondrial genome and, thus, a functional respiratory chain. Similarly, the mitochondrial inhibitor rotenone but not the translation inhibitor cycloheximide, which

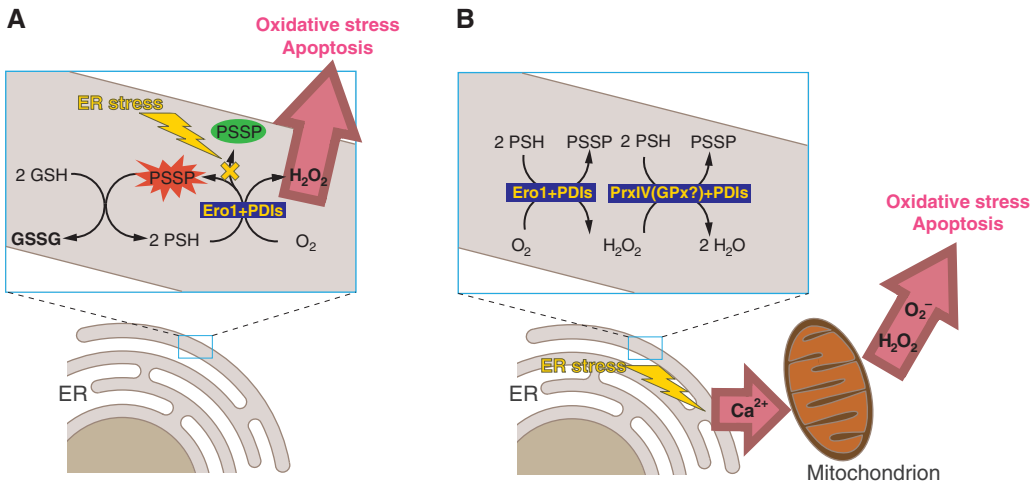

Fig. 4. Models for ER-stress-induced oxidative stress. (**A**) Oxidative folding of nascent polypeptide chains (PSH) in the ER involves the introduction of disulfide bonds through the Ero1–PDI electron relay, which finally reduces molecular oxygen (O_2) to H_2O_2. This process either results in a correctly folded protein (green PSSP) or, in the case of aberrant pairing of cysteines, in a folding-arrested intermediate (red PSSP) that is retained in the ER and eventually isomerized (not depicted) or reduced with GSH-derived electrons for a new round of oxidation. Because native disulfide pairings are hindered under conditions of ER stress, a vicious redox cycle develops and leads to the accumulation of GSSG and H_2O_2, the latter of which can diffuse out of the ER and lead to oxidative stress and apoptosis. (**B**) The formation of disulphide bonds in the ER is a two-step process, in which two disulfides are generated by using the oxidizing power of O_2. The first step occurs as described in panel A. The second step entails the detoxification of H_2O_2 and can be catalyzed by PrxIV (Tavender and Bulleid, 2010) or, possibly, by ER-resident glutathione peroxidases (GPx?) (Raykhel et al., 2007) in conjunction with PDI family members (PDIs). Therefore, H_2O_2 cannot accumulate in the ER lumen. Accordingly, irrespective of native or non-native disulfide synthesis (the latter of which will be prominent during ER stress), the thiol-disulfide homeostasis in the ER remains either stable or becomes more reducing in the stressed ER by an unknown mechanism (Merksamer et al., 2008). ER stress triggers a Ca^{2+} signal from the ER to the mitochondria that can activate the pathway for mitochondria-dependent apoptosis (see Box 2). This scenario is accompanied by the release of superoxide (O_2^-) and H_2O_2 derived from mitochondrial respiration and results in oxidative stress.

lowers oxidative protein folding activity in the ER, potently decreases ROS formation during B cell differentiation (Vene et al., 2010), a process accompanied by a robust ER stress response (Cenci and Sitia, 2007; Shimizu and Hendershot, 2009). As cell death signals that arise from the ER are transmitted to mitochondria (Ferri and Kroemer, 2001) – the main cellular producers of ROS (Murphy, 2009) – it is easily conceivable that ER stress promotes the release of mitochondrial ROS (Fig. 4B).

However, depending on the cell type studied, there are alternative sources of ROS induced by ER stress. In vascular smooth muscle and endothelial cells, ER-resident NADPH oxidase 4 (Nox4) – in response to ER stress signals – generates apoptogenic H_2O_2 that, at least in endothelial cells, acts as a signaling molecule that modulates phosphotyrosine-, mitogen-activated protein kinase (MAPK)-, and ER-stress-signaling pathways (Chen et al., 2008; Pedruzzi et al., 2004; Wu et al., 2010). An additional source of ROS that is essential for ER-stress-induced apoptosis and downstream of ER-stress signaling pathways is NADPH oxidase 2 (Nox2) (Li et al., 2010). *Nox2* is transcriptionally upregulated following Ca^{2+} release from the ER and activation of JNK (Li et al., 2010). Moreover, electron uncoupling in the ER-membrane-embedded microsomal monooxygenase system[4], which is composed of cytochrome P450, NADPH-cytochrome P450 reductase (CPR) and phospholipids, represents a substantial source of ROS during ER stress in hepatocytes (Kim et al., 2009). Microsomal monooxygenase-derived ROS participate in intra- and inter-cellular signaling (Nieto et al., 2002). Finally, the generation of hydroxyl radicals during hypoxic conditions, which are known to also activate ER stress programs, has been detected at the ER membrane and is crucial for the expression of hypoxia-regulated genes (Liu et al., 2004). Thus, it is important to note that ER-derived ROS are not exclusively associated with cellular stress, but also fulfill physiological roles in redox signaling across organelle and cell boundaries (Margittai and Sitia, 2010).

In summary, it is clear that unfolded protein stress in the ER can elicit intracellular ROS formation that contributes to ER-stress-induced apoptosis. Different, cell-type-specific sources of ROS that are activated by ER stress have been identified, but H_2O_2 production by Ero1 is unlikely to directly affect the intracellular redox balance (see also below). Instead, Ca^{2+} that is released from the ER upon ER stress and taken up by mitochondria (Box 2) can activate a well-characterized cell death program (Deniaud et al., 2008) that includes the liberation of mitochondrial ROS.

ER-resident peroxidases and tight ER redox control

The redox stability of the ER is perhaps best illustrated by the finding that a 20-fold overexpression of Ero1α has no discernible effect on both the GSSG:GSH ratio and the redox state of ER oxidoreductases unless a regulatory cysteine in Ero1α is mutated (Appenzeller-Herzog et al., 2008)[5]. Thus, because endogenous Ero1α levels increase only 1.5-fold upon ER stress in macrophages (Li et al., 2009), the ER-stress-triggered, Ero1α-dependent induction of macrophage death through the modulation of IP_3R1 (see above and Box2) most probably relies on a mechanism that is different from ER over-oxidation. For instance, by competing with

[4]Microsomal monooxygenase metabolises and detoxifies predominantly exogenous compounds in the liver. In the absence of substrate oxidation it can, however, also produce H_2O_2 or O_2^-, which is referred to as electron uncoupling.

[5]Ero1α overexpression can, however, lead to a slight increase in the steady-state concentration of ER-luminal H_2O_2 (Enyedi et al., 2010).

IP_3R1 for binding to the PDI-like protein ERp44, Ero1α could promote the dissociation of this inhibitory protein from IP_3R1 and, thereby, facilitate apoptogenic Ca^{2+} release from the ER (Cortini and Sitia, 2010). Although speculative at present, it is formally possible that a similar mechanism accounts for Ero1-dependent accumulation of mitochondrial ROS upon induction of ER stress in *C. elegans* (Harding et al., 2003), in which both IP_3R and ERp44 are conserved.

How is H_2O_2 handled in the ER? Whereas in unstressed endothelial cells, steady-state H_2O_2 levels in the ER and cytosol are indistinguishably low (Wu et al., 2010), the situation in HeLa cells appears to be different with substantially more Ero1α-derived H_2O_2, which is strictly confined to the ER lumen (Enyedi et al., 2010). These observations suggest that H_2O_2 that arises from the generation of disulfide-bonds in the ER – although in principle capable of crossing the ER membrane[6] – is locally detoxified and does not leave the ER. Indeed, PDI^{red} can reduce H_2O_2 to water in vitro (Karala et al., 2009), and ER-resident peroxidases that can catalyze this reaction have also been reported (Raykhel et al., 2007; Tavender et al., 2008). The best characterized ER peroxidase is peroxiredoxin IV (PrxIV). Like other peroxiredoxins, PrxIV is specifically oxidized by H_2O_2 and, when H_2O_2 levels are high, its active site can become hyperoxidized and inactivated. Consistent with the notion that PrxIV metabolizes Ero1α-derived H_2O_2, increased levels of hyper-oxidized PrxIV are detected under conditions of augmented Ero1α activity in cells (Tavender and Bulleid, 2010). Endogenous PrxIV also protects human fibrosarcoma HT1080 cells from ER-stress-induced cell death (Tavender and Bulleid, 2010), which, however, is not the case during B lymphocyte differentiation (Bertolotti et al., 2010). In these cells, excess ER-luminal H_2O_2 can additionally be reduced by nascent immunoglobulins, as indicated by increased levels of disulfide-linked high molecular weight immunoglobulin polymers in the absence of PrxIV (Bertolotti et al., 2010).

Upon reduction of H_2O_2, the oxidized di-cysteine active sites of peroxiredoxins are recycled through reaction with a reduced thioredoxin-like protein. In the case of PrxIV, this reactivation is accomplished by reduced PDI family members, most prominently by ERp46, P5 and PDI, that then can funnel the resulting disulfide into oxidative protein folding (Tavender et al., 2010; Zito et al., 2010b) (Fig. 4B). Thus, these new findings have established an entire redox pathway for the degradation of H_2O_2 through the oxidation of protein thiols in the ER that is capable of de novo disulfide-bond generation. Provided that a source of H_2O_2 other than from Ero1 is available, such as, for instance, the mitochondrial respiratory chain (Yang et al., 2007), this pathway can operate independently of Ero1. Indeed, mammalian cells that carry two non-functional copies of the genes that encode Ero1α and Ero1β are viable (Appenzeller-Herzog et al., 2010; Zito et al., 2010a), unless the expression of endogenous PrxIV is silenced (Zito et al., 2010b). Furthermore, in eukaryotic organisms, such as *S. cerevisiae* and *C. elegans*, that do not harbor a PrxIV ortholog Ero1 is essential.

Taken together, the oxidative equivalents of H_2O_2 are likely to be efficiently converted to disulfides in the ER, possibly along several detoxification pathways. In agreement with the existence of alternative pathways, PrxIV has recently been shown to be non-essential in mouse (Iuchi et al., 2009). The disulfide content in the ER, however, is tightly regulated through GSSG-driven oxidation of PDI^{red} and feedback-regulation of Ero1, which will also stall further generation of H_2O_2 (see above).

[6]Exogenously added H_2O_2 rapidly gains access to a sensor molecule in the ER (Enyedi et al., 2010), and Nox4-derived, ER-luminal H_2O_2 can oxidize cysteines in the cytosol-oriented peripheral membrane enzyme protein-tyrosine phosphatase 1B (Chen et al., 2008).

Conclusions and perspectives

The ER harbors sophisticated redox-control mechanisms that effectively buffer the thiol-disulfide units in this compartment against hyper-oxidation. Apart from the ability of Ero1 oxidases to adapt their activity through the formation of regulatory disulfide bonds (Appenzeller-Herzog et al., 2008; Baker et al., 2008; Sevier et al., 2007), a new understanding of the role of GSSG as a homeostatic redox housekeeper has recently been highlighted (Appenzeller-Herzog et al., 2010). Moreover, as worked out for PrxIV, ER-localized peroxidases have important roles in ER redox control by degrading ER-luminal H_2O_2 as well as contributing to the supply of oxidizing equivalents for biosynthetic disulfide-bond formation (Tavender and Bulleid, 2010; Tavender et al., 2010; Zito et al., 2010b).

Further progress will come from recordings of redox conditions in organelles, such as the ER, mitochondria and the cytosol, by using genetically encoded redox sensors in different physiological settings that recapitulate proapoptotic ER stress. A first glimpse of the results we might expect for mammalian models is provided by a study that used an ER-targeted redox probe in *S. cerevisiae* (Merksamer et al., 2008). It will be of particular interest to learn about the molecular mechanisms underlying the hypo-oxidizing conditions in the ER upon induction of ER stress that were uncovered in that study (Merksamer et al., 2008). Other open questions concern the determinants for (and the physiological consequences of) different H_2O_2 levels in the ER lumen of different cell types (Enyedi et al., 2010; Wu et al., 2010) and the identity of the principal source of H_2O_2 that fuels PrxIV-mediated disulfide production in Ero1-deficient cells (Zito et al., 2010b). Furthermore, the development of new assays to monitor the oxidation state of specific ER proteins that are involved in apoptotic signaling, such as IP_3R1, will be instrumental.

Since most of our knowledge is restricted to the thiol-disulfide system, an important research area will undoubtedly be the exploration of interrelationships and of kinetic barriers between the many redox systems that are present in the ER (Csala et al., 2010). Studies that have so far addressed these connections (Piccirella et al., 2006; Saaranen et al., 2010; Schulman et al., 2010; Uehara et al., 2006) might just represent the tip of the iceberg, and pave the way for exciting new redox links and regulatory loops to be uncovered in the future.

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