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Original Contribution

GPx8 peroxidase prevents leakage of H_2O_2 from the endoplasmic reticulum

Thomas Ramming [a], Henning G. Hansen [b,1], Kazuhiro Nagata [c], Lars Ellgaard [b], Christian Appenzeller-Herzog [a,*]

[a] Division of Molecular and Systems Toxicology, Department of Pharmaceutical Sciences, University of Basel, 4056 Basel, Switzerland
[b] Department of Biology, University of Copenhagen, 2200 Copenhagen N, Denmark
[c] Faculty of Life Sciences, Kyoto Sangyo University, Kyoto 803-8555, Japan

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ABSTRACT

Unbalanced endoplasmic reticulum (ER) homeostasis (ER stress) leads to increased generation of reactive oxygen species (ROS). Disulfide-bond formation in the ER by Ero1 family oxidases produces hydrogen peroxide (H_2O_2) and thereby constitutes one potential source of ER-stress-induced ROS. However, we demonstrate that Ero1α-derived H_2O_2 is rapidly cleared by glutathione peroxidase (GPx) 8. In 293 cells, GPx8 and reduced/activated forms of Ero1α co-reside in the rough ER subdomain. Loss of GPx8 causes ER stress, leakage of Ero1α-derived H_2O_2 to the cytosol, and cell death. In contrast, peroxiredoxin (Prx) IV, another H_2O_2-detoxifying rough ER enzyme, does not protect from Ero1α-mediated toxicity, as is currently proposed. Only when Ero1α-catalyzed H_2O_2 production is artificially maximized can PrxIV participate in its reduction. We conclude that the peroxidase activity of the described Ero1α–GPx8 complex prevents diffusion of Ero1α-derived H_2O_2 within and out of the rough ER. Along with the induction of *GPX8* in ER-stressed cells, these findings question a ubiquitous role of Ero1α as a producer of cytoplasmic ROS under ER stress.

Roughly one-third of the human proteome resides in exocytic endomembrane compartments or travels via exocytic compartments to the cell surface. These proteins are synthesized at and translocated into the endoplasmic reticulum (ER), the largest and most extended compartment of the secretory pathway. The ER lumen provides a unique environment for protein folding that mimics the extracellular space [1]. For instance, reduction–oxidation (redox) conditions are more oxidizing in the ER (and in the extracellular space) than in the cytosol [2,3], thereby favoring the formation of disulfide bonds in proteins. This process, known as oxidative protein folding, is catalyzed by a number of distinct pathways [4,5], the most conserved of which is driven by endoplasmic oxidoreductin 1 (Ero1) oxidases [6]. In human cells, the housekeeping isoform Ero1α introduces disulfide bonds into the disulfide-shuttling enzyme protein disulfide isomerase (PDI) [7,8]. This reaction involves the generation of one molecule of hydrogen peroxide (H_2O_2) for every disulfide formed [9]. Of note, Ero1 activity is essential only in lower eukaryotes, but not, e.g., in flies or mice [6].

Protein misfolding in the ER triggers a cell program called the ER stress response or unfolded protein response (UPR) [10], which in the majority of cases is accompanied by an increase in intracellular reactive oxygen species (ROS) and oxidative damage [11–16]. Importantly, ROS also act upstream of ER stress [15,17–19]. ER stress and ROS therefore constitute a self-perpetuating vicious cycle, which contributes to cell degeneration in the context of ER-stress-centered disorders [20]. The fact that potentially massive amounts of the ROS H_2O_2 are being produced during Ero1α-mediated oxidative protein folding has attracted ample attention [21–23]. Thus, one model for the generation of ER-stress-induced ROS holds that stress-mediated formation of aberrant disulfides results in repeated protein reduction and reoxidation cycles, leading to increased H_2O_2 generation by Ero1 [24–26]. Ero1-derived H_2O_2 is then proposed to pass the ER membrane and spill into the cytoplasm.

In addition to H_2O_2-generating machinery, the ER in mammalian cells harbors three H_2O_2-reducing peroxidases, peroxiredoxin IV (PrxIV), glutathione peroxidase 7 (GPx7), and the transmembrane

Abbreviations: BCNU, carmustine; DRM, detergent-resistant membrane; DTT, dithiothreitol; ER, endoplasmic reticulum; Ero1, endoplasmic oxidoreductin 1; GFP, green fluorescent protein; GSSG, glutathione disulfide; GPx, glutathione peroxidase; MAM, mitochondria-associated membrane; NEM, *N*-ethylmaleimide; Prx, peroxiredoxin; PMSF, phenylmethylsulfonyl fluoride; PNS, postnuclear supernatant; PDI, protein disulfide isomerase; ROS, reactive oxygen species; redox, reduction–oxidation; GS_{tot}, total glutathione; TCA, trichloroacetic acid

* Corresponding author. Fax: +41 61 267 1515.
E-mail address: Christian.Appenzeller@unibas.ch (C. Appenzeller-Herzog).
[1] Present address: Novo Nordisk Foundation Center for Biosustainability, Technical University of Denmark, 2970 Hørsholm, Denmark.

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protein GPx8 [27–29]. PrxIV is a two-cysteine peroxiredoxin that can couple the reduction of H_2O_2 to the oxidation of PDI family members [30–32], but is not induced in response to ER stress [29]. Accordingly, PrxIV can supplement the ER with disulfide bonds and contribute to oxidative protein folding [5,32]. In mice, loss of PrxIV causes a mild phenotype with defects in spermatogenesis [33]. Conversely, GPx7-knockout mice display signs of widespread oxidative injury, develop cancer, and die prematurely [34]. In the same vein, endogenous GPx7 protects esophageal cells from acid-mediated oxidative stress [35] and fibroblasts from pharmacologically induced ER stress [34]. In vitro, GPx7 can react with phospholipid hydroperoxides or H_2O_2 [36] as well as with the reducing substrates PDI family members [27,37,38], glutathione [37], or Grp78 [34]. Little is known about the role of GPx8 in ER physiology, except that, as for GPx7, ectopically expressed GPx8 can bind to Ero1α in cells [27].

In this study, we show that Ero1α-derived H_2O_2 cannot diffuse from ER to cytosol owing to the peroxidase activity of GPx8, which is induced on ER stress. This mechanism is independent of PrxIV and essential to protect cells from Ero1α-mediated hyperoxidation and death. GPx8-centered control of Ero1α-derived H_2O_2 necessitates a reevaluation of the source of ER-stress-induced ROS.

Materials and methods

RNA isolation and qPCR analysis

Total RNA was isolated using TRI reagent (Sigma) and reverse transcribed with Superscript III (Invitrogen) using poly(dT) primers. The resulting cDNA was subjected to qPCR analysis on a Corbett Research Rotor-Gene 6000 (version 1.7) using SYBR Fast qPCR Master Mix (KAPA Biosystems) and the following primer pairs (all 5'–3'): *Prdx4* Fw, CGAAGATTTCCAAGCCAGCGCCC; *Prdx4* Rev, CGAGGGGTATTAATCCAGGCCAAATGGG; *GPx8* Fw, CTACGGAGTAACTTTCCCCATCTTCCACAAG; *GPx8* Rev, CTGCTATGTCAGGCCTGATGACTTCAATGG; *GPx7* Fw, GCAAACTGGTGTCGCTGGAGAAGTACC; *GPx7* Rev, GAAGTCTGGGCCAGGTACTTGAAGG; *KEAP1* Fw, GGACAAACCGCCTTAATTCA; *KEAP1* Rev, CATAGCCTCCAAGGACGTAG; *NQO1* Fw, ATTTGAATTCGGGCGTCTGCTG; *NQO1* Rev, GGGATCCACGGGGACATGAATG; *GCLC* Fw, TCTCTAATAAAGAGATGAGCAACATGC; *GCLC* Rev, TTGACGATAGATAAAGAGATCTACGAA; *NFE2L1* Fw, GTGCGAGAAAGCGAAACG; *NFE2L1* Rev, CCCCAGATCAATATCCTGTCG; *NFE2L2* Fw, GCAGTCATCAAAGTACAAAGCAT; *NFE2L2* Rev, CATCCAGTCAGAAACCAGTGG; *DDIT3* Fw, AAGGCACTGAGCGTATCATGT; *DDIT3* Rev, TGAAGATACACTTCCTTCTTGAACA; *ATF6* Fw, GTCCCAGATATTAATCACGGA; *ATF6* Rev, TATCATACGTTGCTGTCTCCTT; *HERPUD1* Fw, GAGCAGATTCCTCATGGTCAT; *HERPUD1* Rev, GGCCTCGGTCTAAATGGAAA; *GAPDH* Fw, TCCTTGGAGGCCATGTGGGCCAT; *GAPDH* Rev, TGATGACATCAAGAAGGTGGTGAA; *PPIA* Fw, CATCTGCACTGCCAAGACTGA; *PPIA* Rev, TGCAATCCAGCTAGGCATG; *HPRT1* Fw, GGCTCCGTTATGGCGACCCG; *HPRT1* Rev, CGAGCAAGACGTTCAGTCCTGTCC. Genes used as internal standards were *GAPDH* and *HPRT1* (geometric mean calculated using the BestKeeper software [39]) or (for experiments shown in Supplementary Figs. S1A, S1G, and S2E) *PPIA*.

RNA interference

Small interfering RNA (siRNA) transfections were conducted with Lipofectamine RNAiMAX (Invitrogen) using the following siRNAs: negative control siRNA 1022076 (10–60 nM; Qiagen), siPRDX4 HSS173720 (40 nM; Invitrogen), siGPX8 HSS166723 (10 nM; Invitrogen), and siKEAP1 D-012456-04 (10 nM; Thermo Scientific). For combined depletion of GPx8 and PrdxIV, HSS166723 (20 nM) and HSS173720 (40 nM) were mixed.

Ero1α-C104A/C131A cells were seeded in 6-well plates and transfected with siRNAs the following day (day 0). Forty-eight hours posttransfection the cells were trypsinized and reseeded onto 6-well plates (day 2), followed by a second round of transfection (day 3) and subsequent analysis (day 5).

In the case of siRNA-mediated depletion of Keap1, a single transfection was performed and the cells were analyzed 72 h posttransfection.

Alkylation assay of ERp57

The protocol for alkylation of originally oxidized cysteines with 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (Life Technologies) has previously been published [40].

Dithiothreitol (DTT) washout assays

The cellular GSSG:total glutathione (GS_{tot}) ratio after DTT washout was measured using a 5,5'-dithiobis(2-nitrobenzoic acid)/glutathione reductase recycling assay as previously described [41]. Where indicated, BCNU (Sigma) was used at a concentration of 1 mM.

To visualize the redox state of Grx1-roGFP2 after DTT washout, transiently transfected cells were grown on UV-sterilized coverslips and treated as previously published [41]. Subsequently the cells were analyzed by αGFP immunoprecipitation/Western blot as described previously [2]. To generate a mobility marker for the oxidized form of Grx1-roGFP2, transfected cells were treated for 5 min with 5 mM diamide (Sigma).

Sulforhodamine B assay

Ero1α-C104A/C131A cells were seeded in 6-well plates and transfected with siRNA the following day. Forty-eight hours posttransfection the cells were trypsinized and reseeded onto 96-well plates (3 wells per condition). On the following day, cells were either harvested or subjected to a second round of transfection with the respective siRNAs for either 24 or 48 h. Ero1α-C104A/C131A expression was induced for the last 24 h of knockdown. The medium was removed and the proteins were precipitated by addition of 10% trichloroacetic acid (TCA). Staining with 0.4% sulforhodamine B (Sigma) was performed as described elsewhere [42] and OD_{565} measured in a UV Max microplate reader (Molecular Devices).

Fluorescence excitation spectrum analysis

Cells stably transfected with $HyPer_{ER}$ or $HyPer_{cyto}$ were subjected to fluorescence excitation spectrum analysis as described elsewhere [43]. If present, 0.5 mM DTT was added 5 min before analysis. To validate the sensor response, cells treated with either 100 μM H_2O_2 or 10 mM DTT for 5 min were routinely coanalyzed in separate wells.

Indirect immunofluorescence staining

Ero1α-C104A/C131A:$HyPer_{ER}$ or Ero1α-C104A/C131A:$HyPer_{cyto}$ cells were grown for 48 h on glass coverslips, fixed with 4% paraformaldehyde for 20 min at room temperature, quenched with 50 mM NH_4Cl, and either directly mounted in Mowiol 4-88 (Hoechst) (Ero1α-C104A/C131A:$HyPer_{cyto}$) or permeabilized with 0.1% Triton X-100 (Ero1α-C104A/C131A:$HyPer_{ER}$). In the case of the latter, cells were blocked with 1% bovine serum albumin in phosphate-buffered saline and incubated in the same buffer with αPDI for 1 h followed by Hilyte 555-conjugated goat anti-mouse