Free Radical Biology and Medicine 53 (2012) 1451–1458

Contents lists available at SciVerse ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

The H_2O_2-sensitive HyPer protein targeted to the endoplasmic reticulum as a mirror of the oxidizing thiol–disulfide milieu

Ilir Mehmeti, Stephan Lortz, Sigurd Lenzen*

Institute of Clinical Biochemistry, Hannover Medical School, 30623 Hannover, Germany

ARTICLE INFO

Article history:
Received 3 April 2012
Received in revised form
18 July 2012
Accepted 3 August 2012
Available online 10 August 2012

Keywords:
HyPer protein
Disulfide bond formation
Peroxiredoxin IV
Oxidative protein folding
β cells
Free radicals

ABSTRACT

Oxidative protein folding in the endoplasmic reticulum (ER) is associated with the formation of native disulfide bonds, which inevitably results in the formation of hydrogen peroxide (H_2O_2). Particularly in pancreatic β-cells with their high secretory activity and extremely low antioxidant capacity, the H_2O_2 molecules generated during oxidative protein folding could represent a significant oxidative burden. Therefore this study was conducted to elucidate the H_2O_2 generation during disulfide bond formation in insulin-producing RINm5F cells by targeting and specifically expressing the H_2O_2-sensitive biosensor HyPer in the ER (ER-HyPer). In addition the influence of overexpression of the H_2O_2-metabolizing ER-resident peroxiredoxin IV (PRDXIV) on H_2O_2 levels was examined. The ER-HyPer fluorescent protein was completely oxidized, whereas HyPer expressed in cytosol, peroxisomes, and mitochondria was prevalently in the reduced state, indicating a high basal H_2O_2 concentration in the ER lumen. These results could also be confirmed in non-insulin-producing COS-7 cells. Overexpression of PRDXIV in RINm5F cells effectively protected against H_2O_2-mediated toxicity; however, it did not affect the fluorescence signal of ER-HyPer. Moreover, the inhibition of de novo protein synthesis and the associated H_2O_2 generation by cycloheximide had no influence on the ER-HyPer redox state. Taken together, these findings strongly suggest that the H_2O_2-sensitive biosensor reflects exclusively the oxidative milieu in the ER and not the H_2O_2 concentration in the ER lumen.

Introduction

The endoplasmic reticulum (ER) is the main site of biosynthesis and modification of secretory proteins before trafficking to their final destination [1,2]. One of the major posttranslational modifications of secretory proteins is the formation of disulfide bonds, which are essential for their correct folding, stability, and function [3,4]. This oxidative process is catalyzed by ER-resident thiol–disulfide oxidoreductases, such as the disulfide isomerase (PDI) and the flavin adenine dinucleotide-binding enzyme ER oxidoreductin 1 (ERO-1α and ERO-1β in mammals) [5–7]. During disulfide bond formation oxidized PDI accepts two electrons from thiol residues of client proteins, resulting in oxidation of the protein and reduction of PDI. To ensure continuous oxidative protein folding, PDI reoxidation is mandatory and accomplished by ERO-1 with subsequent hydrogen peroxide (H_2O_2) generation as a by-product [8–11]. Because molecular oxygen serves as the terminal electron acceptor during disulfide bond formation, one molecule of H_2O_2 is generated for each disulfide bond formed [8]. Thus, oxidative protein folding in the ER is a substantial source of H_2O_2 and its toxic products, especially in cells with high secretory activity, such as insulin-secreting cells.

Pancreatic β-cells have a highly developed and active ER to secure high rates of insulin biosynthesis [12], generating high amounts of H_2O_2 during the process of disulfide bond formation. In addition, β-cells are particularly prone to accumulating H_2O_2 and they exhibit an extraordinary susceptibility to reactive oxygen species (ROS)-mediated toxicity due to the extremely low H_2O_2-inactivating capacity of the detoxifying enzymes catalase and glutathione peroxidase [13–15]. By targeting the H_2O_2-sensitive biosensor HyPer to mitochondria or peroxisomes we could recently show that H_2O_2 is mainly produced within the mitochondria in response to proinflammatory cytokines [16,17], whereas long-chain nonesterified fatty acids specifically induced peroxisomal H_2O_2 generation [18]. The accumulation of H_2O_2 in those organelles could be efficiently prevented by compartment-specific overexpression of catalase [16–18].

A previous study with non-insulin-secreting cells suggested that the ER generates a higher H_2O_2 concentration than any other cell organelle [19]. Moreover, Geiszt's group showed, by using the H_2O_2-sensitive biosensor HyPer, that the ERO-1α-generated H_2O_2 is strictly confined to the ER lumen [19]. However, the amount of H_2O_2 produced during oxidative folding in insulin-producing cells and the relation between oxidative stress and oxidative protein

* Corresponding author. Fax: +49 511 5323584.
E-mail address: lenzen.sigurd@mh-hannover.de (S. Lenzen).

0891-5849/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.freeradbiomed.2012.08.010

folding have not been studied so far. Therefore, we specifically targeted the H_2O_2-sensitive biosensor HyPer to the ER to quantify H_2O_2 concentrations in the ER lumen in comparison with other cell compartments in insulin-producing RINm5F cells.

Materials and methods

Tissue culture of insulin-producing cells

Insulin-producing RINm5F tissue culture cells (passages 90–105) were cultured in RPMI 1640 medium supplemented with 10 mM glucose, 10% (v/v) fetal calf serum (FCS), penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO_2 as described previously [15,20]. Nonendocrine COS-7 cells (passages 6–15) were grown in Dulbecco's modified Eagle's medium supplemented with 25 mM glucose, 2 mM glutamine, 10% (v/v) FCS, penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO_2. Cells stably expressing the H_2O_2-sensitive biosensor HyPer in cytosol (Cyto-HyPer), mitochondria (Mito-HyPer), or peroxisomes (Peroxi-HyPer) and RINm5F cells overexpressing glutathione peroxidase (GPx) or catalase in the mitochondria (MitoCatalase) or in the cytosol (CytoCatalase) were generated as described before [16,21–23]. The expression of antioxidative enzymes was analyzed by immunoblotting or by catalase enzyme activity measurement [22,23].

Subcloning of the H_2O_2-sensitive sensor HyPer targeted to the endoplasmic reticulum and of peroxiredoxin IV (PRDXIV)

To analyze the ER hydrogen peroxide generation in vivo, the cDNA of the hydrogen peroxide-sensitive fluorescent HyPer protein [24] was amplified using composite primers (HyPer-fw 5′-ATACTGCAGATGGAGATGGCAAGCCAG-3′ and HyPer-rv 5′-ATGCGGCCGCAACCGCCTGTTTTAAAACTTTATC-3′) and subcloned into the *Pst*I and *Not*I restriction sites of the pCMV/myc/ER plasmid (Life Technologies, Darmstadt, Germany). Then the ER-HyPer-specific cDNA region was amplified with the following primers: Hyper-ER-fw 5′-AAGGGATGGAGCTGTATCATCC-3′ and Hyper-ER-rv 5′-CTACAGCTCGTCCTTCTCGCTT-3′. For PRDXIV overexpression in RINm5F cells isolated RNA from human HepG2 cells was reverse transcribed with random hexamers and RevertAid H–M-MuLV reverse transcriptase (Fermentas, St. Leon-Rot, Germany). PRDXIV cDNA was specifically amplified using the following primers: hPRDXIV-fw 5′-ATGGAGGCGCTGCCGCT-3′ and hPRDXIV-rv 5′-TCAATTCAGTTTATCGAAATACTTCAGCTT-3′. Both PCR products were subsequently subcloned into the *Eco*RV restriction site of the pLenti 6.3/V5-MCS plasmid for lentiviral production. All PCRs were performed with the Phusion proofreading polymerase (NEB, Frankfurt, Germany) and all constructs were verified by sequencing.

Preparation of lentiviruses and transduction of insulin-producing RINm5F and COS-7 cells

For stable transduction of RINm5F and COS-7 cells with the ER-HyPer or hPRDXIV constructs lentiviruses were prepared as described before in detail [25]. Briefly, 4×10^6 293T cells were transfected with the packaging plasmid pPAX2 (11.25 μg), the envelope plasmid pcDNA-MDG (3.75 μg), and the transfer plasmid pLenti6.3/V5-MCS-ER-HyPer or pLenti6.3/V5-MCS-hPRDXIV (15 μg) by calcium phosphate precipitation. After 48 h the virus-containing culture medium was collected and centrifuged for 5 min at 700*g* to remove detached cells and cell debris; then the supernatant was filtered through 0.22-μm filters (Millipore Products, Schwalbach, Germany). RINm5F and COS-7 cells were infected with purified ER-HyPer or hPRDXIV (only RINm5F cells) viral supernatant. After 5–6 h of infection the viral supernatant was replaced by fresh medium. The cells were selected for HyPer expression by blasticidin (1 μmol/L) and for PRDXIV expression using zeocin (250 μg/ml) (Life Technologies). Additionally, RINm5F cells stably overexpressing CytoCatalase, MitoCatalase, GPx, or hPRDXIV were infected with the purified ER-HyPer viral particles.

Determination of compartment-specific H_2O_2 formation using HyPer protein

RINm5F control, CytoCatalase, MitoCatalase, GPx, and hPRDXIV cells and COS-7 cells stably expressing the HyPer sensor were seeded at a density of 2.5×10^4 cells per well in a 96-well black plate and allowed to attach for 24 h before incubation with the indicated H_2O_2 concentrations. Immediately after addition of the H_2O_2-containing medium, changes in the fluorescence ratio (excitation, 427 and 504 nm; emission, 520 nm) were quantified fluorimetrically using the Victor2 1420 Multilabel Counter fluorescence reader (PerkinElmer, Wiesbaden, Germany). To determine the redox state of HyPer protein, cells stably expressing HyPer in cytosol, peroxisomes, mitochondria, and ER were incubated with 0.5 mM dithiothreitol (DTT) for 5 min. After measurement of the HyPer fluorescence ratio, the DTT-containing medium was removed, residual DTT was washed out twice with medium, and the cells were exposed to 100 μM H_2O_2 for another 5 min. The fluorescence ratio (504/520 to 427/520 nm) was measured fluorimetrically.

For live cell imaging RINm5F cells stably expressing HyPer protein in various cell organelles were seeded at a density of 20×10^4 onto black 24-well glass-bottom plates (Greiner, Frickenhausen, Germany) and allowed to attach for 24 h before fluorescence imaging. Live cell imaging was performed using a CFP–YFP dual filter (excitation, 427 and 504 nm; emission, 520 nm) with a cellR/Olympus IX 81 inverted microscope system and CellR software (Olympus, Hamburg, Germany) for imaging and analysis.

Exposure to cytokines or cycloheximide (CHX)

RINm5F control and ER-HyPer- and PRDXIV-overexpressing cells were seeded at various concentrations depending on further experimentation and allowed to attach for 24 h. Thereafter the cells were exposed to 600 U/ml human IL-1β or a combination of cytokines (cytokine mixture) consisting of 60 U/ml IL-1β, 185 U/ml human TNF-α, and 14 U/ml IFN-γ (PromoCell, Heidelberg, Germany) for 24 h. CHX (Sigma, St. Louis, MO, USA) was freshly dissolved and cells were treated with various concentrations (2.5, 10, and 50 μg/ml) for the indicated times. The CHX concentrations and incubation times were selected based upon results of previous studies [26–28].

NF-κB reporter gene assay

RINm5F control and PRDXIV-overexpressing cells were transiently transfected with a pSEAP-NF-κB reporter gene vector as described in detail earlier [29]. Twenty-four hours after transfection, the cells were incubated with cytokines for 24 h. Thereafter, secreted alkaline phosphatase was measured using a Phospha-Light System kit (Life Technologies).

Immunocytochemical staining

For immunocytochemical staining, RINm5F cells expressing HyPer, PRDXIV, or catalase were seeded overnight at a density of 7×10^4 cells per well on four-well LabTek chamber slides (Nunc,

Roskilde, Denmark). Thereafter the cells were washed twice with phosphate-buffered saline (PBS) and subsequently fixed with 4% paraformaldehyde at room temperature for 60 min or overnight at 4 °C. After being washed, the cells were permeabilized and blocked with PBS containing 0.2% Triton X-100 and 1% bovine serum albumin (BSA). The cells were incubated with primary antibodies (anti-PDI, ab5484, diluted 1:100, Abcam, Cambridge, UK; anti-PRDXIV, diluted 1:100, R&D Systems, Minneapolis, MN, USA; and anti-bovine catalase, diluted 1:250, Rockland, Gilbertsville, PA, USA) diluted in PBS containing 0.1% Triton X-100 and 0.1% BSA at room temperature for 60 min. Then, the cells were washed with PBS and incubated with specific secondary antibodies that were conjugated with Texas Red (diluted 1:200) or FITC (diluted 1:500; Dianova, Hamburg, Germany) for 60 min in the dark. Afterward the cells were washed and nuclei were counterstained with 300 nM DAPI for 5 min at room temperature. Finally, the cells were washed and mounted with Mowiol/DABCO anti-photobleaching mounting medium (Sigma). Stained cells were examined with an Olympus IX81 inverted microscope (Olympus) and microscopic images were postprocessed using AutoDeblur and AutoVisualize (Autoquant Imaging, New York, NY, USA).

Assessment of cell viability

RINm5F control cells were seeded at 25,000 cells per well in 100 μl culture medium in 96-well plates and allowed to attach for 24 h before they were incubated with various H_2O_2 concentrations. The cells were incubated with H_2O_2 in a concentration range from 1 to 50 μM for 2 h in Hepes (20 mmol/L)-supplemented Krebs–Ringer bicarbonate medium with 5 mmol/L glucose, and after removal of the H_2O_2-containing medium for another 22 h in fresh RPMI 1640 medium. Cell viability was determined thereafter by a microplate-based MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) [30].

Western blot analysis

Whole-cell extracts were prepared in RIPA buffer according to the manufacturer's recommendation (Sigma) and supplemented with Complete protease inhibitor cocktail (Roche Diagnostics, Manheim, Germany). Protein content was determined by the BCA assay (Thermo Fisher Scientific, Rockford, IL, USA). RINm5F cell proteins, 20–50 μg per lane, were separated by 10–12.5% SDS–PAGE and transferred to polyvinylidene difluoride membranes. Nonspecific binding sites of the membranes were blocked with 5% nonfat dry milk for either 1 h at room temperature or overnight at 4 °C. Then, the membranes were incubated with specific primary antibodies for either 3 h at room temperature or overnight at 4 °C. The following antibodies were used: anti-PRDXIV (diluted 1:250; R&D Systems), anti-catalase (diluted 1:5000; Rockland), and actin (sc-1615, diluted 1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The excess of primary antibody was removed by three washes with washing buffer (PBS, 0.1% Tween 20, 0.1% BSA). Subsequently, the membranes were incubated with peroxidase-labeled secondary antibodies at a dilution of 1:20,000 at room temperature for 1 h. The protein bands were visualized by chemiluminescence using the ECL detection system (GE Healthcare, Freiburg, Germany).

Peptide-N-glycosidase F digestion

To remove N-linked glycans from catalase targeted to the ER, 30 μg of total protein was treated with 6 U of peptide-*N*-glycosidase F (PNGase F; Roche Diagnostics) and digested for 3 h at 37 °C in a Thermomixer at 1300 rpm. Thereafter digested proteins were subjected to Western blot analysis.

Data analysis

Data are expressed as means ± SEM. Statistical analyses were performed by ANOVA followed by Dunnett's test for multiple comparisons or by *t* test (unpaired, two-tailed) using the Prism analysis program (GraphPad, San Diego, CA, USA).

Results

Intracellular localization of the H_2O_2-sensitive protein HyPer targeted to the ER in insulin-producing RINm5F cells

The ER is the organelle of the secretory pathway in which the folding and posttranslational modification of membrane and secretory proteins occur. The protein folding is mostly associated with the introduction of native disulfide bonds and generation of H_2O_2 as a by-product. To determine H_2O_2 generation during oxidative folding in living cells, the genetically encoded fluorescence sensor HyPer, with a high submicromolar affinity and specificity to H_2O_2, was specifically targeted to the ER in insulin-producing RINm5F cells. Its subcellular localization was analyzed by immunofluorescent staining. As shown in Fig. 1, costaining approaches with PDI, an ER-specific protein, and DAPI, a nuclear probe, proved that the targeted HyPer protein was exclusively localized in the ER.

Organelle-specific quantification of basal H_2O_2 concentrations in insulin-producing RINm5F cells

Having demonstrated that targeting and expression of HyPer in the ER were successful, we next examined the basal H_2O_2 concentrations in cells stably expressing the HyPer protein in cytosol (Cyto-HyPer), peroxisomes (Peroxi-HyPer), mitochondria (Mito-HyPer), and the ER (ER-HyPer). The H_2O_2-sensitive protein HyPer exhibits two excitation peaks at 420 and 500 nm and only one emission peak at 516 nm. In the presence of H_2O_2 the excitation peak at 420 nm decreases in proportion to the increase at 500 nm. This ratiometric property displays a great advantage in allowing quantification of fluorescence independent of the amount of protein expressed [31]. In RINm5F cells the fluorescence ratios of Cyto-HyPer (0.78 ± 0.03) and Peroxi-HyPer (0.88 ± 0.07) were below 1, whereas in mitochondria a higher fluorescence ratio (Mito-HyPer, 1.18 ± 0.08) could be observed. In contrast, the fluorescence ratio of ER-HyPer was roughly threefold higher than in the other investigated cell organelles, suggesting that the ER is the major source of H_2O_2 generation within the insulin-producing RINm5F cells under resting conditions (Fig. 2A). Overall, the fluorescence ratio of HyPer in all investigated compartments of insulin-producing RINm5F cells was comparable with that measured in COS-7 cells (Fig. 2B).

Sensitivity of HyPer protein in response to exogenous H_2O_2 in various cellular compartments

To determine whether the sensitivity of established ER-HyPer is comparable to that of HyPer specifically expressed in the cytosol, in peroxisomes, and in mitochondria the fluorescence ratio of the various HyPer forms was quantified immediately after H_2O_2 treatment. Exposure of Cyto-HyPer-, Peroxi-HyPer-, and Mito-HyPer-expressing cells to increasing H_2O_2 concentrations caused an exponential increase in the fluorescence ratio (Fig. 3A). These H_2O_2-mediated HyPer fluorescence ratio changes were comparable in all

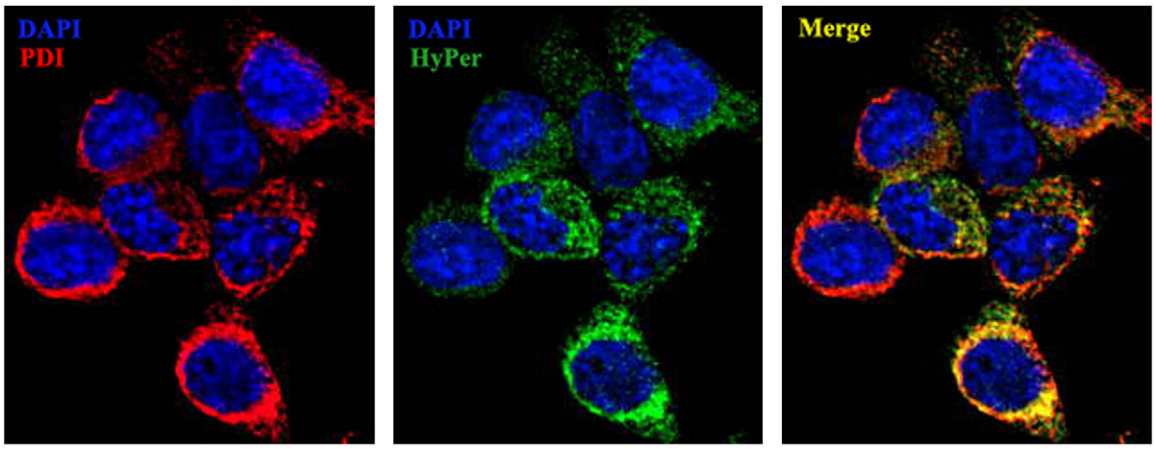

Fig. 1. Intracellular localization of the H_2O_2-sensitive protein HyPer targeted to the endoplasmic reticulum (ER) in insulin-producing RINm5F cells. RINm5F cells stably expressing the hydrogen peroxide-sensitive protein HyPer were seeded on LabTek chamber slides and allowed to attach for 24 h. After fixation and permeabilization the cells were stained for the ER-specific protein disulfide isomerase (PDI, red), followed by counterstaining with DAPI (blue). The H_2O_2-sensitive protein HyPer was detected by excitation at 504 nm and emission at 520 nm (green).

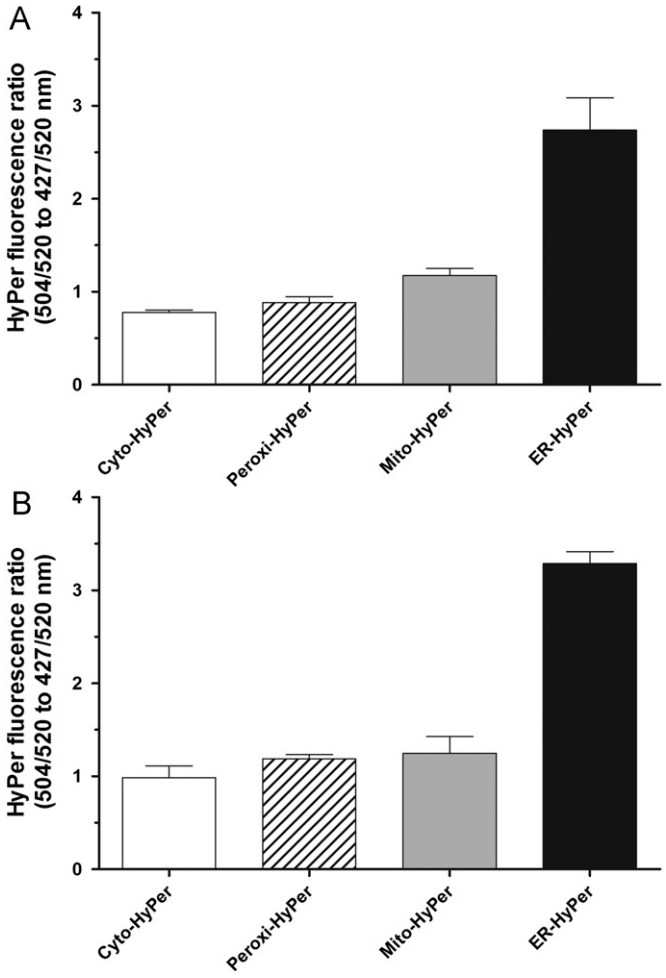

Fig. 2. Organelle-specific quantification of basal H_2O_2 concentrations in insulin-producing RINm5F and nonendocrine COS-7 cells. (A) RINm5F and (B) COS-7 cells stably expressing the H_2O_2-sensitive protein HyPer in cytosol (Cyto-HyPer), peroxisomes (Peroxi-HyPer), mitochondria (Mito-HyPer), and the ER (ER-HyPer) were seeded onto black 96-well plates and allowed to attach overnight. Thereafter the fluorescence ratio (504/520 to 427/520 nm) was measured fluorimetrically. Data are expressed as means ± SEM of four to six independent experiments.

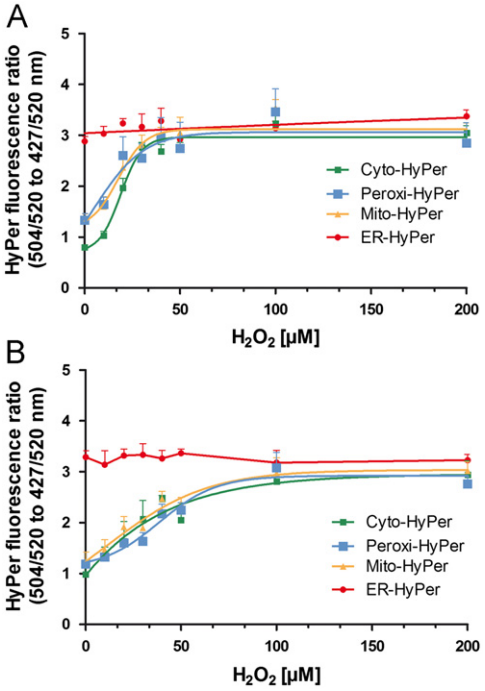

Fig. 3. Sensitivity of HyPer protein in response to exogenous H_2O_2 in various cellular compartments. (A) Insulin-producing RINm5F and (B) nonendocrine COS-7 cells expressing the H_2O_2-sensitive protein HyPer in cytosol (Cyto-HyPer), peroxisomes (Peroxi-HyPer), mitochondria (Mito-HyPer), and the ER (ER-HyPer) were treated with increasing H_2O_2 concentrations ranging from 10 to 200 μM immediately before ratiometric measurement. The fluorescence ratio (504/520 to 427/520 nm) was measured fluorimetrically. Data are expressed as means ± SEM of six to eight independent experiments.

investigated cell clones and showed a strong response in a H_2O_2 concentration range from 10 to 50 μM. However, at higher H_2O_2 concentrations the HyPer protein was completely oxidized, because addition of 100 to 200 μM H_2O_2 did not further increase the fluorescence ratio. In contrast to these three HyPer forms, the fluorescence ratio of ER-HyPer displayed no changes under the same treatment conditions (Fig. 3A). This observation was verified in COS-7 cells, although the fully oxidized state of Cyto-HyPer, Peroxi-HyPer,

and Mito-HyPer was reached at higher H_2O_2 concentrations in these nonsecretory cells (Fig. 3B). This can be explained by the fact that the expression of endogenous antioxidative enzymes in COS-7 cells is markedly higher than in RINm5F cells.

To obtain more information about the redox state of HyPer protein in the ER the response of ER-HyPer to H_2O_2 in relation to HyPer in other cellular compartments was additionally monitored by live cell fluorescence microscopy. As shown in Supplementary Fig. 1 Cyto-HyPer-, Peroxi-HyPer-, or Mito-HyPer-expressing cells revealed a green-yellow fluorescence resulting from an overlay image of the fluorescence signal at 427/520 nm (green) and at 504/520 nm (red), indicating low endogenous H_2O_2 concentrations under control conditions (Supplementary Fig. 1A, C, and E). Upon exposure to 100 μM H_2O_2 the fluorescence intensity at 427/520 nm decreased, whereas that at 504/520 increased, yielding a fluorescence shift from green-yellow to orange-red (Supplementary Fig. 1B, D, and F). Thus, the HyPer protein is responsive in those cellular organelles. In contrast, cells expressing ER-HyPer showed a strong orange-red fluorescence under both control and H_2O_2-treatment conditions (Supplementary Fig. 1G and H), strongly suggesting that the HyPer protein in the ER is fully oxidized and not responsive to externally added H_2O_2. These live cell images corresponded to the quantitative data shown in Fig. 3.

Effects of DTT and cycloheximide on the redox state of HyPer protein in various cell organelles

As an independent approach to testing the functionality of HyPer in the ER, cells expressing the HyPer protein in various cell compartments were treated with the strong reducing agent DTT. Exposure of cells expressing ER-HyPer to 0.5 mM DTT resulted in an extreme decrease in the fluorescence ratio, indicating that the HyPer protein in the ER is in an oxidized state. After removal of the DTT-containing medium, followed by addition of 100 μM H_2O_2, the ER-HyPer fluorescence ratio increased again nearly to the original level, showing that HyPer within the ER is functional. However, the fluorescence ratio of HyPer in cytosol, peroxisomes, or mitochondria did not change significantly in response to DTT, suggesting that HyPer in those cellular organelles is in a reduced state (Fig. 4). We next ascertained whether the inhibition of de novo protein synthesis impairs the observed higher HyPer fluorescence ratio in the ER. Therefore, RINm5F cells expressing ER-HyPer were treated with CHX, an inhibitor of protein biosynthesis. Exposure of ER-HyPer cells to CHX did not affect the ER-HyPer fluorescence ratio over the whole conducted time-course analysis (Supplementary Fig. 5). Following the treatment with DTT resulted in a severe reduction in ER-HyPer fluorescence ratio, which was nearly completely reversible after DTT washout, indicating that the ER-HyPer fluorescence is most likely influenced by the prevalent oxidizing ER milieu.

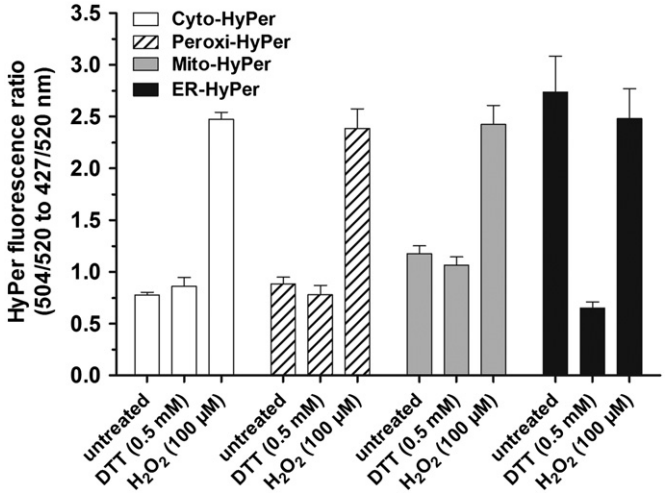

Fig. 4. Effects of dithiothreitol (DTT) on the redox state of HyPer protein in various cell organelles. Insulin-producing RINm5F cells stably expressing the hydrogen peroxide sensor protein HyPer in the cytosol (Cyto-HyPer), peroxisomes (Peroxi-HyPer), mitochondria (Mito-HyPer), and the ER (ER-HyPer) were incubated under control conditions (untreated), with 0.5 mM DTT, and, after removal of DTT-containing medium, with 100 μM H_2O_2 for 5 min. The fluorescence ratio (504/520 to 427/520 nm) was measured fluorimetrically. Data are expressed as means ± SEM of six independent experiments.

Effects of the H_2O_2-detoxifying enzymes catalase and glutathione peroxidase on the ER-HyPer redox state

H_2O_2 is a diffusible molecule that freely permeates through biomembranes [14,32,33]. Thus, lowering the H_2O_2 concentration outside the ER compartment by overexpression of cytosolically (CytoCatalase) or mitochondrially (MitoCatalase) located catalase or GPx should increase the diffusion rate of H_2O_2 out of the ER lumen. However, as shown in Fig. 5, overexpression of the H_2O_2-detoxifying enzymes catalase and GPx had no influence on the fluorescence ratio of ER-HyPer. This observation suggested that either the H_2O_2 generated within the ER is strongly restricted to this organelle or the oxidation of HyPer protein within the ER lumen is H_2O_2 independent.

Effects of overexpression of the ER-specific peroxiredoxin IV on H_2O_2-induced toxicity

To confirm that the high fluorescence ratio of ER-HyPer is a consequence of H_2O_2 in the ER lumen, we first targeted and stably overexpressed the H_2O_2-inactivating enzyme catalase in the ER (Supplementary Fig. 2). However, contrary to our expectations it appeared that catalase in the ER did not exhibit enzyme activity. Its activity was extremely low compared to that of catalase in cytosol or mitochondria (Supplementary Fig. 3). Western blot analyses revealed that catalase in the ER had a higher molecular weight than in other investigated cell organelles (Supplementary

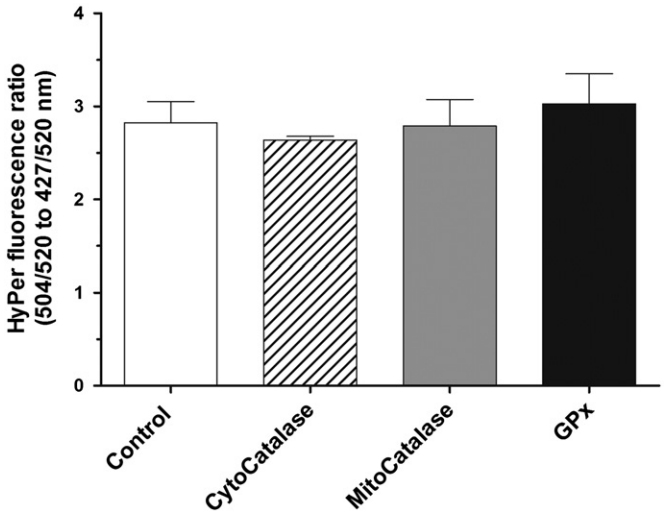

Fig. 5. Effects of the H_2O_2-detoxifying enzymes catalase and glutathione peroxidase on the ER-HyPer fluorescence ratio in insulin-producing RINm5F cells under control conditions. RINm5F control cells (control) and cells stably overexpressing catalase in the cytosol (CytoCatalase) or mitochondria (MitoCatalase) or glutathione peroxidase (GPx) were cotransfected with the ER-HyPer protein. Stably cotransfected cells were seeded onto black 96-well plates and allowed to attach overnight. Thereafter the fluorescence ratio (504/520 to 427/520 nm) was measured fluorimetrically. Data are expressed as means ± SEM of four independent experiments.

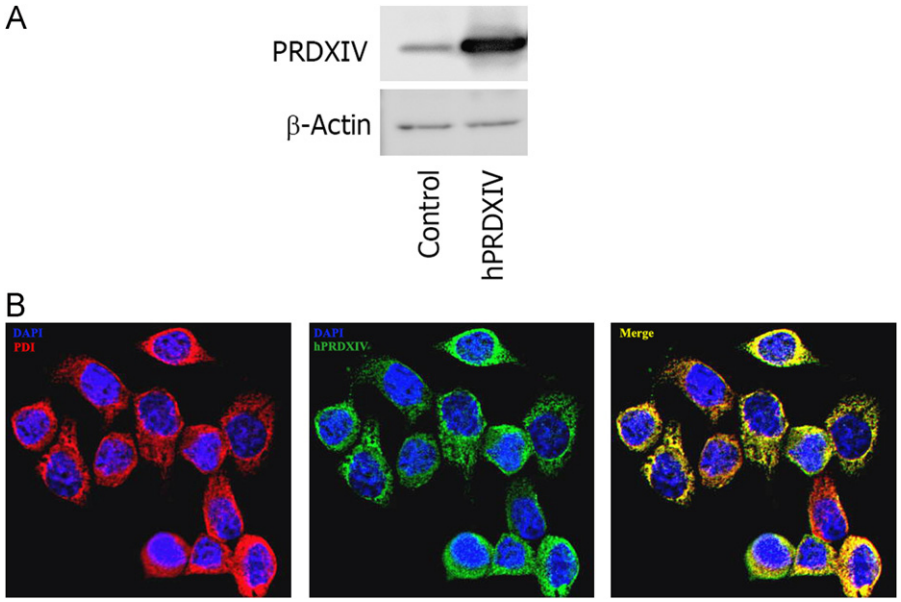

Fig. 6. Stable overexpression of peroxiredoxin IV (hPRDXIV) in insulin-producing RINm5F cells. (A) Protein expression of PRDXIV in RINm5F control cells and cells stably overexpressing PRDXIV was assessed by Western blot analyses. (B) Subcellular localization of the PRDXIV overexpressed in RINm5F cells. RINm5F cells overexpressing PRDXIV were seeded overnight on LabTek chamber slides. After fixation and permeabilization the cells were stained with specific antibodies for the ER-specific protein disulfide isomerase (PDI, red) and for PRDXIV (green) followed by counterstaining with DAPI (blue).

Fig. 4A). To confirm that the increase in molecular weight is due to glycosylation, we next treated the cells expressing catalase in the ER with PNGase F. A PNGase F treatment resulted in a decrease in molecular weight, strongly suggesting that catalase in the ER is N-glycosylated (Supplementary Fig. 4B).

Therefore we stably overexpressed the newly described ER-specific cytoprotective enzyme PRDXIV in insulin-producing RINm5F cells. As shown in Fig. 6A cells stably transfected with human PRDXIV exhibited a marked increase in the level of PRDXIV. Its subcellular localization was analyzed by immunofluorescent staining. Costaining approaches with PDI, a specific ER protein, and DAPI, a nuclear probe, revealed that PRDXIV was exclusively localized in the ER (Fig. 6B). To determine whether PRDXIV protects against H_2O_2-induced toxicity, we initially compared the cell viability of control cells with cells overexpressing PRDXIV after H_2O_2 treatment. Control cells were significantly more susceptible to a H_2O_2-induced decrease in cell viability than cells overexpressing PRDXIV (Fig. 7). Hence overexpression of PRDXIV can detoxify H_2O_2. Moreover, the overexpression of PRDXIV successfully prevented the activation of the unfolded protein response caused by the N-glycosylation inhibitor tunicamycin (data not shown). Because earlier studies have suggested that PRDXIV might directly regulate the activity of the transcription factor NF-κB [34,35], we additionally examined the effect of PRDXIV overexpression on NF-κB activation after cytokine treatment. A 24 h exposure to cytokines resulted in a significant activation of NF-κB in RINm5F control cells. A comparable NF-κB activation was also seen in PRDXIV-overexpressing cells, meaning that NF-κB activity is not modulated by PRDXIV overexpression under these treatment conditions (Supplementary Fig. 6).

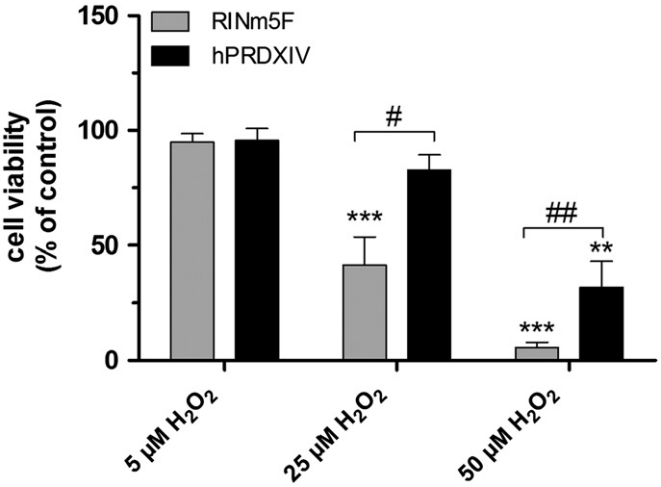

Fig. 7. Effects of peroxiredoxin IV (hPRDXIV) overexpression on H_2O_2 toxicity in insulin-producing RINm5F cells. RINm5F control cells and PRDXIV-overexpressing cells were incubated under control conditions, with 5, 25, or 50 μM H_2O_2 for 2 h in Krebs–Ringer medium, and after removal of the H_2O_2-containing medium for another 22 h in fresh RPMI 1640 medium. Thereafter, the viability of the cells was determined by the MTT assay and expressed as a percentage of untreated cells. Data are expressed as means ± SEM of four independent experiments; $^{**}p < 0.01$, $^{***}p < 0.001$ compared with cells incubated under control conditions (ANOVA/Dunnett's test); $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ compared with cells incubated under the same conditions (*t* test, unpaired, two-tailed).

Effects of overexpression of the ER-specific peroxiredoxin IV on ER-HyPer fluorescence ratio

Given that PRDXIV can protect against H_2O_2-mediated toxicity, we next specifically expressed the ER-HyPer protein in cells overexpressing PRDXIV and examined the ER-HyPer fluorescence. Overexpression of the H_2O_2-metabolizing enzyme PRDXIV had no influence on the fluorescence ratio of ER-HyPer. The quantified ER-HyPer fluorescence ratio in PRDXIV-overexpressing cells was comparable to that in control cells (Fig. 8), indicating that oxidation of the ER-HyPer protein is most probably H_2O_2 independent.

Discussion

The ER provides a unique oxidizing environment for the formation of intra- and intermolecular disulfide bonds of most

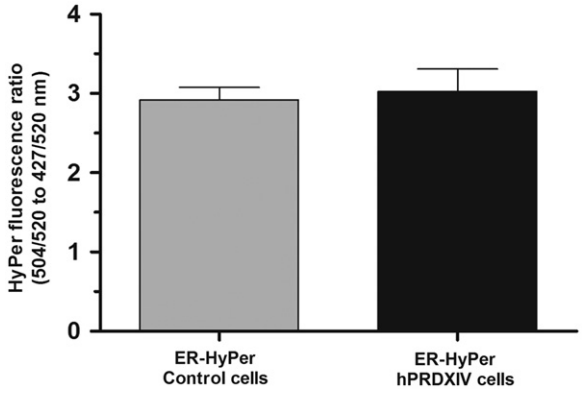

Fig. 8. Effects of peroxiredoxin IV (hPRDXIV) overexpression on ER-HyPer fluorescence ratio in insulin-producing RINm5F cells. RINm5F cells stably expressing ER-HyPer (control) and PRDXIV–ER-HyPer cotransfected cells were seeded onto black 96-well plates and allowed to attach overnight. Thereafter the fluorescence ratio (504/520 to 427/520 nm) was measured fluorimetrically. Data are expressed as means ± SEM of four independent experiments.

secretory proteins. This oxidative process is accelerated by the ER-resident oxidoreductases, such as PDI and ERO-1 proteins (ERO-1p in yeast, ERO-1α and ERO-1β in mammals), resulting finally in generation of equivalent amounts H_2O_2 [8,9,36,37].

Earlier studies in yeast and *Caenorhabditis elegans* have emphasized the great potential of ERO-1 for ROS generation in living cells [38,39]. Accordingly, it has been estimated that approximately 25% of the ROS generated intracellularly may result from disulfide bond formation in the ER [6]. However, for different mammalian cell types conflicting data exist with respect to quantification of ER luminal H_2O_2 generation. Whereas in endothelial cells the basal H_2O_2 concentrations quantified by the H_2O_2-sensitive biosensor HyPer in both cellular compartments, ER and cytosol, were comparably low [40], in HeLa cells the fluorescence ratio of HyPer—which represents the H_2O_2 concentration—in the ER was threefold higher than in cytosol [19]. Because of the observation that the fluorescence ratio of HyPer protein in the ER lumen was markedly higher than that of HyPer targeted to the cytoplasmic surface of the ER, Enyedi and collaborators [19] suggested that the ERO-1-mediated formation of H_2O_2—although it is highly biomembrane permeative [14,32,33]—is strictly confined to the ER lumen. Hence, the highest intracellular H_2O_2 concentrations prevail in the ER.

Considering that proinsulin is the major biosynthetic product of pancreatic β-cells and its oxidative folding comprises three disulfide bonds [41], this may represent a significant oxidative burden to the particularly weakly protected insulin-producing β-cell [14]. These circumstances tempted us to examine the H_2O_2 production during the disulfide bond formation within the ER in living insulin-producing β-cells. Stable expression of the H_2O_2-sensitive HyPer protein targeted to various cell organelles revealed that the fluorescence ratio of HyPer in the ER was significantly higher than in all other investigated cell organelles, indicating high basal H_2O_2 concentrations in that organelle. These findings are in agreement with those recently reported for HeLa cells [19] and our findings obtained in secretory inactive COS-7 cells. Importantly, the fluorescence signal of the genetically encoded biosensor HyPer is based on formation of an intramolecular disulfide bond between cysteine 199 and cysteine 208 of the regulatory domain OxyR within the HyPer protein [24]. Thus, the observed high HyPer fluorescence ratio within the ER could arise from thiol–disulfide oxidoreductase-mediated oxidation of HyPer, independent of the luminal H_2O_2 concentration as hypothesized earlier by Malinouski and collaborators [42].

A genetic manipulation of ER-resident oxidoreductase levels does not necessarily provide proof of H_2O_2-mediated oxidation of HyPer protein in the ER, because deletion of ERO-1 would compromise the thiol–disulfide exchange reaction and consequently the H_2O_2 generation. This could be exemplified in DTT washout experiments, because DTT interferes with the ER redox homeostasis [43], but can also directly reduce the essential disulfide bonds of the HyPer protein. Indeed, addition of the disulfide-bond-reducing dithiol DTT to insulin-producing cells stably expressing the HyPer protein in various organelles led to a strong reduction of the HyPer fluorescence ratio in the ER, which was recovered upon removal of DTT. Moreover, inhibition of protein translation by cycloheximide had no effect on the HyPer redox state within the ER.

If the oxidized status of HyPer in the ER is specifically H_2O_2-mediated, overexpression of the H_2O_2-inactivating enzyme catalase either in the cytosol or in the mitochondria should reduce the observed ER-HyPer fluorescence ratio, because H_2O_2 is highly biomembrane permeative [14,32,33]. However, neither cytosolically nor mitochondrially located catalase or GPx had an influence on the ER-HyPer fluorescence, whereas the H_2O_2-induced oxidation of Peroxi-HyPer, Cyto-HyPer, and Mito-HyPer in those catalase-overexpressing cells was reduced [16,18,21]. To strengthen this observation the oxidoreductase catalase was specifically targeted to the ER. However, in contrast to catalase in peroxisomes [18,20] or mitochondria [22], the specific enzyme activity of catalase in the ER was unexpectedly low and insufficient for H_2O_2 decomposition. A possible reason for the low catalase enzyme activity could be a posttranslational modification of ER-targeted catalase, such as N-linked glycosylation.

Recently a new ER-resident antioxidant enzyme, peroxiredoxin IV, has been described, which metabolizes the H_2O_2 produced during the disulfide bond formation within the ER and attenuates the tunicamycin-induced toxicity [44,45]. Indeed, overexpression of ER-specific PRDXIV resulted in a significant protection against H_2O_2-mediated toxicity in insulin-producing cells in our study, showing the successful elimination of H_2O_2 by PRDXIV as was described earlier for PRDXs I, II, and III [46,47]. However, the overexpression of PRDXIV did not affect the high fluorescence ratio of the HyPer protein in the ER, strongly suggesting that oxidation of HyPer in the ER is not H_2O_2-mediated as convincingly demonstrated in other cellular compartments [16,18,42]. Thus, ER-HyPer is not a suitable indicator of the H_2O_2 concentration prevailing in the ER. Nevertheless, the ER-HyPer biosensor could be employed for visualization of the compartment-specific redox state in real time, as previously proposed by Malinouski et al. [42]. However, one should consider that the HyPer biosensor is H_2O_2 sensitive and its application as a redox sensor is only possible at unchanging H_2O_2 concentrations to exclude misinterpretation of the obtained fluorescence ratios. Importantly, in contrast to earlier studies in non-insulin-producing cells [34,35], no regulatory effect of PRDXIV overexpression on cytokine-mediated NF-κB activation was detectable in RINm5F cells. This is confirmatory to previous results showing that other H_2O_2-degrading enzymes have no significant influence on cytokine-mediated activation of NF-κB in RINm5F cells [29].

Overall, our findings demonstrate that the oxidation of the H_2O_2-sensitive HyPer protein in the ER reflects the ER luminal oxidizing thiol–disulfide milieu and not the H_2O_2 generation. Thus, the HyPer protein, at variance from earlier contentions [19], is an unsuitable tool for H_2O_2 monitoring during disulfide bond formation in the ER. Furthermore, the present results clearly show that overexpression of ER-resident PRDXIV is able to protect insulin-producing cells against oxidative injury when exposed to external H_2O_2, indicating that PRDXIV serves as a H_2O_2 scavenger in the ER lumen.

Acknowledgments

This work was supported by a grant from the European Union (BetaBAT, Grant Agreement 277713) in the Framework Programme 7. The skillful technical assistance of Martin Wirth is gratefully acknowledged.

Appendix A. Supplementary Information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2012.08.010.

References

[1] Kaufman, R. J.; Back, S. H.; Song, B.; Han, J.; Hassler, J. The unfolded protein response is required to maintain the integrity of the endoplasmic reticulum, prevent oxidative stress and preserve differentiation in beta-cells. *Diabetes Obes. Metab* **12(Suppl. 2):**99–107; 2010.
[2] Zhang, K.; Kaufman, R. J. From endoplasmic-reticulum stress to the inflammatory response. *Nature* **454:**455–462; 2008.
[3] Anfinsen, C. B.; Redfield, R. R. Protein structure in relation to function and biosynthesis. *Adv. Protein Chem.* **11:**1–100; 1956.
[4] Frand, A. R.; Cuozzo, J. W.; Kaiser, C. A. Pathways for protein disulphide bond formation. *Trends Cell Biol.* **10:**203–210; 2000.
[5] Appenzeller-Herzog, C.; Riemer, J.; Christensen, B.; Sorensen, E. S.; Ellgaard, L. A novel disulphide switch mechanism in Ero1alpha balances ER oxidation in human cells. *EMBO J* **27:**2977–2987; 2008.
[6] Tu, B. P.; Weissman, J. S. Oxidative protein folding in eukaryotes: mechanisms and consequences. *J. Cell Biol.* **164:**341–346; 2004.
[7] Zito, E.; Chin, K. T.; Blais, J.; Harding, H. P.; Ron, D. ERO1-beta, a pancreas-specific disulfide oxidase, promotes insulin biogenesis and glucose homeostasis. *J. Cell Biol.* **188:**821–832; 2010.
[8] Gross, E.; Sevier, C. S.; Heldman, N.; Vitu, E.; Bentzur, M.; Kaiser, C. A.; Thorpe, C.; Fass, D. Generating disulfides enzymatically: reaction products and electron acceptors of the endoplasmic reticulum thiol oxidase Ero1p. *Proc. Natl. Acad. Sci. USA* **103:**299–304; 2006.
[9] Malhotra, J. D.; Kaufman, R. J. Endoplasmic reticulum stress and oxidative stress: a vicious cycle or a double-edged sword? *Antioxid. Redox Signaling* **9:**2277–2293; 2007.
[10] Margittai, E.; Sitia, R. Oxidative protein folding in the secretory pathway and redox signaling across compartments and cells. *Traffic* **12:**1–8; 2011.
[11] Tu, B. P.; Weissman, J. S. The FAD- and O_2-dependent reaction cycle of Ero1-mediated oxidative protein folding in the endoplasmic reticulum. *Mol. Cell* **10:**983–994; 2002.
[12] Harding, H. P.; Ron, D. Endoplasmic reticulum stress and the development of diabetes: a review. *Diabetes* **51(Suppl. 3):**S455–461; 2002.
[13] Lenzen, S.; Drinkgern, J.; Tiedge, M. Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic. Biol. Med.* **20:**463–466; 1996.
[14] Lenzen, S. Oxidative stress: the vulnerable beta-cell. *Biochem. Soc. Trans.* **36:**343–347; 2008.
[15] Tiedge, M.; Lortz, S.; Drinkgern, J.; Lenzen, S. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* **46:**1733–1742; 1997.
[16] Gurgul-Convey, E.; Mehmeti, I.; Lortz, S.; Lenzen, S. Cytokine toxicity in insulin-producing cells is mediated by nitro-oxidative stress-induced hydroxyl radical formation in mitochondria. *J. Mol. Med. (Berlin)* **89:**785–798; 2011.
[17] Mehmeti, I.; Gurgul-Convey, E.; Lenzen, S.; Lortz, S. Induction of the intrinsic apoptosis pathway in insulin-secreting cells is dependent on oxidative damage of mitochondria but independent of caspase-12 activation. *Biochim. Biophys. Acta* **1813:**1827–1835; 2011.
[18] Elsner, M.; Gehrmann, W.; Lenzen, S. Peroxisome-generated hydrogen peroxide as important mediator of lipotoxicity in insulin-producing cells. *Diabetes* **60:**200–208; 2011.
[19] Enyedi, B.; Varnai, P.; Geiszt, M. Redox state of the endoplasmic reticulum is controlled by Ero1L-alpha and intraluminal calcium. *Antioxid. Redox Signaling* **13:**721–729; 2010.
[20] Lortz, S.; Tiedge, M.; Nachtwey, T.; Karlsen, A. E.; Nerup, J.; Lenzen, S. Protection of insulin-producing RINm5F cells against cytokine-mediated toxicity through overexpression of antioxidant enzymes. *Diabetes* **49:**1123–1130; 2000.
[21] Gehrmann, W.; Elsner, M. A specific fluorescence probe for hydrogen peroxide detection in peroxisomes. *Free Radic. Res.* **45:**501–506; 2011.
[22] Gurgul, E.; Lortz, S.; Tiedge, M.; Jörns, A.; Lenzen, S. Mitochondrial catalase overexpression protects insulin-producing cells against toxicity of reactive oxygen species and proinflammatory cytokines. *Diabetes* **53:**2271–2280; 2004.
[23] Tiedge, M.; Lortz, S.; Munday, R.; Lenzen, S. Complementary action of antioxidant enzymes in the protection of bioengineered insulin-producing RINm5F cells against the toxicity of reactive oxygen species. *Diabetes* **47:**1578–1585; 1998.
[24] Belousov, V. V.; Fradkov, A. F.; Lukyanov, K. A.; Staroverov, D. B.; Shakhbazov, K. S.; Terskikh, A. V.; Lukyanov, S. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat. Methods* **3:**281–286; 2006.
[25] Zufferey, R.; Dull, T.; Mandel, R. J.; Bukovsky, A.; Quiroz, D.; Naldini, L.; Trono, D. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J. Virol.* **72:**9873–9880; 1998.
[26] Azzu, V.; Brand, M. D. Degradation of an intramitochondrial protein by the cytosolic proteasome. *J. Cell Sci.* **123:**578–585; 2010.
[27] Darville, M. I.; Terryn, S.; Eizirik, D. L. An octamer motif is required for activation of the inducible nitric oxide synthase promoter in pancreatic beta-cells. *Endocrinology* **145:**1130–1136; 2004.
[28] Stewart, D. P.; Koss, B.; Bathina, M.; Perciavalle, R. M.; Bisanz, K.; Opferman, J. T. Ubiquitin-independent degradation of antiapoptotic MCL-1. *Mol. Cell. Biol.* **30:**3099–3110; 2010.
[29] Azevedo-Martins, A. K.; Lortz, S.; Lenzen, S.; Curi, R.; Eizirik, D. L.; Tiedge, M. Improvement of the mitochondrial antioxidant defense status prevents cytokine-induced nuclear factor-kappaB activation in insulin-producing cells. *Diabetes* **52:**93–101; 2003.
[30] Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65:**55–63; 1983.
[31] Belousov, V. V.; Fradkov, A. F.; Lukyanov, K. A.; Staroverov, D. B.; Shakhbazov, K. S.; Terskikh, A. V.; Lukyanov, S. Genetically encoded fluorescent indicator for intracellular hydrogen peroxide. *Nat. Methods* **3:**281–286; 2006.
[32] Giorgio, M.; Trinei, M.; Migliaccio, E.; Pelicci, P. G. Hydrogen peroxide: a metabolic by-product or a common mediator of ageing signals? *Nat. Rev* **8:**722–728; 2007.
[33] Halliwell, B.; Gutteridge, J. M. C. *Free Radicals in Biology and Medicine*. London: Oxford Univ. Press; 2007.
[34] Haridas, V.; Ni, J.; Meager, A.; Su, J.; Yu, G. L.; Zhai, Y.; Kyaw, H.; Akama, K. T.; Hu, J.; Van Eldik, L. J.; Aggarwal, B. B. TRANK, a novel cytokine that activates NF-kappa B and c-Jun N-terminal kinase. *J. Immunol.* **161:**1–6; 1998.
[35] Jin, D. Y.; Chae, H. Z.; Rhee, S. G.; Jeang, K. T. Regulatory role for a novel human thioredoxin peroxidase in NF-kappaB activation. *J. Biol. Chem.* **272:**30952–30961; 1997.
[36] Appenzeller-Herzog, C. Glutathione- and non-glutathione-based oxidant control in the endoplasmic reticulum. *J. Cell Sci* **124:**847–855; 2011.
[37] Wang, L.; Li, S. J.; Sidhu, A.; Zhu, L.; Liang, Y.; Freedman, R. B.; Wang, C. C. Reconstitution of human Ero1-Lalpha/protein-disulfide isomerase oxidative folding pathway in vitro: position-dependent differences in role between the a and a′ domains of protein-disulfide isomerase. *J. Biol. Chem.* **284:**199–206; 2009.
[38] Harding, H. P.; Zhang, Y.; Zeng, H.; Novoa, I.; Lu, P. D.; Calfon, M.; Sadri, N.; Yun, C.; Popko, B.; Paules, R.; Stojdl, D. F.; Bell, J. C.; Hettmann, T.; Leiden, J. M.; Ron, D. An integrated stress response regulates amino acid metabolism and resistance to oxidative stress. *Mol. Cell* **11:**619–633; 2003.
[39] Haynes, C. M.; Titus, E. A.; Cooper, A. A. Degradation of misfolded proteins prevents ER-derived oxidative stress and cell death. *Mol. Cell* **15:**767–776; 2004.
[40] Wu, R. F.; Ma, Z.; Liu, Z.; Terada, L. S. Nox4-derived H_2O_2 mediates endoplasmic reticulum signaling through local Ras activation. *Mol. Cell. Biol.* **30:**3553–3568; 2010.
[41] Shimizu, Y.; Hendershot, L. M. Oxidative folding: cellular strategies for dealing with the resultant equimolar production of reactive oxygen species. *Antioxid. Redox Signaling* **11:**2317–2331; 2009.
[42] Malinouski, M.; Zhou, Y.; Belousov, V. V.; Hatfield, D. L.; Gladyshev, V. N. Hydrogen peroxide probes directed to different cellular compartments. *PLoS One* **6:**e14564; 2011.
[43] Braakman, I.; Helenius, J.; Helenius, A. Manipulating disulfide bond formation and protein folding in the endoplasmic reticulum. *EMBO J* **11:**1717–1722; 1992.
[44] Tavender, T. J.; Bulleid, N. J. Peroxiredoxin IV protects cells from oxidative stress by removing H_2O_2 produced during disulphide formation. *J. Cell Sci* **123:**2672–2679; 2010.
[45] Tavender, T. J.; Sheppard, A. M.; Bulleid, N. J. Peroxiredoxin IV is an endoplasmic reticulum-localized enzyme forming oligomeric complexes in human cells. *Biochem. J.* **411:**191–199; 2008.
[46] Wolf, G.; Aumann, N.; Michalska, M.; Bast, A.; Sonnemann, J.; Beck, J. F.; Lendeckel, U.; Newsholme, P.; Walther, R. Peroxiredoxin III protects pancreatic beta cells from apoptosis. *J. Endocrinol.* **207:**163–175; 2010.
[47] Bast, A.; Wolf, G.; Oberbaumer, I.; Walther, R. Oxidative and nitrosative stress induces peroxiredoxins in pancreatic beta cells. *Diabetologia* **45:**867–876; 2002.