

Peroxiredoxin 4 Improves Insulin Biosynthesis and Glucose-induced Insulin Secretion in Insulin-secreting INS-1E Cells*

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Background: Peroxiredoxin 4 facilitates *de novo* disulfide bond formation by metabolizing hydrogen peroxide.

Results: Overexpression of peroxiredoxin 4 improved insulin synthesis and glucose-induced insulin secretion.

Conclusion: Increasing the constitutively low expression of peroxiredoxin 4 enhances the ER folding capacity and improves β -cell function.

Significance: Peroxiredoxin 4 contributes to the preservation of β -cell function under conditions of high insulin requirement.

Oxidative folding of (pro)insulin is crucial for its assembly and biological function. This process takes place in the endoplasmic reticulum (ER) and is accomplished by protein disulfide isomerase and ER oxidoreductin 1 β , generating stoichiometric amounts of hydrogen peroxide (H_2O_2) as byproduct. During insulin resistance in the prediabetic state, increased insulin biosynthesis can overwhelm the ER antioxidative and folding capacity, causing an imbalance in the ER redox homeostasis and oxidative stress. Peroxiredoxin 4 (Prdx4), an ER-specific antioxidative peroxidase can utilize luminal H_2O_2 as driving force for reoxidizing protein disulfide isomerase family members, thus efficiently contributing to disulfide bond formation. Here, we examined the functional significance of Prdx4 on β -cell function with emphasis on insulin content and secretion during stimulation with nutrient secretagogues. Overexpression of Prdx4 in glucose-responsive insulin-secreting INS-1E cells significantly metabolized luminal H_2O_2 and improved the glucose-induced insulin secretion, which was accompanied by the enhanced proinsulin mRNA transcription and insulin content. This β -cell beneficial effect was also observed upon stimulation with the nutrient insulin secretagogue combination of leucine plus glutamine, indicating that the effect is not restricted to glucose. However, knockdown of Prdx4 had no impact on H_2O_2 metabolism or β -cell function due to the fact that Prdx4 expression is negligibly low in pancreatic β -cells. Moreover, we provide evidence that the constitutively low expression of Prdx4 is highly susceptible to hyperoxidation in the presence of high glucose. Overall, these data suggest an important role of Prdx4 in maintaining insulin levels and improving the ER folding capacity also under conditions of a high insulin requirement.

Pancreatic β -cells are highly specialized in the synthesis and secretion of insulin. The biosynthesis of (pro)insulin, the major product of β -cells, takes place in the endoplasmic reticulum (ER),²

which provides a unique oxidizing environment for proper protein folding and formation of intra- and interchain disulfide bonds (1, 2). Before trafficking to the Golgi apparatus and storage in secretory granules, (pro)insulin must be folded into a three-dimensional structure that is stabilized by three disulfide bonds: two between the A and B chains and one within the A chain (3, 4). This folding process is catalyzed by a large family of ER oxidoreductases, including protein disulfide isomerase (PDI) and the flavin adenine dinucleotide-binding (FAD) enzyme ER oxidoreductin 1 (ERO-1) (5, 6). The PDI-mediated introduction of a disulfide bond into a nascent polypeptide results in the oxidation of the polypeptide and the reduction of the PDI active site. Reoxidation of PDI is catalyzed by members of the ERO-1 family (Ero1 α and Ero1 β in mammals), which in turn deliver the electrons in a FAD-dependent reaction to molecular oxygen, thereby generating stoichiometric amounts of H_2O_2 for every disulfide bond produced (7–9). It has been estimated that ERO-1-mediated reoxidation of PDI could account for ~25% of intracellular H_2O_2 production (9). In pancreatic β -cells with an extremely high protein-folding load (3) and an unfolded protein response (UPR)-inducible ERO-1 β oxidase (10, 11), the H_2O_2 generated during the process of oxidative protein folding could represent a major source of this reactive oxygen species (ROS). Pancreatic β -cells easily accumulate H_2O_2 and are very prone to H_2O_2 -induced toxicity due to the low expression level of H_2O_2 -detoxifying enzymes such as catalase and glutathione peroxidases (GPx) (12–15). However, only three antioxidative enzymes, GPx7, GPx8, and peroxiredoxin 4 (Prdx4) occur within the ER (16–18). Prdx4 is a typical 2-cysteine peroxiredoxin that utilizes luminal H_2O_2 to reoxidize PDI (19, 20), thus contributing to disulfide formation while preventing H_2O_2 -mediated oxidative damage in the ER. In this respect, it has recently been shown that PDI is more efficiently reoxidized by Prdx4 than by ERO-1 (19) and that Prdx4 complements the ERO1 function (20, 21). In addition, overexpression of Prdx4 in mice resulted in a significant protection of β -cells against streptozotocin-induced diabetes and

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² The abbreviations used are: ER, endoplasmic reticulum; CHOP, CCAAT-enhancer-binding protein homologous protein; DCF, dichlorofluorescein;

ERO-1, ER oxidoreductin 1; Prdx4, peroxiredoxin 4; GPx, glutathione peroxidase; PDI, protein disulfide isomerase; ROS, reactive oxygen species; XBP1, X-box binding protein 1; UPR, unfolded protein response; ANOVA, analysis of variance.

against the progression of nonalcoholic steatohepatitis and insulin resistance in the liver (22, 23). However, the significance of Prdx4 in the disulfide transfer chain promoting efficient oxidative folding of (pro)insulin must still be clarified.

Hence, we investigated the potential role of Prdx4 in improving β -cell function with emphasis on insulin content and secretion during stimulation with glucose and other nutrient secretagogues. Furthermore, we provide evidence that the constitutively low endogenous expression of Prdx4 in insulin-secreting cells is highly susceptible to inactivation in response to high glucose concentrations.

EXPERIMENTAL PROCEDURES

Tissue Culture of Insulin-producing INS-1E Cells—Insulin-secreting INS-1E tissue culture cells (kindly provided by C. Wollheim, University of Geneva Medical Center, Geneva, Switzerland) were cultured in RPMI 1640 medium supplemented with 10 mmol/liter glucose, 10% (v/v) fetal calf serum (FCS), 10 mmol/liter HEPES, 1 mmol/liter sodium pyruvate, 50 μ mol/liter 2-mercaptoethanol, penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO₂ as described previously (24).

Overexpression and Suppression of Peroxiredoxin 4 in INS-1E cells—For stable overexpression of Prdx4, isolated RNA from human HepG2 cells was reverse transcribed with random hexamers and RevertAid H-M-MuLV reverse transcriptase (Fermentas, St. Leon-Rot, Germany). Prdx4 cDNA was amplified using specific hPrdx4 forward (5'-ATGGAGGCGCTGCGCT-3') and hPrdx4 reverse (5'-TCAATTCAGTTTATCGAAATACTTTCAGCTT-3') primers. The PCR product was subsequently subcloned into the EcoRV restriction site of the pLenti 6.3/V5-MCS plasmid for lentiviral production. The PCR reaction was performed with the Phusion proofreading polymerase (New England Biolabs), and all constructs were verified by sequencing as described previously (25). Two short hairpin RNAs (shRNA) directed against rat Prdx4 mRNA and a non-targeting shRNA as control were generated by Sirion Biotec (Sirion Biotec GmbH, Munich, Germany).

Lentivirus Preparation and Transduction of INS-1E Cells—For stable transduction of INS-1E cells with the hPrdx4 and shRNA constructs (shRNA 275 and shRNA 477), lentiviruses were prepared as described previously in detail (26). Briefly, 4 \times 10⁶ 293T cells were transfected with the packaging plasmid pPAX2 (11.25 μ g), the envelope plasmid pcDNA-MD6 (3.75 μ g), and the transfer plasmids pLenti6.3/V5-MCS-hPrdx4, pcDH-shRNA-rPrdx4-275, and pcDH-shRNA-rPrdx4-477 (15 μ g) by calcium phosphate precipitation. 48 h after transfection, the lentiviral particles were harvested from the culture medium and purified by ultrafiltration columns at 4 °C and 3,000 \times g for 25 min (Amicon Ultra Ultracel-100K; Millipore, Schwalbach, Germany). INS-1E cells were seeded at a density of 1 \times 10⁵ cells per well onto a six-well plate and allowed to attach for 24 h before transfection with purified lentiviral particles. After 16 h of infection, the viral supernatant was replaced by fresh medium. Cells were selected for hPrdx4 expression by zeocin (250 μ g/ml) (Invitrogen) and for shRNAs (shRNA 275 and shRNA 477) using puromycin (0.25 μ g/ml) (InvivoGen).

Immunofluorescence Staining—Immunofluorescence staining was performed as described previously (25). Briefly, INS-1E cells overexpressing Prdx4 were seeded overnight at a density of 1 \times 10⁵ cells per well on four-well LabTek chamber slides (Nunc, Roskilde, Denmark). Thereafter the cells were washed twice with PBS and fixed with 4% paraformaldehyde overnight at 4 °C. After washing, the cells were permeabilized and blocked with PBS containing 0.2% Triton X-100 and 1% BSA. The cells were incubated with primary antibodies (anti-PDI, ab5484, diluted 1:100, Abcam, Cambridge, UK, and anti-Prdx4, diluted 1:100, R&D Systems, Minneapolis, MN) 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 nmol/liter 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, Hamburg, Germany), and microscopic images were post-processed using AutoDeblur and AutoVisualize (Autoquant Imaging).

Western Blot Analysis—Whole cell extracts were prepared in radioimmune precipitation assay buffer according to the manufacturer's recommendation (Sigma) supplemented with complete protease inhibitor mixture (Roche Diagnostics, Mannheim, Germany). Protein content was determined by the BCA assay (Thermo Fisher Scientific, Rockford, IL). 20 μ g of total protein were separated by a 12.5% SDS-PAGE and electroblotted to polyvinylidene fluoride membranes. Nonspecific binding sites of the membranes were blocked with 5% nonfat dry milk for 1 h at room temperature. The membranes were incubated with specific primary antibodies overnight at 4 °C. The following antibodies were used: anti-Prdx4 (diluted 1:250), anti-Prdx-SO₃ (ab16830, diluted 1:2000), and anti- β -actin (sc-1615, diluted 1:250, Santa Cruz Biotechnology, Santa Cruz, CA). 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). The protein band intensity was quantified related to β -actin through densitometry with the Gel-Pro Analyzer program (version 6.0, Media Cybernetics, Silver Spring, MD).

Alkylation of Free Thiols by N-Ethylmaleimide—To prevent disulfide exchange reactions during protein preparation whole cell extracts were lysed in the presence of 10 mmol/liter thiol-alkylating agent N-ethylmaleimide (27) (Sigma-Aldrich). Thereafter, total proteins were subjected to Western blot analysis as described above.

Real-time Quantitative RT-PCR—Total RNA from INS-1E cells was isolated using the Qiagen RNeasy Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For cDNA synthesis, random hexamers were used to prime the reaction of the RevertAidH-M-MuLV reverse transcriptase (Fermentas). The reactions were performed using the Quanti-

Tect SYBR Green technology (Qiagen) and the ViiA 7 real-time PCR system (Invitrogen). Samples were first incubated for 5 min at 50 °C and then denatured at 95 °C for 10 min followed by 40 PCR cycles comprising of a melting step at 95 °C for 30 s, an annealing step at 60 °C for 30 s, and an extension step at 72 °C for 30 s. Primers for quantitative RT-PCR were used at an annealing temperature of 60 °C and gave an amplicon of 79–200 base pairs. The optimal parameters for the PCR reactions were empirically established, and the purity and specificity of the amplified PCR product in each experiment was verified by melting curves. All analyzed transcripts showed C_t values, which were at least 10 C_t values lower than the blank values. Each measurement was performed in triplicate. The housekeeping genes β -actin, peptidylprolyl isomerase A, and ribosomal protein L32 were used for normalization. Data analysis was performed with qBasePLUS (Biogazelle, Zulte, Belgium).

Assessment of Cell Viability after H_2O_2 Treatment—INS-1E cells were seeded at 40,000 cells per well in 100 μ l of culture medium onto 96-well plates and allowed to attach for 24 h before they were incubated with different H_2O_2 concentrations. The cells were incubated with H_2O_2 in a concentration range from 1–50 μ mol/liter for 2 h in 20 mmol/liter HEPES-supplemented Krebs-Ringer bicarbonate medium with 5 mmol/liter glucose, and after removal of the H_2O_2 containing medium for another 22 h in fresh medium. Cell viability was determined thereafter by a microplate-based MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma-Aldrich) (28).

Assessment of ER Luminal ROS Generation by Flow Cytometry—The intracellular ROS generation was assessed using dichlorodihydrofluorescein diacetate (Invitrogen), a chemically reduced, acetylated form of fluorescein (29). INS-1E cells were seeded at a density of 1×10^6 cells per well in a six-well plate and allowed to attach for 24 h, before they were preincubated with 10 μ mol/liter DCF for 30 min. Thereafter, the DCF-containing medium was removed, and cells were treated with DTT at a final concentration of 5 mmol/liter for different times. Thereafter, the cells were trypsinized and collected by centrifugation at $700 \times g$ for 5 min. After washing with ice-cold PBS, 20,000 cells were analyzed by flow cytometry (CyFlow ML, Partec, Münster, Germany) using the FL-1 channel (488 nm excitation/520 nm emission). Data were analyzed by FlowJo software (Tree Star, Ashland, OR).

Assessment of Insulin Secretion—Modified INS-1E cells were seeded at a density of 0.5×10^5 cells per well in a six-well plate and allowed to grow for 48 h. Then, the cells were pre-incubated for 1 h in bicarbonate-buffered Krebs-Ringer solution without glucose, supplemented with 0.1% albumin, and thereafter stimulated for 2 h with 3, 10, or 30 mmol/liter glucose or with leucine (10 mmol/liter) plus glutamine (2 mmol/liter). After incubation, the medium was removed and gently centrifuged to remove detached cells. Secreted insulin in the supernatant fraction was determined by radioimmunoassay using rat insulin as standard, and the resulting values were normalized to DNA content. For determination of insulin content, cells were homogenized by sonication in phosphate-buffered saline and measured by radioimmunoassay in the homogenization buffer.

Assessment of Cell Proliferation—The proliferation rate of INS-1E cells was quantified by the colorimetric 5-bromo-2-deoxyuridine (BrdU) cell proliferation ELISA (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's manual.

Data Analysis—Data are expressed as means \pm S.E. 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).

RESULTS

Stable Modulation of Prdx4 Expression in Insulin-secreting INS-1E Cells—To evaluate the role of the ER-specific antioxidative enzyme Prdx4 on β -cell function and survival, we established stable INS-1E cell lines in which the Prdx4 expression was either overexpressed or knocked down. For knockdown of Prdx4 expression, lentiviral constructs encoding two different shRNAs against Prdx4 were used. In addition, Mock-transfected cells were generated to control for pleiotropic effects (Fig. 1A).

Immunoblotting revealed that cells stably transduced with a lentiviral construct carrying human Prdx4 exhibited a significant increase in the hPrdx4 expression level (576%), whereas both shRNAs directed against Prdx4 led to a marked decrease in endogenous Prdx4 expression (shRNA 275, 56%; shRNA 477, 78%) compared with non-modified or Mock-transfected INS-1E cells (Fig. 1A). Co-immunostaining of Prdx4 overexpressing cells with PDI, an ER-specific protein, and DAPI, a nuclear probe, revealed that Prdx4 was exclusively localized in the ER (Fig. 1B).

Effects of Prdx4 Overexpression or Knockdown on ER Stress, Homeostasis, and Cell Proliferation Rate—To determine whether modulation of Prdx4 expression itself results in an activation of UPR and alteration of ER redox homeostasis, the expression of two primary UPR components CHOP and XBP1 spliced and two of the oxidative ER folding machinery enzymes PDI and ERO-1 β was examined in all generated cell lines. As shown in Fig. 2A, neither overexpression nor knockdown of Prdx4 had an effect on PERK (double-stranded RNA activated protein kinase (PKR)-like endoplasmic reticulum kinase) or inositol-requiring enzyme 1, representing the most conserved UPR signaling pathway. The expression level of the two transcription factors CHOP and XBP1 spliced was comparable with that in Mock-transfected cells (Fig. 2A). Moreover, the expression of PDI and ERO-1 β (Fig. 2B) and the cell proliferation rate (Fig. 2C) were not affected by stable modulation of Prdx4 expression, indicating that modulation of Prdx4 expression did not induce ER stress nor altered the ER homeostasis and also had no deleterious effect on cell growth.

Effects of Prdx4 Overexpression or Knockdown on H_2O_2 Toxicity—Prdx4 is the only ER-resident typical two-cysteine peroxidase with antioxidative properties. To analyze this capability, Mock-transfected cells and cells stably overexpressing or suppressing Prdx4 were treated with increasing H_2O_2 concentrations, and the cell viability was measured. As shown in Fig. 3, exogenously added H_2O_2 induced a significant concentration-dependent loss of cell viability in control and Prdx4 knockdown (shRNA 274 and shRNA 477) cells. Overexpression of Prdx4

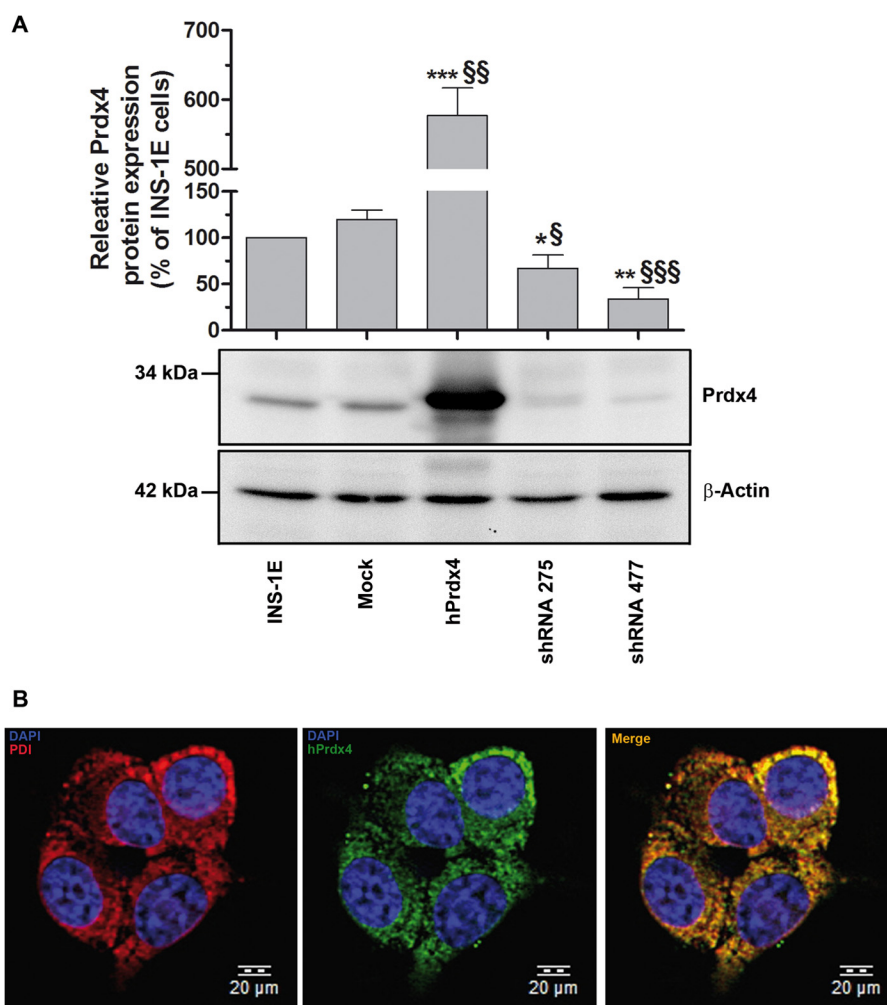


FIGURE 1. Stable modulation of peroxiredoxin 4 (Prdx4) expression in insulin-secreting INS-1E cells. *A*, Prdx4 expression in unmodified (INS-1E), Mock-transfected (Mock), Prdx4-overexpressing (hPrdx4), and knockdown (shRNA 275 and shRNA 477) INS-1E cells was quantified by immunoblot analysis and normalized to β -actin as housekeeping protein. Prdx4 protein expression in unmodified INS-1E was set as 100%. A representative blot is shown for Prdx4 expression. Data are means \pm S.E. of four independent experiments; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with unmodified INS-1E; §, $p < 0.05$; §§, $p < 0.01$; §§§, $p < 0.001$ compared with Mock cells (ANOVA/Dunnett's test; Mock versus hPrdx4, *t* test, unpaired, two-tailed). *B*, subcellular localization of Prdx4 overexpressed in INS-1E cells. INS-1E cells stably overexpressing Prdx4 were seeded overnight on LabTek chamber slides. After fixation and permeabilization, the cells were stained with specific antibodies for the ER-specific PDI (red) and for Prdx4 (green) followed by counterstaining with DAPI (blue). When hPrdx4 occurs within the ER, the green (hPrdx4) and red (PDI) colors merge, and yellow appears in the overlay image (merge).

provided significant protection against H_2O_2 -mediated toxicity (Fig. 3). The half-maximal effective concentration (EC_{50}) of H_2O_2 toxicity was ~ 30 μ mol/liter in Mock-transfected and Prdx4 knockdown cells. In Prdx4-overexpressing cells, the EC_{50} value of H_2O_2 was increased to 45 μ mol/liter and was thus significantly ($p < 0.01$) higher than in Mock-transfected cells, indicating that the reduced H_2O_2 toxicity after the overexpression of the ER-specific Prdx4 is due to its ability to eliminate H_2O_2 .

Effects of Prdx4 Overexpression or Knockdown on DTT-induced H_2O_2 Generation—As an independent approach for verification of the antioxidative effect of Prdx4 in the ER, Mock-transfected cells and cells stably overexpressing or suppressing Prdx4 were exposed to DTT to generate H_2O_2 within the ER. It is known that ERO-1 preferentially oxidizes DTT, thereby producing equimolar H_2O_2 (7, 30). Exposure of Mock-transfected cells to 5 mmol/liter DTT resulted in a significant generation of ROS, determined by DCF, within 15 min and persisted for up to 60 min (Fig. 4). As in Mock-transfected cells, the DTT-induced

DCF oxidation was also observed in Prdx4-suppressing cell lines. However, overexpression of Prdx4 significantly reduced DTT-mediated ROS generation (Fig. 4).

Effects of Prdx4 Overexpression or Knockdown on Insulin Content and Secretion—Having demonstrated that lentivirus-induced overexpression of Prdx4 is specifically localized within the ER and efficiently detoxifies H_2O_2 , we next investigated the effects of Prdx4 overexpression on insulin secretion in response to glucose (3, 10, or 30 mmol/liter) or leucine (10 mmol/liter) plus glutamine (2 mmol/liter). Stimulation of Mock-transfected INS-1E cells with glucose resulted in a significant 3-fold increased rate of insulin secretion compared with basal glucose (3 mmol/liter; Fig. 5A). Leucine (10 mmol/liter) together with glutamine (2 mmol/liter) caused a four times higher insulin secretion than at basal glucose concentration (3 mmol/liter; Fig. 5A). In Prdx4-overexpressing INS-1E cells, basal insulin secretion at 3 mmol/liter glucose was not significantly increased compared with Mock-transfected cells (Fig. 5A). However, at higher glucose concentrations (10 and 30 mmol/

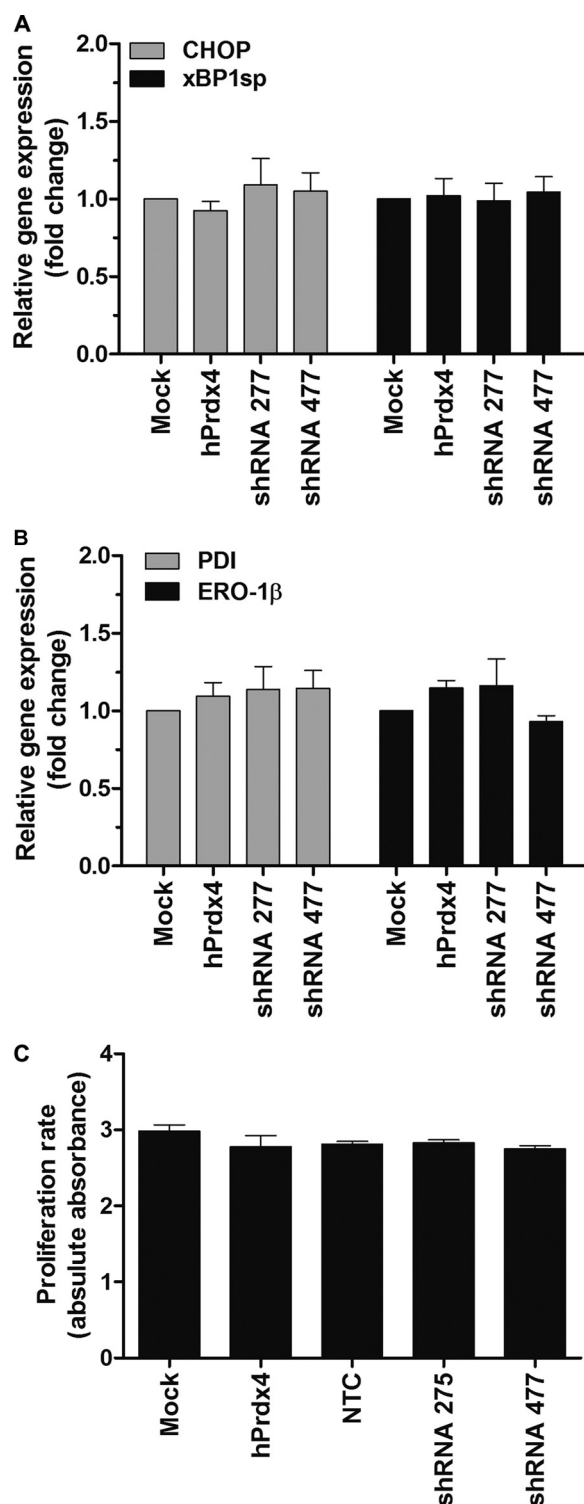


FIGURE 2. Overexpression or knockdown of Prdx4 does not induce ER stress, change ER homeostasis, or affect proliferation rate in insulin-secreting INS-1E cells. A, gene expression of the ER stress markers CHOP and spliced xBP1 (xBP1sp) in INS-1E stably overexpressing and suppressing Prdx4. B, gene expression of the ER-specific folding catalysts PDI and ERO-1 β in INS-1E stably overexpressing and suppressing Prdx4. Total RNA was isolated 24 h after cell culture and analyzed by quantitative RT-PCR with gene specific primer sets. The relative expression levels were normalized to the housekeeping genes β -actin, peptidylprolyl isomerase A, and ribosomal protein L32. Data are means \pm S.E. of three independent experiments. C, proliferation rate in INS-1E cells stably overexpressing, suppressing Prdx4, and in cells expressing non-targeting (NTC) shRNA as control. INS-1E cells were seeded onto 96-well plates and allowed to attach overnight. Thereafter, the proliferation

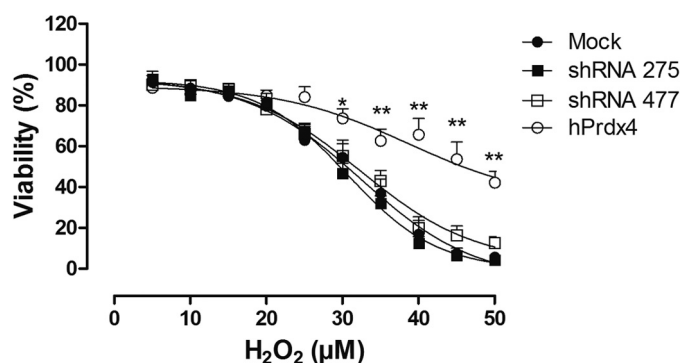


FIGURE 3. Overexpression of Prdx4 protects insulin-secreting INS-1E cells against H₂O₂-induced toxicity. Mock-transfected cells (filled circles), Prdx4 overexpressing (hPrdx4, open circles), and knockdown cells (shRNA 275, filled squares; shRNA 477, open squares) were incubated under control conditions and with H₂O₂ at concentration ranging from 1–50 μ M/liter for 2 h in Krebs-Ringer medium and after removal of the H₂O₂-containing medium for another 22 h in fresh culture medium without H₂O₂. Thereafter, cell viability was determined by the MTT assay and expressed as a percentage of untreated cells. Data are means \pm S.E. of six independent experiments; *, $p < 0.05$; **, $p < 0.01$, compared with cells incubated under the same conditions (t test, unpaired, two-tailed).

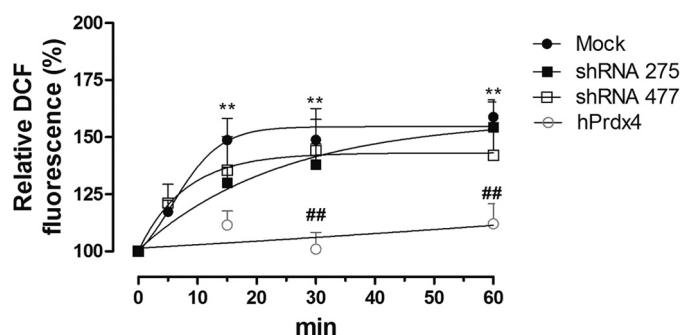


FIGURE 4. Overexpression of Prdx4 prevents DTT-mediated ROS generation in insulin-secreting INS-1E cells. Mock-transfected cells (filled circles), Prdx4 overexpressing (hPrdx4, open circles), and knockdown cells (shRNA 275, filled squares; shRNA 477, open squares) were loaded with 10 μ M/liter of DCFH-H2 dye for 30 min and then cultured with 5 mmol/liter DTT for the indicated times. Thereafter, the intracellular DCF fluorescence intensity was measured by flow cytometry. Data are means \pm S.E. of four independent experiments; **, $p < 0.01$ compared with cells incubated under control conditions (ANOVA/Dunnett's test); ##, $p < 0.01$ compared with cells incubated at the same time point (t test, unpaired, two-tailed).

liter), insulin secretion was significantly increased in Prdx4-overexpressing cells compared with Mock-transfected cells (Fig. 5A). An augmented insulin secretion could also be observed in Prdx4-overexpressing cells incubated with leucine (10 mmol/liter) plus glutamine (2 mmol/liter), indicating that the stimulatory effect of Prdx4 overexpression on insulin secretion was achieved also by other nutrient secretagogues (Fig. 5A). In addition, the insulin content was assessed in both Mock-transfected and Prdx4-overexpressing cells. Insulin content was significantly higher in INS-1E cells stably overexpressing Prdx4 than in Mock-transfected cells at all glucose concentrations tested (Fig. 5B). Although insulin content was decreased in the Prdx4-overexpressing cells after exposure of leucine (10 mmol/liter) plus glutamine (2 mmol/liter), the remaining insu-

rate was quantified by a BrdU-specific ELISA and expressed as a percentage of the proliferation rate of Mock-transfected cells. Data are means \pm S.E. of four independent experiments.

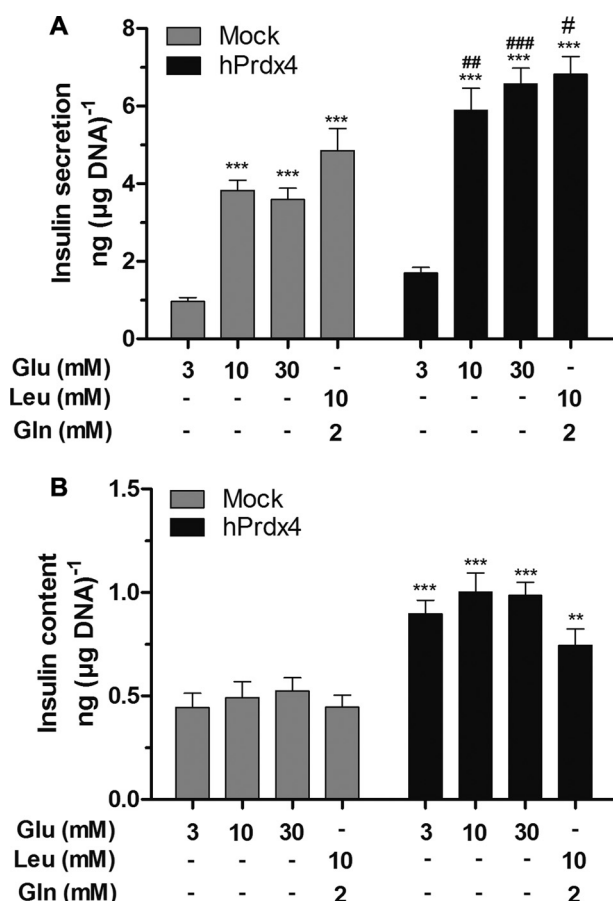


FIGURE 5. Overexpression of Prdx4 enhances insulin content and glucose-induced insulin secretion in insulin-secreting INS-1E cells. INS-1E control cells (gray bars) and cells overexpressing Prdx4 (black bars) were seeded 48 h before stimulation with secretagogues. After starvation at zero glucose for 1 h, cells were incubated with 3, 10, or 30 mmol/liter glucose or leucine (10 mmol/liter) plus glutamine (2 mmol/liter) for 2 h. Thereafter, insulin secretion (A) and insulin content (B) were measured by radioimmunoassay. Data are means \pm S.E. of 8–12 independent experiments; **, $p < 0.01$; ***, $p < 0.001$ compared with cells incubated at 3 mmol/liter glucose (Glu; ANOVA/Dunnett's test); #, $p < 0.05$; ##, $p < 0.01$; ###, $p < 0.001$ compared with Mock-transfected cells incubated under the same conditions (t test, unpaired, two-tailed).

lin content was still significantly higher than in Mock-transfected cells (Fig. 5B).

To further characterize the impact of Prdx4 on the insulin secretory capacity of INS-1E cells, insulin secretion and content was assessed also in Prdx4 knockdown cells. In knockdown Prdx4 INS-1E cell lines, both glucose-induced insulin secretion and insulin content were not significantly affected when compared with control cells (Fig. 6, A and B).

Effects of Prdx4 Overexpression or Knockdown on Insulin 1 and Insulin 2 Gene Expression—To determine whether the observed elevated insulin content and the increased glucose-induced insulin secretion might be a result of an improved synthesis of insulin, the mRNA expression level of insulin 1 and insulin 2 genes was quantified. In agreement with the elevated insulin content, the expression of the insulin 1 and insulin 2 was significantly higher in Prdx4-overexpressing cells than in control cells (Fig. 7, A and B), suggesting that overexpression of Prdx4 raises the insulin translation rate in INS-1E cells. However, knockdown of the endogenous Prdx4 expression had no

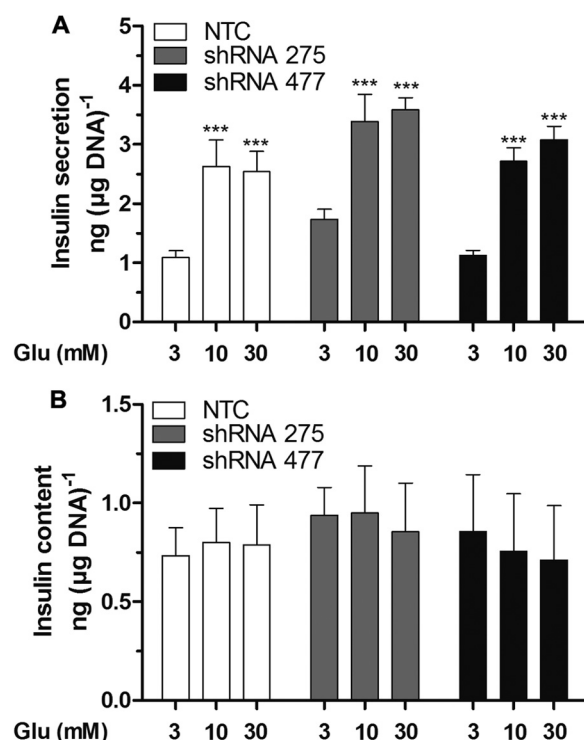


FIGURE 6. Knockdown of Prdx4 does not affect insulin content and glucose-induced insulin secretion in insulin-secreting INS-1E cells. Cells stably transfected with a non-targeting control (NTC) shRNA (open bars) and cells knocking down Prdx4 with shRNA 275 (gray bars) or with shRNA 477 (black bars) were seeded 48 h before incubation with glucose. After starvation for 1 h at zero glucose (Glu), cells were incubated with 3, 10, or 30 mmol/liter glucose for 2 h. Thereafter, insulin secretion (A) and insulin content (B) were determined by radioimmunoassay. Data are means \pm S.E. of six independent experiments; ***, $p < 0.001$ compared with cells incubated at 3 mm glucose (ANOVA/Dunnett's test).

significant influence on transcriptional insulin gene expression when compared with control cells (Fig. 7, C and D).

Prdx4 Gene Expression in Different Rat Tissues and in Insulin-producing Tissue Culture Cells—Because knockdown of Prdx4 had neither beneficial nor deleterious effects on β -cell function and survival, we quantified the endogenous expression of Prdx4 in different rat tissues (Table 1). The gene expression level of Prdx4 in liver was set as 100%. As shown in Table 1, Prdx4 was expressed at a higher level in liver followed by pancreas, testis, and heart muscle. In all other investigated tissues, including pancreatic islets, thymus, spleen, kidney, and intestine, Prdx4 expression was extremely low when compared with that in liver ($< 10\%$; Table 1). Hence, Prdx4 is highly abundant in exocrine pancreas but only negligibly expressed in pancreatic islets.

Effects of Glucose on Prdx4 Sulfenylation—Prdx4 is a typical two-cysteine peroxiredoxin whose peroxidase activity is based on a peroxidatic and resolving cysteine residue. In the presence of H_2O_2 , the peroxidatic cysteine is selectively oxidized to sulfenic acid, which then reacts with an adjacent resolving cysteine to form an intermolecular disulfide bond that is reduced by the thioredoxin/thioredoxin reductase system or PDI (6, 30). However, at very high H_2O_2 concentrations, sulfenic acid can be overoxidized to sulfinic acid or even irreversibly to sulfonic acid (31). Thus, Prdx4 can be easily inactivated by H_2O_2 and thereby lose its antioxidative activity. To determine whether prolonged

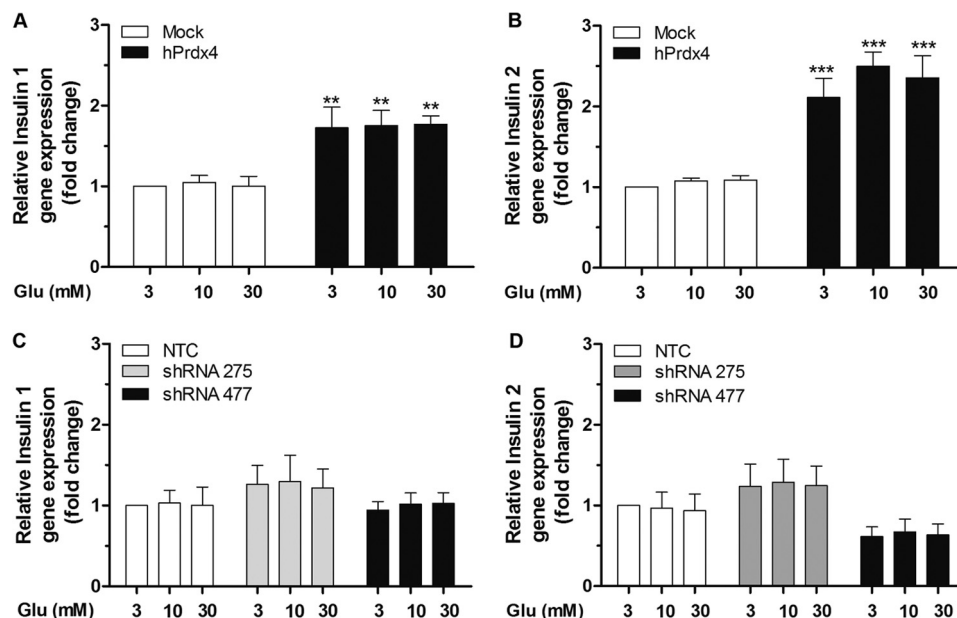


FIGURE 7. Overexpression, but not knockdown of Prdx4 enhances the gene expression of insulin 1 and insulin 2 in insulin-secreting INS-1E cells. Gene expression of insulin 1 (A) and insulin 2 (B) in Mock-transfected (open bars) and Prdx4 overexpressing cells (black bars). Gene expression of insulin 1 (C) and insulin 2 (D) in cells stably transfected with a non-targeting control (NTC) shRNA (open bars) and cells knocking down Prdx4 with shRNA 275 (gray bars) or with shRNA 477 (black bars). All cells were starved for 1 h at zero glucose and then incubated with 3, 10, or 30 mmol/liter glucose (Glu). After 2 h, total RNA was isolated, and gene expression was analyzed by quantitative RT-PCR with insulin 1-specific (A and C) and insulin 2-specific (B and D) primer sets. The relative expression levels were normalized to the housekeeping genes β -actin, peptidylprolyl isomerase A, and ribosomal protein L32. Data are means \pm S.E. of 8–12 independent experiments; **, $p < 0.01$, ***, $p < 0.001$ compared with cells incubated at 3 mmol/liter glucose (ANOVA/Dunnett's test).

TABLE 1

Prdx4 gene expression in rat tissues and in insulin-producing tissue culture cells

Prdx4 expression in different rat tissues and in insulin-producing INS-1E cells was assessed by quantitative real-time RT-PCR and normalized to the housekeeper genes β -actin, peptidylprolyl isomerase A, and ribosomal protein L32. Results are means \pm S.E. with the numbers of experiments given in parentheses. Gene expression of the liver was set as 100%.

Tissue	Peroxisredoxin 4 gene expression (% of liver)
Liver	100 \pm 7 (7)
Pancreas	38 \pm 5 (7) ^a
Testis	29 \pm 4 (6) ^a
Heart muscle	27 \pm 6 (8) ^a
Skeletal muscle	9 \pm 0 (5) ^a
Pancreatic islets	8 \pm 1 (7) ^a
Thymus	7 \pm 1 (7) ^a
Spleen	6 \pm 1 (6) ^a
Kidney	6 \pm 1 (8) ^a
Intestine	5 \pm 0 (5) ^a
INS-1E cells	16 \pm 2 (6) ^a

^a $p < 0.001$ compared with liver (ANOVA/Dunnett's test for multiple comparisons).

high glucose exposure would possibly give rise to an overoxidation of the constitutively poorly expressed Prdx4, INS-1E cells were treated with glucose (3, 10, or 30 mmol/liter) for 24 h. As shown in Fig. 8, exposure of INS-1E cells to glucose (30 mmol/liter) resulted in a significant increase in the level of overoxidized Prdx4 compared with basal glucose (3 mmol/liter), strongly suggesting that Prdx4 is highly susceptible to oxidative inactivation.

DISCUSSION

Oxidative protein folding is crucial for the structural stability and proper function of most secretory and membrane proteins (5, 32). Disulfide bond formation in the ER is accompanied by the formation of equimolar amounts of H_2O_2 as a byproduct

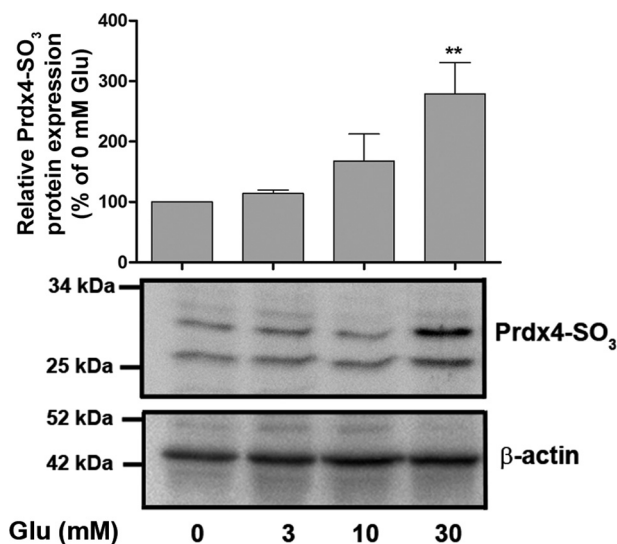


FIGURE 8. High glucose concentrations induce sulfenylation of Prdx4 in insulin-secreting INS-1E cells. INS-1E cells were incubated with different glucose concentrations (3, 10, or 30 mmol/liter) for 24 h. Untreated and treated cells were washed and lysed in the presence of the alkylating agent *N*-ethylmaleimide (20 mmol/liter) to prevent further thiol oxidation. Total protein lysates were resolved by SDS-PAGE, followed by immunoblotting with an antibody that recognizes the sulfonic forms of Prdx4-SO₃. The Prdx4-SO₃ protein expression was normalized to the housekeeping protein β -actin. The protein expression level of Prdx4-SO₃ incubated at 0 mmol/liter glucose (Glu) was set as 100%. Data are means \pm S.E. of five independent experiments. **, $p < 0.01$ compared with cells incubated at 0 mmol/liter glucose (ANOVA/Dunnett's test).

(7). In particular in specialized secretory cells, such as pancreatic β -cells with insulin as the major biosynthetic product accounting for \sim 50% of total protein synthesis (33, 34), the H_2O_2 generated during the oxidative folding of (pro)insulin could be potentially harmful to the β -cell. However, recently, it

has been suggested that exogenously added or enzymatically produced H_2O_2 could contribute either non-enzymatically or via an enzymatic reaction to native disulfide formation (35, 36). This catalytic reaction can be accomplished by three recently described ER-localized peroxidases, Prdx4, GPx7, and GPx8 (16, 19, 20). Among them, Prdx4 is ubiquitously expressed and enriched in highly active secretory tissues (37). Furthermore, Prdx4 exhibits an extraordinary reactivity toward H_2O_2 with a second-order rate constant of $2.2 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (27), indicating that H_2O_2 preferentially reacts with the peroxidatic cysteine within Prdx4 rather than with cysteines in GPx7, GPx8, and PDI or other ER proteins (16, 35). The essential role of disulfide bond formation in assembly and processing of insulin (38) and the ability of Prdx4 to couple H_2O_2 metabolism with disulfide bond formation (19, 20) motivated us to characterize the functional significance of the ER-specific Prdx4 on β -cell function with emphasis on insulin content and secretion during stimulation with nutrient secretagogues.

In the present study, we could show that stable overexpression of the ER-specific Prdx4 in the glucose-responsive INS-1E insulin-secreting cell line provided a significant protection against H_2O_2 -mediated toxicity and successfully prevented the DTT-mediated ROS generation, indicating that Prdx4 overexpression can help to efficiently scavenge H_2O_2 as previously demonstrated for peroxiredoxin I, II, III, and VI (39–41). These findings are in agreement with those recently reported for non-insulin-secreting HEK293 cells (30) and our findings obtained in non-glucose-responsive insulin-producing RINm5F cells (25). Importantly, modulation of Prdx4 expression in insulin-secreting INS-1E cells had no conspicuous effect on ER stress components, folding machinery or cell proliferation, clearly indicating that neither Prdx4 overexpression nor Prdx4 suppression affect the UPR or ER homeostasis in INS-1E cells.

In particular, the protein-folding machinery in the ER of the pancreatic β -cells is extremely challenged due to the high (pro)insulin synthesis rate, which presumably reflects the high insulin demand during insulin resistance in the prediabetic state (1). It has been proposed that such an increase in insulin biosynthesis leads to accumulation of misfolded and unfolded proinsulin in the ER lumen (42, 43) and consequently to the induction of UPR markers such as of the pancreas-specific oxidoreductase ERO-1 β (11, 44), presumably to cope with the increased (pro)insulin folding demand. However, interestingly, Prdx4 is not induced by ER stress (18, 45), indicating that ER stress-mediated induction of the H_2O_2 -generating ERO-1 β could have detrimental effects on insulin biosynthesis and glucose-induced insulin secretion as demonstrated recently (46). In addition to its antioxidative action in the ER, it has recently been shown that Prdx4 plays an important role in the oxidation of PDI proteins (19, 47). Depletion of Prdx4 resulted in a lack of disulfide bond formation and reduced cell viability in the double ERO-1 α /ERO-1 β knock-out mice, indicating that Prdx4 is an important ERO-1-independent disulfide bond catalyst (20). Indeed, Prdx4 overexpression resulted in a significantly higher β -cell insulin content and glucose-induced insulin secretion. The increased insulin content could be the result of improved insulin biosynthesis and optimized oxidative folding because the gene expression of insulin1 and insulin2 was significantly ele-

vated. This β -cell beneficial effect did not only apply to the physiological stimulus glucose but also to the nutrient insulin secretagogue combination of leucine plus glutamine. Thus, this effect is not restricted to glucose.

Because Prdx4 overexpression did not significantly affect insulin secretion at basal glucose concentrations but increased insulin content, we surmised that Prdx4 overexpression solely increased the ER protein folding capacity without affecting the glucose recognition apparatus. This assumption is supported by the observation that Prdx4 overexpression did not affect the expression level of β -cell-specific transcription factors Pdx-1, MafA, and NeuroD (data not shown). However, the mechanism how overexpression of hPrdx4 influences the insulin 1 and 2 mRNA expressions is unclear and remains to be further investigated. Knockdown of Prdx4 expression did not significantly influence H_2O_2 -mediated toxicity and β -cell function, suggesting that either the constitutive expression of Prdx4 as such is very low in INS-1E cells or other parallel ER antioxidative systems could complement Prdx4 as recently proposed in other cell systems (45, 48). Indeed, we found that Prdx4 expression is very high in exocrine pancreas, confirming an earlier observation (49), but only negligibly expressed in pancreatic islets and INS-1E cells when compared with liver.

Despite the important role in antioxidant defense and oxidative protein folding, Prdx4 as a typical two-cysteine peroxiredoxin is susceptible to hyperoxidation (30). In the presence of H_2O_2 , the peroxidatic cysteine is selectively oxidized to sulfenic acid, which then reacts with an adjacent resolving cysteine to form an intermolecular disulfide bond that is reduced by the thioredoxin/thioredoxin reductase system or PDI proteins (6, 30, 50). However, at high H_2O_2 concentrations, sulfenic acid can be overoxidized to sulfinic acid or even irreversibly to sulfonic acid (31). Such an overoxidation has been proposed to result in the inactivation of peroxidase activity and in the formation of higher order molecular aggregates that exhibit protein chaperone function (51). Consistent with those observations, we found that Prdx4 is highly susceptible to oxidative inactivation following prolonged high glucose exposure. This overoxidation could be successfully prevented by specific targeting and expression of a functional catalase in the ER.³

In conclusion, the present results demonstrate that endogenous Prdx4 expression in pancreatic β -cells is rather low and highly susceptible to hyperoxidation in the presence of high glucose. Increasing Prdx4 expression results in an efficient metabolism of luminal H_2O_2 and in a greater insulin secretory capacity due to increased proinsulin mRNA transcription and insulin content.

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