Peroxiredoxin III protects pancreatic β cells from apoptosis

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Abstract

Type 1 diabetes mellitus is characterized by a progressive autoimmune destruction of insulin-producing β cells. Macrophages and T lymphocytes release cytokines, which induce the synthesis of oxygen and nitrogen radicals in the pancreatic islets. The resulting cellular and mitochondrial damage promotes β cell death. β cells are very sensitive to the autoimmune free radical-dependent attack due to their low content of antioxidant enzymes such as glutathione peroxidase and catalase. A focal point of β cell protection should be the control of the mitochondrial redox status, which will result in the preservation of metabolic stimulus-secretion coupling. For this reason, there is a considerable interest in the mitochondrial peroxiredoxin III (PRX III), a thioredoxin-dependent peroxide reductase, which was shown to be able to protect against both oxidative and nitrosative stress.

Using the Tet-On-system, we generated stably transfected rat insulinoma cells over- or under-expressing PRX III in a doxycyclin-dependent manner to analyze the effect of increased or decreased amounts of cellular PRX III, following treatment with several stressors. We provide evidence that PRX III protects pancreatic β cells from cell stress induced by accumulation of hydrogen peroxide, or the induction of inducible nitric oxide synthase or caspase-9 and -3 by pro-inflammatory cytokines or streptozotocin. Basal insulin secretion was markedly decreased in cells expressing lower levels of PRX III. We suggest PRX III may be a suitable target for promoting deceleration or even prevention of stress-associated apoptosis in pancreatic β cells and the manifestation of insulin-dependent diabetes mellitus.

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Introduction

Autoimmune destruction of pancreatic β cells during the development of type 1 diabetes is a complex process involving both cellular and humoral elements of cytotoxicity (Mandrup-Poulsen *et al.* 1990). It is characterized by insulinitis, an infiltration of pancreatic islets with lymphocytes, macrophages and neutrophil granulocytes releasing various pro-inflammatory cytokines (Eizirik & Mandrup-Poulsen 2001). Chronic exposure to cytokines such as tumour necrosis factor- α (TNF- α), interleukin 1 β (IL1 β), and interferon- γ (INF- γ) induces the synthesis of reactive oxygen species (ROS) and nitrogen species (RNS) via inducible nitric oxide synthase (iNOS; Rabinovitch & Suarez-Pinzon 1998, Stirling *et al.* 2005), which inhibit insulin secretion (Kiely *et al.* 2007). Oxygen free radicals, such as $O_2^{\cdot-}$, and nitrogen free radicals, such as $NO^{\cdot}$, may act separately or interact to generate peroxynitrite (e.g. $O_2^{\cdot-} + NO^{\cdot} \rightarrow ONOO^{\cdot}$), which is a powerful oxidant. These radicals can inactivate

mitochondrial and cytosolic proteins, leading to a decrease in oxidative phosphorylation, decreased glycolysis, and consequently decreased ATP levels as well as impaired insulin secretion. The resulting mitochondrial damage promotes β cell death (Azevedo-Martins *et al.* 2003), which *in vivo* will be followed by the manifestation of insulin-dependent diabetes mellitus (IDDM, also known as type 1 diabetes). Antioxidant enzymes such as glutathione-dependent peroxidase (Gpx) and catalase (Cat) are able to inactivate ROS and prevent oxidative stress (Radi *et al.* 1991, Knopp *et al.* 1999). The insulin-producing β cells are very sensitive against autoimmune attack due to their low content of these antioxidant enzymes (Lenzen *et al.* 1996, Tiedge *et al.* 1997). Several groups have attempted to prevent β cell destruction by over-expression of Cat, Gpx, mitochondrial Mn Superoxide dismutase (SOD), cytoplasmic Cu/ZnSOD, or thioredoxin with mixed results (Hotta *et al.* 1998, Bernabe *et al.* 2001, Sandström *et al.* 2002, Azevedo-Martins *et al.* 2003, Gurgul *et al.* 2004, Li *et al.* 2006, Harmon *et al.* 2009, Michalska *et al.* 2010). In animal models,

scavengers of ROS and RNS have been shown to prevent β cell death induced by the diabetogenic drug streptozotocin (STZ) or by the pro-inflammatory cytokines. *In vivo*, over-expression of Cu/ZnSOD in diabetic animals provides protection against the development of alloxan- or STZ-induced diabetes. Moreover, addition of scavenging agents such as SOD, Catalase (Cat), guanidinoethyldisulphide, and metal chelators prior to alloxan, STZ, or cytokine exposure has prevented β cell death in isolated islets (Gandy *et al.* 1982, Abdel-Rahman *et al.* 1992, Suarez-Pinzon *et al.* 2001). Lower concentrations of H_2O_2 , however, seem to be involved in the regulation of physiological processes, e.g. receptor-mediated cell signaling pathways, cell proliferation, and transcriptional activation (Pi *et al.* 2007).

We have focused our work on the role of peroxiredoxins (PRXs) in protection from oxidative stress. In contrast to other antioxidant enzymes, insulin-producing cells are not compromised with respect to PRXs. PRXs comprise a family of highly conserved thioredoxin-dependent peroxide reductases (Chae *et al.* 1999, Kang *et al.* 2005), the only inducible antioxidant enzymes, which are known to protect against both oxidative and nitrosative stress (Hattori *et al.* 2003). PRXs are small proteins, which are able to form homo- and heterodimers as well as oligomers. They possess a highly conserved N-terminal Cys-residue. This sulfhydryl group enables the peroxidase activity, which results in disulfide bond formation, intramolecular or between two PRXs, and is followed by regeneration via thioredoxin. The function of mammalian PRXs is linked to the modulation of cytokine-induced H_2O_2 levels, which have been shown to mediate signaling cascades leading to cell proliferation, differentiation, and apoptosis. In addition, they play an important role in peroxide detoxification in cells in cooperation with glutathione peroxidases and catalases (Wood *et al.* 2003).

A focal point of β cell protection should be the control of the mitochondrial redox status. For this reason, there is a considerable interest in PRX III, which is localized in the mitochondria and uses the mitochondrial thioredoxin-2 as electron donor (Watabe *et al.* 1997, Araki *et al.* 1999). Numerous synonyms, such as AOP-1 for antioxidant protein 1 (Mitsumoto *et al.* 2001), mer5 because it was originally isolated from murine erythroleukemia cells (Yamamoto *et al.* 1989), or SP-22 as substrate protein for mitochondrial ATP-dependent protease (Lee *et al.* 2007), have been used to describe this multifunctional enzyme. *Prx III* can be induced by various stimuli (Araki *et al.* 1999, Nonn *et al.* 2003, Lee *et al.* 2007). It is able to regulate the physiological H_2O_2 concentration within the cells and to prevent apoptosis (Nonn *et al.* 2003). Chang *et al.* (2004) postulated that PRX III protects cardiolipin from lipid peroxidation. Cardiolipin is an anionic phospholipid, which binds cytochrome *c* on the inner mitochondrial membrane. If cardiolipin is peroxidized by $\cdot OH$, cytochrome *c* is released from mitochondria to the cytosol, followed by the activation of specific caspases leading to apoptotic cell death (Petrosillo *et al.* 2003). We suggest that

PRX III could be a useful target to prevent cell death induced by oxidative stress and thus could prevent or at least delay the onset of the clinical symptoms of type 1 diabetes. To investigate whether this enzyme is able to protect insulin-producing cells against inflammatory stimuli while maintaining the functionality of these cells, we generated and analyzed stably transfected rat insulinoma cells (RINm5F) over- or under-expressing *Prx III* in a doxycyclin-dependent manner, using the Tet-On-system, which we have previously demonstrated to be important for analysis of the functional role of Pdx-1 (Lottmann *et al.* 2001).

Materials and Methods

Animals and materials

C57/BL6 mice used for islet preparation were purchased from the Department of Pathophysiology of the Greifswald University (Greifswald, Germany). Rat insulinoma cells RINm5F were a kind gift from Sigurd Lenzen from the Medical School Hannover. Mouse insulinoma cells β TC6-F7 were from Shimon Efrat (Tel Aviv, Israel). Collagenase type IV and mouse antibody against glucagon or monoclonal rabbit antibody against iNOS were obtained from Sigma-Aldrich. Monoclonal mouse antibody against insulin was obtained from Chemicon (Terneucula, CA, USA). The rabbit polyclonal antibody directed against GAPDH was purchased from Abcam (Cambridge, UK). A rabbit polyclonal antibody directed against PRX III from rat or mouse was generated by SeqLab (Göttingen, Germany). Monoclonal rabbit antibody against cleaved poly (ADP-ribose) polymerase (PARP) was purchased from Cell Signaling (Danvers, MA, USA). The secondary antibodies anti-mouse-Cy2 and anti-rabbit-Cy3 were obtained from Dianova (Hamburg, Germany). Taq polymerase was from GE Healthcare (Munich, Germany). Cytokines were obtained from Roche. STZ was from Calbiochem Novabiochem (Nottingham, UK), and alloxan was from ICN (Northeim, Germany). Doxycyclin was purchased from BD Biosciences (Heidelberg, Germany). Genitcin G418 was obtained from PAA (Pasching, Austria). All other chemicals were from commercially available sources.

Immunohistochemistry

Paraffin slices of pancreas from Balb/c mice were successively incubated for 5 min in xylol, 100% (v/v), 95% (v/v), and 75% (v/v) ethanol, double distilled water and 30 min in immunofluorescence buffer (0.2% (w/v) BSA, 0.05% (w/v) saponin, 0.1% (w/v) sodium azide in PBS, pH 7.4) followed by an overnight incubation at 4 °C with polyclonal rabbit anti-PRX III and monoclonal mouse anti-glucagon or anti-insulin antibody diluted 400-fold in IF buffer. After washing with IF buffer, the immunoreacted primary antibodies were visualized with red fluorescent Cy3-conjugated goat anti-rabbit IgG and green fluorescent Cy2-conjugated goat anti-mouse IgG,

diluted 600-fold in IF buffer, by incubation for 2 h at room temperature in darkness. After washing with IF buffer again, the slices were covered with DAKO Fluorescent Mounting Medium and observed by fluorescent microscopy (400 $\times$ magnification) with an IX-70 microscope (Olympus Europa GmbH, Hamburg, Germany). Control staining with preimmune sera gave no immunoreactive signals.

Cell culture

Cells were propagated in RPMI-1640 medium (rat insulinoma cells RINm5F) or DMEM (mouse insulinoma cells β TC6-F7) supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin. The cells were cultured at 37 °C in a humidified atmosphere containing 95% (v/v) air and 5% (v/v) CO₂.

Isolation of pancreatic islets

The isolated pancreas from 3-month-old male or female C57/BL6 mice were inflated with Gey-Gey buffer (6.5 g/l NaCl, 2.27 g/l NaHCO₃, 0.37 g/l KCl, 0.212 g/l MgCl₂ 6H₂O, 0.74 g/l MgSO₄·7H₂O, 0.421 g/l Na₂HPO₄, 0.04 g/l KH₂PO₄, and 1.5 mg/l CaCl₂ aerated with CO₂/O₂ for 20 min) and were chopped into small pieces. After a short centrifugation (300 g for 2 s), the pellet was resuspended in Gey-Gey buffer containing 2 mg collagenase XI (Sigma) per pancreas and was shaken in a water bath at 37 °C followed by a subsequent centrifugation step (300 g for 5 s). The pelleted islets were suspended in fresh buffer, picked, and cultured for 24 h in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, 100 μ g/ml streptomycin and with or without cytokines (250 U/ml TNF- α , 2.5 U/ml IL1 β , and 125 U/ml IFN- γ) at 37 °C in a humidified atmosphere containing 95% (v/v) air and 5% (v/v) CO₂.

Semi-quantitative reverse transcription-PCR

Total RNA was isolated using a commercial kit from Qiagen. cDNA was prepared by reverse transcription (RT) of 2 μ g total RNA using 0.5 μ g BamTT-primer (3'-CGC GGA TCC TTT TTT TTT TTT TTT TTT-5') and SuperScript II reverse transcriptase. *Prx III* was amplified with Taq DNA polymerase in a DNA thermal cycler (Eppendorf, Germany) using the following gene-specific primers: *Prx III* for: 5'-TAG CTA GCT AGA ATT CCT TGC GTG CTC TGA AGA TGG-3' and *Prx III* rev: 5'-TAG CTA GCT AGG ATC CGC CTA CTG ATG GAC CTT CTC-3' with an expected product of 791 bp. The conditions for each cycle were 30 s at 94 °C, 40 s at 57 °C and 60 s at 72 °C (25 cycles).

Tet-On-system

PCR products containing amplified complete cDNA of *Prx III* were inserted into pUHD10-3 (Clontech) at the EcoRI

and BamHI restriction sites. For the PCR, the primers *Prx III* for EcoRI (5'-TAG CTA GCT AGA ATT CCT TGC GTG CTC TGA AGA TGG-3') and *Prx III* rev BamHI (5'-TAG CTA GCT AGG ATC CGC CTA CTG ATG GAC CTT CTC-3') were used for sense orientation, while *Prx III* for BamHI (5'-TAG CTA GCT AGG ATC CCT TGC GTG CTC TGA AGA TGG-3') and *Prx III* rev EcoRI (5'-TAG CTA GCT AGA ATT CGC CTA CTG ATG GAC CTT CTC-3') were used for antisense orientation.

From both the constructs, a 1573 bp fragment, containing the complete transcriptional unit, was cut out by digestion with XhoI and KasI. After a fill in of the 5'-overhangs, it was inserted into the vector pUHD172-1neo with blunt end (Clontech) at PvuII restriction site. The neo cassette of pUHD172-1neo was removed before and inserted again after this cloning step using XhoI. For stable transfection of RINm5F cells with Lipofectamine 2000 (Invitrogen), the construct was linearized with BspHI. The transfected cells were selected using 500 μ g/ml G418. The expression of *Prx III* mRNA in sense or antisense orientation was induced by 1 μ g/ml doxycyclin for 4 days. In preliminary studies, the expression of the Tet repressor or the time-dependent induction of the *Prx III* construct was detected by northern blots (data not shown).

Western blot

RINm5F cells were scraped with 100 μ l lysis buffer (25 mmol/l Tris-HCl pH 7.4, 50 mmol/l NaF, 0.1 mol/l NaCl, 5 mmol/l EGTA, 1 mmol/l EDTA, 10 mmol/l Na₄P₂O₇, 1% (v/v) Triton X-100, 9.2% (w/v) sucrose, and protease inhibitors), shock frozen in liquid nitrogen, and centrifuged (5 min, 13 400 g, 4 °C).

Isolated islets were lysed by the addition of 50 μ l RIPA buffer (50 mmol/l Tris-HCl, pH 7.4; 1% (v/v) NP-40; 0.25% (w/v) Na deoxycholate; 150 mmol/l NaCl; 1 mmol/l EDTA; and protease inhibitors) to 100 islets and were frozen in liquid nitrogen, followed by a centrifugation step after thawing.

Proteins (10 or 20 μ g) were separated by 12.5% (w/v) SDS-PAGE and electroblotted onto nitrocellulose membranes using a semidry blotter. The blot was blocked with Roti-Block (Roth, Karlsruhe, Germany) for 1 h at room temperature and subsequently incubated in a 1:2000 dilution of a polyclonal rabbit anti-PRX III or anti-GAPDH antiserum in IF buffer with 5% (w/v) BSA, 0.05% (w/v) saponin, 0.1% (w/v) sodium azide in PBS, pH 7.4 in a 1:10 000 dilution of monoclonal rabbit anti-iNOS antibody (Sigma-Aldrich) or 1:1000 dilution of monoclonal rabbit antibody directed against cleaved PARP-1 (Cell Signaling Technology). After washing the blot with PBS/Tween 0.05% (v/v), a HRP-conjugated goat anti-rabbit IgG (1:10 000) was used as secondary antibody. Detection of bound antibody was performed with LumiGLO reagent and peroxide (Cell Signaling Technology).

Intracellular oxidant production

Cells (7×10^4) grown in 35 mm dishes were treated for 4 days with/without 1 $\mu\text{g/ml}$ doxycyclin and for 1 h with 1 mmol/l H_2O_2 . The cells were trypsinized and incubated for 2 h with 20 μM DCFH-DA (Molecular Probes/Invitrogen Darmstadt, Germany) in 300 μl PBS at 37 °C. 2',7'-dichlorofluorescein-diacetate (DCFH-DA) is permeable, and it is cleaved by endogenous esterases to DCFH. The intracellular oxidation of DCFH by ROS generates the green fluorescent DCF, which is not further permeable and can be analyzed by a FACScan, measuring the emission at 530 nm. The fluorescence intensity is proportional to the amount of intracellular ROS.

Mitochondrial transmembrane potential ($\Delta\psi_m$)

Cells (7×10^4), grown in 35 mm dishes, were treated for 4 days with/without 1 $\mu\text{g/ml}$ doxycyclin and for 48 h with a mix of 100 U/ml TNF- α , 50 U/ml IL1 β , and 100 U/ml IFN- γ . The trypsinized cells were centrifuged (400 g) together with the supernatant to harvest all the cells, adherent and detached. Next, the supernatant was aspirated, and the cells were resuspended in 1 ml medium with 50 nM 3,3'-dihexyloxycarbocyanine iodide (DiOC₆(3); Mobitec SA, Eupen, Belgium) and incubated for 30 min at 37 °C. After incubation time, the cells were centrifuged (1 min, 500 g), resuspended in 300 μl PBS, and analyzed (at least 10 000 cells) using a FACSCalibur and CellQuest software. Data were gated to exclude debris.

Activation of caspase-3 and -9

Cells (7×10^4) grown in 35 mm dishes were treated for 4 days with/without 1 $\mu\text{g/ml}$ doxycyclin and for 9 h with 100 U/ml TNF- α , 50 U/ml IL1 β , 100 U/ml IFN- γ , 4 mmol/l STZ, or 4 mmol/l alloxan. The trypsinized cells were centrifuged (400 g) together with the supernatant to harvest all the cells, adherent and detached. The sediment was resuspended in 100 μl lysis buffer (10 mmol/l Tris-HCl, 10 mmol/l NaH_2PO_4 , 10 mmol/l $\text{Na}_4\text{P}_2\text{O}_7$, 130 mmol/l NaCl, and 1% (v/v) Triton X-100) before the addition of 130 μl caspase buffer (20 mmol/l HEPES pH 7.5, 0.1 M NaCl, 2 mmol/l dithiothreitol, 10% (v/v) glycerol, 25 $\mu\text{g/ml}$ caspase substrate). We used Ac-DEVD-AFC (Bachem, Heidelberg, Germany) as substrate for caspase-3 and Ac-LEHD-AFC (Bachem) for caspase-9. After an incubation step for 2 h at 37 °C, the release of fluorescent AFC (390 nm excitation and 510 nm emission) by activated caspases was determined using a Wallac Victor fluorescence photometer (Perkin Elmer, Waltham, MA, USA).

RIA

Insulin secretion was analyzed from 3×10^6 wild-type or stably transfected RINm5F cells treated for 4 days with/without 1 $\mu\text{g/ml}$ doxycyclin in 2 ml RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml

penicillin, 100 $\mu\text{g/ml}$ streptomycin using a rat insulin RIA kit (Linco Research, St Charles, MO, USA) according to the manufacturer's protocol. The samples were counted by a multi-crystal gamma counter LB2104 (Berthold) for 1 min.

ELISA

Glucose-stimulated insulin secretion was analyzed by using the Mercodia Ultrasensitive Insulin ELISA kit (Mercodia, AB, Uppsala, Sweden). Briefly, wild-type or stably transfected RINm5F cells were seeded into 24-well plates (3×10^5 cells/well per 2 ml) and cultured for 4 days with 1 $\mu\text{g/ml}$ doxycyclin in 2 ml RPMI-1640 medium containing 3.3 mmol/l glucose and supplemented with 10% (v/v) FCS. The cells were cultured in the presence or absence of 80 $\mu\text{mol/l}$ H_2O_2 for a further 24 h. Thereafter, the medium was replaced by 1 ml RPMI containing either 3.3 or 20 mmol/l glucose, and after 2 h, the cell-free supernatants were collected for determination of insulin secreted into the medium.

Cell viability

Cell viability was determined by applying the LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes) following the instructions of the manufacturer. Briefly, wild-type or stably transfected RINm5F cells were seeded into 96-well plates (1×10^4 cells/well per 0.2 ml; four replicates each) and cultured for 4 days with/without 1 $\mu\text{g/ml}$ doxycyclin in 2 ml RPMI-1640 medium supplemented with 10% (v/v) FCS. The cells were cultured for an additional 60 min in the presence or absence of 1 mmol/l H_2O_2 . Then, the medium was replaced by 200 μl PBS containing 20 $\mu\text{mol/l}$ ethidium homodimer-1 and 80 $\mu\text{mol/l}$ calcein AM, and the cells were incubated for 20 min at 37 °C. Cells were examined by fluorescence microscopy using an Axiovert 135 TV (Carl Zeiss, Jena, Germany) and the optical filter set No. 23 (Carl Zeiss). The number of green (viable) and red (dead) cells was counted from three randomly chosen areas in each case.

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 3 software. Means $\pm$ S.E.M. were calculated from at least three different experiments. Data were analyzed using a nonparametric Mann-Whitney test. Differences were considered statistically significant at $P < 0.05$.

Results

Prx III is expressed in insulin-producing cells

First, we demonstrated the presence of PRX III in insulin-secreting cells using immunofluorescence and RT-PCR (Fig. 1).

Figure 1 (A) Immunohistochemistry. Paraffin slices of Balb/c mouse pancreas were stained with antisera against PRX III (a, c, d, and f, red), insulin (b and c, green), or glucagon (e and f, green). In the overlay (c and f), colocalization of red and green signals resulted in an orange color. Control staining with preimmune sera gave no immunoreactive signals. (B) Semi-quantitative RT-PCR. cDNA was prepared by reverse transcription of 2 μ g total RNA from mouse insulinoma cells β TC6-F7. *Prx III* was amplified with an expected product of 791 bp (lane 2). Molecular weight marker (lane 1).

Using paraffin slices of pancreas from Balb/c mice (Fig. 1A), we demonstrated that the green fluorescence associated with either insulin (b and c) or glucagon (e and f) was localized to the central islet core (b and c) or to the surrounding α cells (e and f). The red fluorescent signals represented PRX III (a and d). Immunotargeted signals were not visible when the corresponding preimmune sera were used (data not shown). The colocalization of PRX III with insulin resulted in yellow immunostaining in the overlay (c). There was no colocalization of PRX III and glucagon detectable in the overlay (f). PRX III was predominantly expressed in β cells, only slight background signals were observed in exocrine cells of the pancreas.

To confirm the expression of *Prx III* in insulin-producing cells, mouse insulinoma cells β TC6-F7 were cultured for 24 h. The mRNA was examined by semi-quantitative RT-PCR (Fig. 1B). We detected *Prx III* mRNA in the insulinoma cells with an expected RT-PCR product of 791 bp.

Cytokine stress increased the expression of *Prx III* in isolated islets

To study whether the PRX III system is induced after stress, islets of Langerhans from C57/BL6 mice were exposed to a mixture of cytokines (100 U/ml TNF- α , 50 U/ml IL1 β , and 100 U/ml IFN- γ). The subsequent western blots revealed an increased amount of PRX III in cytokine-stressed cells compared with the control cells incubated without cytokines (Fig. 2).

Exogenously triggered expression of *Prx III* in stably transfected cells

To determine the importance of PRX III in β cells, we established stably transfected insulinoma cells, in which *Prx III* is inducibly upregulated or downregulated in dependence of doxycyclin. For this purpose, we applied the inducible Tet-On-system. We transfected RINm5F cells with a construct composed of two transcriptional units. In the first unit, a regulator protein called reverse tetracycline-controlled transactivator (rtTA) is expressed constitutively. In the second unit, a tetracycline response element (TRE) fused to a minimal promoter drives the gene of interest. Only in the presence of doxycyclin, the over-expressed rtTA binds to the TRE and activates the transcription of the target gene. In our cell system, we over-expressed *Prx III* using the cDNA in sense orientation or suppressed it via the expression of an antisense RNA. Next, we verified the functionality of the stably transfected cells (Fig. 3). We regulated the expression of the *Prx III* construct by incubation in the presence or absence of doxycyclin for 4 days. Northern blot analyses with a digoxigenin-labeled DNA probe revealed a regulation of

Figure 2 Western blot. Isolated islets from C57/BL6 mice were treated for 24 h with cytokines (250 U/ml TNF- α , 2.5 U/ml IL1 β , and 125 U/ml IFN- γ). Proteins (20 μ g) were subjected to western blot analysis with anti-PRX III. Anti-GAPDH was utilized as a loading control. The results of four independent experiments were statistically analyzed (** $P < 0.005$).

Figure 3 Tet-On-system. (A) Western blot. Proteins (20 μ g), prepared from wild-type (wt) or stably transfected RINm5F cells over- (sense) or under- (antisense) expressing *Prx III*, which had been treated with/without 1 μ g/ml doxycyclin (dox) for 4 days, were estimated by western blotting with an antibody directed against PRX III. GAPDH was employed as loading control. (B) Statistical analysis. Gray scale values of all experiments ($n \geq 3$) were statistically analyzed, and significant differences between '+' and '-' dox were indicated (* $P < 0.05$ and ** $P < 0.005$).

transfected *Prx III* in the induced cells compared to untreated cells and untransfected cells (data not shown). Furthermore, the expression level of *Prx III* was analyzed by western blotting (Fig. 3). The amount of PRX III protein was altered by the expression of the sense or the antisense construct in the stably transfected cells after treatment with doxycyclin for 4 days compared to the untreated and untransfected cells. Over-expression of the sense construct resulted in a significant increase in protein amounts of PRX III (nearly twofold), while the antisense construct highly significantly diminished the *Prx III* expression to about 50%. We additionally observed that the Tet-On-system was leaky. Nevertheless, there was a clear difference between induced and noninduced cells: doxycyclin adjusted the PRX III amount from 120 to 177% in the sense clone and from 69.5 to 48.5% in the antisense clone. So a difference of 177 vs 48.5% between the sense and antisense clones as physiological consequences of upregulation and downregulation of *Prx III* is observed. The cells were incubated with doxycyclin for 4 days (a period which had been found to be optimal) and subsequently treated with diverse stressors.

PRX III lowers intracellular oxidant production after stress with H_2O_2 and protects from cell death

Different pro-apoptotic stimuli enhance the production of oxygen free radicals in cells. The intracellular redox status was assessed by the level of intracellular ROS, using the oxidation-sensitive fluorescent marker DCFH-DA (Fig. 4).

After incubation for 1 h with 1 mmol/l H_2O_2 (a concentration that causes a strong stress situation), the intensity of fluorescence of intracellular oxidized DCF was analyzed by a flow cytometer at 530 nm. After treating cells with H_2O_2 , an increased intracellular oxidation of DCFH could be observed, which was partially but not significantly reduced in *Prx III* over-expressing cells (Fig. 4A). In cells transfected with *Prx III* antisense construct, the amount of oxidized DCFH was approximately doubled in the presence of H_2O_2 , while PRX III depletion by doxycyclin led to a dramatic, nearly fourfold, increase in oxidized DCFH (Fig. 4B).

Cell viability was analyzed using the LIVE/DEAD Viability/Cytotoxicity kit (Molecular Probes). The prolonged exposure to doxycyclin (96 h) did not compromise cell viability to a significant extent, as <0.4% of cells appeared nonviable (stained red by ethidium bromide homodimer) irrespective of the presence (+ dox) or absence (– dox) of doxycyclin. When *Prx III* antisense transfectants were subjected to strong oxidative stress by exposure to 1 mmol/l H_2O_2 for 60 min, cell death was increased to $18.7 \pm 1.1\%$ (– dox) or $17.6 \pm 1.9\%$ (+ dox) (Fig. 4C and D). Over-expression of *Prx III* leads to a clear reduction in the amount of nonviable cells (+ dox: $6.18 \pm 0.9\%$, $P < 0.01$; – dox: $13.99 \pm 2.3\%$, $P = NS$; Fig. 4C and D).

PRX III prevented the induction of iNOS after stress with cytokines

The iNOS can produce large amounts of NO^* , which leads to cytochrome *c* release from mitochondria, DNA fragmentation, and apoptosis. Stably transfected RINm5F cells were induced for 4 days with doxycyclin and treated for 24 h with cytokines. The western blot analysis with anti-iNOS demonstrated that *Prx III* over-expression reduced the induction of iNOS after stress with IL1 β or the pro-inflammatory cytokine mix (Fig. 5A). However, down-regulation of *Prx III* resulted in an additional increase in iNOS expression due to these stressors (Fig. 5B).

PRX III maintained the mitochondrial membrane potential during stress

We next investigated whether PRX III was able to prevent apoptosis. There are two different apoptotic pathways, intrinsic and extrinsic. During the intrinsic pathway of apoptosis, peroxidation of cardiolipin in the mitochondrial inner membrane results in an impaired mitochondrial membrane potential and in the release of cytochrome *c*, which triggers the activation of caspase-9. To analyze such

Figure 4 Effects of the expression of *Prx III* on intracellular oxidant concentration and cell viability. Stably transfected RINm5F cells, expressing the (A) sense or (B) antisense construct, were treated for 4 days with/without 1 μ g/ml doxycyclin (dox) and for 1 h with 1 mmol/l H₂O₂. The trypsinized cells were incubated for 2 h with 20 μ M DCFH-DA in 300 μ l PBS at 37 °C. The intracellular oxidant production was analyzed by a FACScan. (C and D) LIVE/DEAD assay demonstrating that *Prx III* expression protects from cell death due to a 60-min exposure to 1 mmol/l H₂O₂. Viability of cells not exposed to H₂O₂ was >96.6% irrespective of the cells being wild-type or transfected with *Prx III* sense or antisense constructs, or the presence or absence of doxycyclin respectively (* P <0.01).

processes, we used DiOC₆(3), a fluorescent marker of $\Delta\psi_m$. The stably transfected RINm5F cells were induced for 4 days with doxycyclin and stressed for 48 h with pro-inflammatory cytokines. After staining with DiOC₆(3), the cells were analyzed for $\Delta\psi_m$ on a FACSCalibur. Unstressed cells were visible as a peak in the right side of the diagram (M1; Fig. 6), while stressed cells with an impaired mitochondrial membrane potential were detectable on the left side. Over-expression of *Prx III* resulted in a decrease in apoptotic cells after stress and in an increased peak within the M1 region (Fig. 6).

PRX III prevented the activation of caspase-3 and -9 after stress

While only the intrinsic apoptotic pathway involves mitochondria and activates caspase-9, caspase-3 is activated via both ways. To analyze whether PRX III is able to protect against mitochondrial damages induced by diabetogenic substances and pro-inflammatory cytokines, stably transfected cells were incubated in the presence (black bars) or absence (white bars) of doxycyclin for 4 days followed by a treatment with IL1 β (Fig. 7A–D) for 9 h. The caspase-3 and -9 activities were decreased by enhanced amounts of PRX III (A and C). Downregulation of this mitochondrial enzyme resulted in

increased caspase-3 and -9 activities (B and D). Similar experiments with cells treated with STZ (E–H) or TNF- α or IFN- γ (data not shown) resulted in comparable results. Thus, we have demonstrated that enhanced expression of *Prx III* reduced apoptosis, while downregulation of this mitochondrial enzyme resulted in increased caspase-3 and -9 activities and thus apoptosis.

PRX III prevented the cleavage of PARP-1

PARP is an abundant nuclear enzyme that mediates the repair of DNA single strand breaks via the activation and recruitment of DNA repair enzymes. In apoptotic cells, PARP is cleaved by activated caspase-3. We analyzed cleaved PARP as an apoptosis marker using stably transfected RINm5F cells, which were induced for 4 days with doxycyclin and treated for 24 h with pro-inflammatory cytokines or STZ. We utilized western blot analyses with an anti-cleaved PARP-1 antibody. In RINm5F cells over-expressing *Prx III*, the cleavage of PARP-1 was significantly reduced after cytokine- or STZ-induced stress (Fig. 8A), while downregulation of *Prx III* resulted in increased PARP cleavage in the cytokine- or STZ-treated cells (Fig. 8B).

Figure 5 Western blot. Stably transfected RINm5F cells with the *Prx III* construct in (A) sense or (B) antisense orientation were induced for 4 days with/without 1 μ g/ml doxycycline (dox) and treated for 24 h with cytokines (100 U/ml TNF- α , 50 U/ml IL1 β , and 100 U/ml IFN- γ). Protein (20 μ g) was extracted and examined by western blots with an antibody directed against iNOS. GAPDH was utilized as a loading control. The intensity of the signals generated in three independent experiments and compared to GAPDH was statistically analyzed (* $P < 0.05$).

PRX III maintains the insulin secretion during stress

Finally, RIA of released insulin over 24 h, when cells were incubated in RPMI media, confirmed that PRX III deficiency resulted in impaired basal insulin secretion. Over-expression of *Prx III* slightly increased the basal secretion of insulin, while the downregulation of *Prx III* resulted in significantly reduced basal insulin secretion and thus concentration in the culture medium (Fig. 9A).

Analyses of the glucose-stimulated insulin release revealed that – as expected – wild-type RINm5F cells show a weak glucose-dependent increase in insulin secretion only.

However, exposure of cells to H₂O₂ significantly reduced insulin release from wild-type cells. This effect of H₂O₂ was also apparent in *Prx III* antisense cells, but was completely abrogated in cells over-expressing *Prx III* (sense). Under high glucose conditions (20 mmol/l), insulin secretion was enhanced in *Prx III* antisense cells. However, this effect was not observed in *Prx III* sense cells (Fig. 9B).

Figure 6 Analysis of mitochondrial membrane potential ($\Delta\psi_m$). Stably transfected RINm5F cells over-expressing *Prx III* were induced for 4 days with/without 1 μ g/ml doxycycline (dox) and stressed for 48 h with cytokines (100 U/ml TNF- α , 50 U/ml IL1 β , and 100 U/ml IFN- γ) and stained with DiOC₆(3). The treated cells were analyzed by FACSCalibur and CellQuest software. Non-stressed cells induced for 4 days with/without 1 μ g/ml doxycycline were used as a control.

Figure 7 Determination of caspase-9 and -3 activities. Cell lysates from stably transfected RINm5F cells, over- (A, C, E, and G) or under-expressing (B, D, F, and H) *Prx III*, were treated for 4 days with/without 1 μ g/ml doxycycline (dox) and for 24 h with 50 U/ml IL1 β (A–D) or 4 mmol/l streptozotocin (STZ) (E–H) to determine caspase-9 (A, B, E, and F) and -3 (C, D, G, and H) activities, which was assayed by measuring the enzymatic hydrolysis of the added substrates LEHD-AFC or DEVD-AFC respectively. The released amount of AFC was determined with the fluorescence microplate reader Wallac 1420 Victor at 390 nm (excitation) and 510 nm (emission). Values were related to the protein content and were statistically analyzed (* $P < 0.05$).

Discussion

Considering that during pathogenesis of type 1 (insulin-dependent) diabetes, the insulin-producing β cells are destroyed by immune-mediated oxidative and nitrosative stress (Mandrup-Poulsen *et al.* 1990), and taking into account that β cells are more sensitive to oxidative stress than many other cells (Lenzen *et al.* 1996, Tiedge *et al.* 1997), we analyzed whether PRXs are present in these cells and whether they are sufficient to prevent dysfunction and cell damage. Previously we reported that the expression of *Prx I* and *II* was detectable in α as well as in β cells (Bast *et al.* 2002). It is well known that β cells express low levels of most antioxidant enzymes such as Cu/ZnSOD, MnSOD, catalase, and Gpx (Grankvist *et al.* 1981, Lenzen *et al.* 1996, Tiedge *et al.* 1997) than liver and kidney cells. Moreover, in diabetes-prone BB rats and diabetic patients, a further reduction in the islet antioxidative status was observed, thus making β cells particularly susceptible to damage by radicals following exposure to cytokines or activated immune cells (L'Abbe & Trick 1994, Maxwell *et al.* 1997). Indeed, we have found that *Prx I* expression was dramatically reduced in the islets of diabetogenic NOD mice (data not shown). Here we investigated whether the mitochondrial thioredoxin-2-PRX III system could be helpful in preventing oxidative stress-induced damage or destruction of insulin-producing cells. The analysis of *Prx III* expression in pancreatic tissue revealed expression restricted to insulin-producing β cells (Fig. 1), which was enhanced after pro-inflammatory cytokine-induced stress (Fig. 2).

During the pathogenesis of IDDM, macrophages and T cells recruited to the islets play an essential role due to their synthesis and secretion of pro-inflammatory cytokines IL1 β , TNF- α and IFN- γ as well as ROS and RNS (Delaney *et al.* 1997, Eizirik & Mandrup-Poulsen 2001). It has been previously shown that the experimental upregulation of different antioxidant enzymes in insulin-producing β cells protects against cytokine-mediated toxicity through prevention of oxidative damage (Hotta *et al.* 1998, Azevedo-Martins *et al.* 2003, Gurgul *et al.* 2004). To analyze PRX III in this respect, we generated stably transfected rat insulinoma cells over- or under-expressing this mitochondrial enzyme induced by doxycycline (Fig. 3). The Tet-On-system that was successfully used to control *Prx III* expression did not influence the viability, but was marginally leaky (Fig. 3). Thus, we cannot fully exclude the possibility that the observed activation of the transfected *Prx III* constructs in the absence of doxycycline slightly altered the impact of our analyses (Figs 4 and 7–9). However, we believe these effects to be marginal.

We utilized different stressors and measurements of apoptosis, viability and functionality to demonstrate the protective function and properties of PRX III. We analyzed the effects of individual pro-inflammatory cytokines, a mixture of them, hydrogen peroxide, and the diabetogenic substance STZ. The most impressive effects

Figure 8 Western blot. Stably transfected RINm5F cells (A) over- or (B) under-expressing *Prx III* after induction for 4 days with 1 μ M doxycyclin (dox) were treated for 24 h with cytokines (100 U/ml TNF- α , 50 U/ml IL1 β , and 100 U/ml IFN- γ) or 4 mmol/l streptozotocin (STZ). Proteins (20 μ g) were subjected to western blot analysis with anti-cleaved PARP-1. The results compared to GAPDH were statistically analyzed (* P <0.05, *** P <0.0001).

were summarized in this article. Only in the cases where the individual cytokines acted differently, we have presented all results obtained (Figs 5 and 7). Similar results were only briefly described.

Oxygen free radicals are potent executors of cell death associated with pro-inflammatory cytokine effects and are known to be involved in β cell destruction in type 1 diabetes (Verge *et al.* 1995, Rabinovitch *et al.* 1999, Chang *et al.* 2004). PRX III acts as an effective guardian against excessive oxygen radical generation in mitochondria (Miranda-Vizuet *et al.* 2000, Nonn *et al.* 2003, Chang *et al.* 2004). In our experiments with cells over-expressing PRX III, we were not able to detect a significant accumulation of ROS after stress with H₂O₂ or any effect by induction with doxycyclin. This may be due to the leaky Tet-On-system (Fig. 4A). The measurement of viability of the cells under these strong stress conditions revealed that doxycyclin does not provoke cell

death *per se*, and that over-expressing *Prx III* protects from cell death due to exposure to high concentrations of H₂O₂ (Fig. 4C and D). Noteworthy are the effects when we transfected with antisense clone, which resulted in a fourfold increase in intracellular reactive oxygen intermediates (Fig. 4B). The same effect was induced by repression of thioredoxin-2, which is used together with thioredoxin reductase-2 for regeneration of PRX III (Tanaka *et al.* 2002). In addition to increasing oxidative stress, the cytokine IL1 β plays a key role in NO⁻-dependent destruction of β cells (McDaniel *et al.* 1996). It induces a broad range of signaling pathways including upregulation of iNOS via nuclear factor 'kappa-light-chain-enhancer' of activated β -cells (NF- κ B) and signal transducers and activators of transcription (STAT)

Figure 9 Insulin secretion. (A) Stably transfected cells, expressing the sense or antisense construct, were treated for 4 days with/without 1 μ M doxycyclin (dox). Basal insulin secretion of 24 h was analyzed in the culture medium using a rat insulin RIA kit (for wild-type -dox 100% is equivalent to 267.5 ng/ml). The samples were counted by a gamma counter for 1 min. (B) Glucose-stimulated insulin secretion and its sensitivity to 80 μ M H₂O₂ were determined in wild-type cells as well as in *Prx III* sense and *Prx III* antisense cells. The results obtained from at least three independent experiments measured in duplicates were statistically analyzed (* P <0.01, # P <0.05, and ** P <0.005).

activation. Our results indicate that a mitochondrial generation of ROS may further amplify the direct cytokine-dependent induction of iNOS, which can be partially suppressed but not prevented by PRX III (Fig. 5). This is in agreement with our previous results of experiments with wild-type cells and different antioxidants (Michalska *et al.* 2010). In a synergism with the ROS, NO $\cdot$ forms various toxic radicals that modify cellular structures such as lipids, nucleic acids, and proteins, especially iron-containing enzymes of the mitochondrial respiratory chain and Krebs cycle. The resulting production of ROS by the impaired respiratory chain mediates the loss of membrane potential and release of cytochrome *c*. In the cytosol, cytochrome *c* induces the oligomerization of the apoptotic protease-activating factor 1, which recruits the pro-caspase-9. By this mechanism, the apoptosome is assembled, which leads to autoprocessing of caspase-9, increasing its catalytic activity, which is important for the activation of effector caspase-3 (Adrain & Martin 2001). We demonstrated that stably transfected RINm5F cells over-expressing the peroxidase PRX III were protected against ROS-induced damaging mechanisms as cell death (Fig. 4), iNOS induction (Fig. 5), loss of mitochondrial membrane potential (Fig. 6), and activation of caspase-9 and -3 (Fig. 7) after stress with pro-inflammatory cytokines, H₂O₂ or STZ. The increased apoptotic signals measured in the antisense clone verified these results. Similar observations were made in HeLa cells suppressing *Prx III* (Chang *et al.* 2004) or in WEHI7.2 thymoma cells over-expressing *Prx III* (Nonn *et al.* 2003). A further method to analyze apoptosis is the detection of cleaved PARP as a well-known substrate of activated caspase-3 (Nargi-Aizenman *et al.* 2002). We were able to demonstrate that over-expression of *Prx III* significantly suppressed the cleavage of PARP-1, while a downregulation of *Prx III* resulted in an almost twofold increase in the PARP-derived 24 kDa fragment (Fig. 8).

Insulin synthesis and secretion are closely connected to mitochondrial function. The increased ATP level from metabolized glucose represents an important stimulus-secretion signal for the function of pancreatic β cells (Maechler *et al.* 1997). Oxidative stress leads to mitochondrial dysfunction and reduced metabolic stimulus-secretion coupling. However, probably because of the requirement of small amounts of H₂O₂ in normal cell function, it could be critical to express appropriate levels of antioxidant proteins (Pi *et al.* 2007). In *Prx III* over-expressing cells, insulin secretion was positively affected (Fig. 9), demonstrating that upregulation of *Prx III* is not detrimental to β cell function. In cells where the expression of *Prx III* was suppressed by doxycyclin, insulin secretion was lowered significantly (Fig. 9). However, the price for the protection of β cells by antioxidant enzymes could be the loss of glucose sensitivity. Over-expression of *Prx III* resulted in a high secretion of insulin without further increase after stimulation with glucose (Fig. 9B).

Thus, our work has demonstrated that over-expression of the mitochondrial PRX III can protect against oxidative and nitrosative stress induced by cytokines or chemical generators of oxygen free radicals and NO $\cdot$. Positive features of PRX III have been previously published: e.g. *Prx III* gene expression is inducible by oxidative stress (Mitsumoto *et al.* 2001, Lee *et al.* 2007). Through the interaction of PRX III with leucine zipper-bearing kinase, activation of NF κ B can be modified (Masaki *et al.* 2003). Furthermore, *Prx III* was identified as a gene regulated by the transcription factor c-Myc, which plays an important role in the regulation of the cell cycle, cell proliferation, and differentiation, as well as in apoptosis (Wonsey *et al.* 2002). It may not be surprising therefore to find high expression levels in tumor cells and several human cancers, where it may protect the growing tumor against hypoxia-induced apoptosis due to increased H₂O₂ production (Nonn *et al.* 2003, Chua *et al.* 2010). Indeed, in HeLa cells the amount of PRX III is 30-fold higher than glutathione peroxidase (Chang *et al.* 2004).

Our results are also in line with the recently published data from transgenic PRX III over-expressing mice. Cells from these mice had increased resistance to stress-induced cell death and apoptosis. Interestingly, the mice showed improved glucose homeostasis, as evidenced by their reduced levels of blood glucose and increased glucose clearance. They are also protected against hyperglycemia and glucose intolerance induced by high-fat diet feeding. The results from Chen *et al.* (2008) further showed that the inhibition of glycogen synthase kinase-3 (GSK3) may play a role in mediating the improved glucose tolerance phenotype and indicate that reduction in mitochondrial H₂O₂ by over-expressing PRX III improves glucose tolerance.

So we propose that PRXs could be an attractive target to protect β cell function and mass *in vivo* and thus delay manifestation of IDDM.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

- Abdel-Rahman MS, Elrakhawly FI & Iskander FA 1992 Protection of B cells against the effect of alloxan. *Toxicology Letters* **63** 155–164. (doi:10.1016/0378-4274(92)90007-7)
- Adrain C & Martin SJ 2001 The mitochondrial apoptosome: a killer unleashed by the cytochrome seas. *Trends in Biochemical Sciences* **26** 390–397. (doi:10.1016/S0968-0004(01)01844-8)

- Araki M, Nanri H, Ejima K, Murasato Y, Fujiwara T, Nakashima Y & Ikeda M 1999 Antioxidant function of the mitochondrial protein SP-22 in the cardiovascular system. *Journal of Biological Chemistry* **274** 2271–2278. (doi:10.1074/jbc.274.4.2271)
- Azevedo-Martins AK, Lortz S, Lenzen S, Curi R, Eizirik DL & Tiedge M 2003 Improvement of the mitochondrial antioxidant defense status prevents cytokine-induced nuclear factor- κ B activation in insulin-producing cells. *Diabetes* **52** 93–101. (doi:10.2337/diabetes.52.1.93)
- Bast A, Wolf G, Oberbäumer I & Walther R 2002 Oxidative and nitrosative stress induces peroxiredoxins in pancreatic beta cells. *Diabetologia* **45** 867–876. (doi:10.1007/s00125-002-0846-1)
- Bernabe JC, Tejedo JR, Rincon P, Cahuana GM, Ramirez R, Sobrino F & Bedoya F 2001 Sodium nitroprusside-induced mitochondrial apoptotic events in insulin-secreting RINm5F cells are associated with MAP kinases activation. *Experimental Cell Research* **269** 222–229. (doi:10.1006/excr.2001.5315)
- Chae HZ, Kim HJ, Kang SW & Rhee SG 1999 Characterization of three isoforms of mammalian peroxiredoxin that reduce peroxides in the presence of thioredoxin. *Diabetes Research and Clinical Practice* **45** 101–112. (doi:10.1016/S0168-8227(99)00037-6)
- Chang TS, Cho CS, Park S, Yu S, Kang SW & Rhee SG 2004 Peroxiredoxin III, a mitochondrion-specific peroxidase, regulates apoptotic signaling by mitochondria. *Journal of Biological Chemistry* **279** 41975–41984. (doi:10.1074/jbc.M407707200)
- Chen L, Na R, Gu M, Salmon AB, Liu Y, Liang H, Qi W, Van Remmen H, Richardson A & Ran Q 2008 Reduction of mitochondrial H_2O_2 by overexpressing peroxiredoxin 3 improves glucose tolerance in mice. *Aging Cell* **7** 866–878. (doi:10.1111/j.1474-9726.2008.00432.x)
- Chua PJ, Lee EH, Yu Y, Yip GW, Tan PH & Bay BH 2010 Silencing the peroxiredoxin III gene inhibits cell proliferation in breast cancer. *International Journal of Oncology* **36** 359–364. (doi:10.3892/ijo.00000507)
- Delaney CA, Pavlovic D, Hoorens A, Pipeleers DG & Eizirik DL 1997 Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* **138** 2610–2614. (doi:10.1210/en.138.6.2610)
- Eizirik DL & Mandrup-Poulsen T 2001 A choice of death – the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* **44** 2115–2133. (doi:10.1007/s001250100021)
- Gandy SE, Buse MG & Crouch RK 1982 Protective role of superoxide dismutase against diabetogenic drugs. *Journal of Clinical Investigation* **70** 650–658. (doi:10.1172/JCI110659)
- Grankvist K, Marklund SL & Täljedal IB 1981 CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. *Biochemical Journal* **199** 393–398.
- Gurgul E, Lortz S, Tiedge M, Jörns A & Lenzen S 2004 Mitochondrial catalase overexpression protects insulin-producing cells against toxicity of reactive oxygen species and proinflammatory cytokines. *Diabetes* **53** 2271–2280. (doi:10.2337/diabetes.53.9.2271)
- Harmon JS, Bogdani M, Parazzoli SD, Mak SS, Oseid EA, Berghmans M, Leboeuf RC & Robertson RP 2009 β -Cell-specific overexpression of glutathione peroxidase preserves intranuclear MafA and reverses diabetes in db/db mice. *Endocrinology* **150** 4855–4862. (doi:10.1210/en.2009-0708)
- Hattori F, Murayama N, Noshita T & Oikawa S 2003 Mitochondrial peroxiredoxin-3 protects hippocampal neurons from excitotoxic injury in vivo. *Journal of Neurochemistry* **86** 860–868. (doi:10.1046/j.1471-4159.2003.01918.x)
- Hotta M, Tashiro F, Ikegami H, Niwa H, Ogihara T, Yodoi J & Miyazaki J 1998 Pancreatic beta cell-specific expression of thioredoxin, an anti-oxidative and antiapoptotic protein, prevents autoimmune and streptozotocin-induced diabetes. *Journal of Experimental Medicine* **188** 1445–1451. (doi:10.1084/jem.188.8.1445)
- Kang SW, Rhee SG, Chang TS, Jeong W & Choi MH 2005 2-Cys peroxiredoxin function in intracellular signal transduction: therapeutic implications. *Trends in Molecular Medicine* **11** 571–578. (doi:10.1016/j.jmolmed.2005.10.006)
- Kiely A, McClenaghan NH, Flatt PF & Newsholme P 2007 Pro-inflammatory cytokines increase glucose, alanine and triacylglycerol utilization but inhibit insulin secretion in a clonal pancreatic beta-cell line. *Journal of Endocrinology* **195** 113–123. (doi:10.1677/JOE-07-0306)
- Knopp E, Arndt TL, Eng KL, Caldwell M, LeBoeuf RC, Deeb SS & O'Brien KD 1999 Murine phospholipid hydroperoxide glutathione peroxidase: cDNA sequence, tissue expression, and mapping. *Mammalian Genome* **10** 601–605. (doi:10.1007/s003359901053)
- L'Abbe MR & Trick KD 1994 Changes in pancreatic glutathione peroxidase and superoxide dismutase activities in the prediabetic diabetes-prone BB rat. *Proceedings of the Society for Experimental Biology and Medicine* **207** 206–212.
- Lee W, Wells T & Kantorow M 2007 Localization and H_2O_2 -specific induction of PRDX3 in the eye lens. *Molecular Vision* **13** 1469–1474.
- Lenzen S, Drinkgern J & Tiedge M 1996 Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radical Biology and Medicine* **20** 463–466. (doi:10.1016/0891-5849(96)02051-5)
- Li X, Chen H & Epstein PN 2006 Metallothionein and catalase sensitize to diabetes in nonobese diabetic mice: reactive oxygen species may have a protective role in pancreatic beta-cells. *Diabetes* **55** 1592–1604. (doi:10.2337/db05-1357)
- Lottmann H, Vanselow J, Hessabi B & Walther R 2001 The Tet-On system in transgenic mice: inhibition of the mouse pdx-1 gene activity by antisense RNA expression in pancreatic beta-cells. *Journal of Molecular Medicine* **79** 321–328. (doi:10.1007/s001090100229)
- Maechler P, Kennedy ED, Pozzan T & Wollheim CB 1997 Mitochondrial activation directly triggers the exocytosis of insulin in permeabilized pancreatic beta-cells. *EMBO Journal* **16** 3833–3841. (doi:10.1093/emboj/16.13.3833)
- Mandrup-Poulsen T, Helqvist S, Wogensens LD, Molvig J, Pociot F, Johannesen J & Nerup J 1990 Cytokine and free radicals as effector molecules in the destruction of pancreatic beta cells. *Current Topics in Microbiology and Immunology* **164** 169–193.
- Masaki M, Ikeda A, Shiraki E, Oka S & Kawasaki T 2003 Mixed lineage kinase LZK and antioxidant protein-1 activate NF- κ B synergistically. *European Journal of Biochemistry* **270** 76–83. (doi:10.1046/j.1432-1033.2003.03363.x)
- Maxwell SR, Thomason H, Sandler D, Leguen C, Baxter MA, Thorpe GH, Jones AF & Barnett AH 1997 Antioxidant status in patients with uncomplicated insulin-dependent and non-insulin-dependent diabetes mellitus. *European Journal of Clinical Investigation* **27** 484–490. (doi:10.1046/j.1365-2362.1997.1390687.x)
- McDaniel ML, Kwon G, Hill JR, Marshall CA & Corbett JA 1996 Cytokines and nitric oxide in islet inflammation and diabetes. *Proceedings of the Society for Experimental Biology and Medicine* **211** 24–32.
- Michalska M, Wolf G, Walther R & Newsholme P 2010 The effects of pharmacologic inhibition of NADPH oxidase or iNOS on pro-inflammatory cytokine, palmitic acid or H_2O_2 -induced mouse islet or clonal pancreatic beta cell dysfunction. *Bioscience Reports* **30** 445–453. (doi:10.1042/BSR20090138)
- Miranda-Vizuete A, Damdimopoulos AE & Spyrou G 2000 The mitochondrial thioredoxin system. *Antioxidants & Redox Signaling* **2** 801–810. (doi:10.1089/ars.2000.2.4-801)
- Mitsumoto A, Takanezawa Y, Okawa K, Iwamatsu A & Nakagawa Y 2001 Variants of peroxiredoxins expression in response to hydrogen peroxide stress. *Free Radical Biology and Medicine* **30** 625–635. (doi:10.1016/S0891-5849(00)00503-7)
- Nargi-Aizenman JL, Simbulan-Rosenthal CM, Kelly TA, Smulson ME & Griffin DE 2002 Rapid activation of poly(ADP-ribose) polymerase contributes to Sindbis virus and staurosporine-induced apoptotic cell death. *Virology* **293** 164–171. (doi:10.1006/viro.2001.1253)
- Nonn L, Berggren M & Powis G 2003 Increased expression of mitochondrial peroxiredoxin-3 (thioredoxin peroxidase-2) protects cancer cells against hypoxia and drug-induced hydrogen peroxide-dependent apoptosis. *Molecular Cancer Research* **1** 682–689.
- Petrosillo G, Ruggiero FM & Paradis G 2003 Role of reactive oxygen species and cardiolipin in the release of cytochrome c from mitochondria. *FASEB Journal* **17** 2202–2208. (doi:10.1096/fj.03-0012com)

- Pi J, Bai Y, Zhang Q, Wong V, Floering LM, Daniel K, Reece JM, Deeney JT, Andersen ME, Corkey BE *et al.* 2007 Reactive oxygen species as a signal in glucose-stimulated insulin secretion. *Diabetes* **56** 1783–1791. (doi:10.2337/db06-1601)
- Rabinovitch A & Suarez-Pinzon WL 1998 Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. *Biochemical Pharmacology* **55** 1139–1149. (doi:10.1016/S0006-2952(97)00492-9)
- Rabinovitch A, Suarez-Pinzon W, Strynadka K, Ju Q, Edelstein D, Brownlee M, Korbitt GS & Rajotte RV 1999 Transfection of human pancreatic islets with an anti-apoptotic gene (bcl-2) protects beta-cells from cytokine-induced destruction. *Diabetes* **48** 1223–1229. (doi:10.2337/diabetes.48.6.1223)
- Radi R, Turrens JF, Chang LY, Bush KM, Crapo JD & Freeman BA 1991 Detection of catalase in rat heart mitochondria. *Journal of Biological Chemistry* **266** 22028–22034.
- Sandstrom J, Jonsson LM, Edlund H, Holmberg D & Marklund SL 2002 Overexpression of extracellular-SOD in islets of nonobese diabetic mice and development of diabetes. *Free Radical Biology and Medicine* **33** 71–75. (doi:10.1016/S0891-5849(02)00859-6)
- Storling J, Binzer J, Andersson AK, Zullig RA, Tonnesen M, Lehmann R, Spinas GA, Sandler S, Billestrup N & Mandrup-Poulsen T 2005 Nitric oxide contributes to cytokine-induced apoptosis in pancreatic beta cells via potentiation of JNK activity and inhibition of Akt. *Diabetologia* **48** 2039–2050. (doi:10.1007/s00125-005-1912-2)
- Suarez-Pinzon WL, Mabley JG, Strynadka K, Power RF, Szabo C & Rabinovitch A 2001 An inhibitor of inducible nitric oxide synthase and scavenger of peroxynitrite prevents diabetes development in NOD mice. *Journal of Autoimmunity* **16** 449–455. (doi:10.1006/jaut.2001.0507)
- Tanaka T, Hosoi F, Yamaguchi-Iwai Y, Nakamura H, Masutani H, Ueda S, Nishiyama A, Takeda S, Wada H, Spyrou G *et al.* 2002 Thioredoxin-2 (TRX-2) is an essential gene regulating mitochondria-dependent apoptosis. *EMBO Journal* **21** 1695–1703. (doi:10.1093/emboj/21.7.1695)
- Tiedge M, Lortz S, Drinkgern J & Lenzen S 1997 Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* **46** 1733–1742. (doi:10.2337/diabetes.46.11.1733)
- Verge CF, Gianani R, Yu L, Pietropaolo M, Smith T, Jackson RA, Soeldner JS & Eisenbarth GS 1995 Late progression to diabetes and evidence for chronic beta-cell autoimmunity in identical twins of patients with type I diabetes. *Diabetes* **44** 1176–1179. (doi:10.2337/diabetes.44.10.1176)
- Watabe S, Hiroi T, Yamamoto Y, Fujioka Y, Hasegawa H, Yago N & Takahashi SY 1997 SP-22 is a thioredoxin-dependent peroxide reductase in mitochondria. *European Journal of Biochemistry* **249** 52–60. (doi:10.1111/j.1432-1033.1997.t01-1-00052.x)
- Wonsey DR, Zeller KI & Dang CV 2002 The c-Myc target gene PRDX3 is required for mitochondrial homeostasis and neoplastic transformation. *PNAS* **99** 6649–6654. (doi:10.1073/pnas.102523299)
- Wood ZA, Schroder E, Robin Harris J & Poole LB 2003 Structure, mechanism and regulation of peroxiredoxins. *Trends in Biochemical Sciences* **28** 32–40. (doi:10.1016/S0968-0004(02)00003-8)
- Yamamoto T, Matsui Y, Natori S & Obinata M 1989 Cloning of a housekeeping-type gene (MER5) preferentially expressed in murine erythroleukemia cells. *Gene* **80** 337–343. (doi:10.1016/0378-1119(89)90297-7)

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