Localization and regulation of pancreatic selenoprotein P

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Abstract

Progressive loss of pancreatic β -cell mass is a crucial feature of type 2 diabetes mellitus. As β -cells express very low amounts of the antioxidant enzymes catalase and glutathione peroxidase (GPx), they appear to be particularly vulnerable to oxidative damage in the pathogenesis of diabetes. Here, we investigated the pancreatic expression pattern and regulation of selenoprotein P (Sepp1), which may serve as an additional antioxidant enzyme inside and outside of cells. Sepp1 was detected in rodent pancreas by immunofluorescence and real-time RT-PCR. Regulation of Sepp1 biosynthesis in INS-1 rat insulinoma cells was investigated by real-time RT-PCR, luciferase gene reporter assay, and immunoblotting. *Sepp1* and *Gpx1* gene expressions in rat pancreas were 58 and 22% respectively of the liver values. Pancreatic *Sepp1* expression was restricted to the endocrine tissue, with Sepp1 being present in the α - and β -cells of mouse islets. In INS-1 insulinoma cells, *Sepp1* expression was stimulated by the selenium compound sodium selenate and diminished in the presence of high glucose (16.7 vs 5 mM) concentrations. *Sepp1* mRNA stability was also lowered at 16.7 mM glucose. Moreover, *Sepp1* mRNA levels were decreased in isolated murine islets cultured in high-glucose (22 mM) medium compared with normal glucose (5.5 mM) medium. Pancreatic *Sepp1* expression was elevated upon treatment of mice with the β -cell toxin streptozotocin. This study shows that pancreatic islets express relatively high levels of Sepp1 that may fulfill a function in antioxidant protection of β -cells. Downregulation of *Sepp1* expression by high glucose might thus contribute to glucotoxicity in β -cells.

Key Words

- ▶ pancreatic islet
- ▶ β -cell
- ▶ diabetes
- ▶ selenium
- ▶ selenoprotein
- ▶ oxidative stress

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Introduction

Insulin resistance is a hallmark in the pathogenesis of type 2 diabetes mellitus (T2DM). Pancreatic β -cells have the capacity to compensate for insulin resistance by a reactive increase in insulin secretion over a long period.

However, chronic nutrient overload may eventually result in β -cell failure, characterized by an impaired insulin secretory capacity upon glucose stimulation and enhanced apoptosis (Donath *et al.* 2003, Kahn 2003,

Schinner *et al.* 2005). Oxidative/nitrosative stress induced by reactive oxygen and nitrogen species (ROS/RNS) including superoxide, hydrogen peroxide (H_2O_2), nitric oxide (NO), and peroxynitrite is increasingly recognized to contribute to β -cell dysfunction and destruction in T2DM. The majority of cellular ROS production derives from electron leakage in the mitochondrial respiratory chain and from membrane-bound NAD(P)H oxidases (Nox), producing superoxide that can subsequently dismutate to H_2O_2 (Drews *et al.* 2010). Hyperglycemia results in increased superoxide generation in rat islets *in vitro* and *in vivo* (Bindokas *et al.* 2003, Tang *et al.* 2007). Pro-inflammatory cytokines that are produced in the adipose tissue of insulin-resistant individuals (Hotamisligil *et al.* 1993, Donath *et al.* 2003) have been reported to enhance superoxide and NO levels in β -cells through stimulation of Nox and inducible NO synthase (iNOS; Darville & Eizirik 1998, Morgan *et al.* 2007, Drews *et al.* 2010). The reaction of NO with superoxide generates peroxynitrite, a potent mediator of β -cell destruction (Lakey *et al.* 2001).

Oxidative stress due to imbalance of oxidants and antioxidants may result in damage of cellular macromolecules and cell death (Sies 1986). Interestingly, pancreatic islets are among the weakest endowed tissues in terms of their antioxidant defense status. Expression and activity of the H_2O_2 -reducing enzymes catalase and glutathione peroxidase (GPx) are particularly low in islets, exhibiting around 5% of the values in liver. Superoxide dismutases (SOD) are moderately expressed in islets (Tiedge *et al.* 1997). On the other hand, β -cells express relatively high levels of peroxiredoxins, an additional enzymatic antioxidant system that is inducible by oxidative stress (Bast *et al.* 2002).

Selenoprotein P (Sepp1 (SEPP1)) is another protein with a putative antioxidant function, even though it is primarily known as plasma protein that supports peripheral tissues with the essential micronutrient selenium (Se; Burk & Hill 2009, Steinbrenner & Sies 2009). Two enzymatic activities have been demonstrated for Sepp1, a peroxynitrite reductase and a phospholipid hydroperoxide GPx (PH-GPx) activity (Arteel *et al.* 1998, Takebe *et al.* 2002). While hepatocytes produce and secrete the bulk of Sepp1 circulating in plasma, *Sepp1* mRNA is present ubiquitously in most tissues (Burk & Hill 2009). Knock-down of Sepp1 biosynthesis in astrocytes and in adipocytes has been shown to increase their sensitivity for oxidative damage (Steinbrenner *et al.* 2006, Zhang & Chen 2011). *Sepp1* mRNA has also been detected in the mouse pancreas (Lee *et al.* 2008), where it has been

described to be enriched in β -cells compared with α -cells of islets (Niwa *et al.* 1997).

Here, we provide evidence that expression of Sepp1 in pancreas is restricted to the islets of Langerhans. We investigated Sepp1 regulation by glucose in INS-1 rat insulinoma cells and in mice islets *in vitro* and by treatment of mice with the β -cell toxin streptozotocin (STZ) *in vivo*.

Materials and methods

Reagents and antibodies

Se compounds, glucose, STZ, and actinomycin D were from Sigma. Reagents for SDS-PAGE were from Roth (Karlsruhe, Germany). PCR primers were synthesized by Life Technologies Invitrogen. The primary antibody against Sepp1 and the secondary HRP-coupled anti-goat IgG antibody were from Santa Cruz Biotechnology. The primary antibody against β -actin was from Sigma, and the secondary HRP-coupled anti-mouse IgG antibody was from Dianova (Hamburg, Germany).

Cell culture

The rat insulinoma cell line INS-1 (Asfari *et al.* 1992) was a kind gift of Dr J Seissler (Ludwig Maximilians University, Munich, Germany). INS-1 cells were cultured at 37 °C in a humidified 5% CO_2 atmosphere in RPMI 1640 medium (Sigma) supplemented with 10% FCS (PAA; Pasching, Austria), 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin (PAA), 2 mM glutamax (Invitrogen), and 50 μM 2-mercaptoethanol (Sigma) as described previously (Schinner *et al.* 2008). For experiments, cells were cultured in serum-free medium supplemented with 1 μM Se (sodium selenite, sodium selenate, or selenomethionine) and glucose (5, 11, or 16.7 mM) for 24 h. Cytotoxicity of the applied Se compounds was assessed by determining the percentage of metabolically active INS-1 cells using the CellTiter 96 AQueous nonradioactive cell proliferation assay (Promega) according to the manufacturer's protocol.

Sample collection from animals

Animals were obtained from the animal facility of the University Hospital Duesseldorf. The protocols were conducted in accordance with the principles of laboratory care. Islets were prepared from 2-month-old C57BL/6 mice. Mice were killed and islets were isolated using the intraductal collagenase digestion technique as described

previously (Schinner *et al.* 2008). Islets were purified, handpicked, and thereafter incubated for 24 h in Krebs–Ringer buffer (Sigma) containing different glucose concentrations. Samples of pancreas, liver, and kidney were taken from Wistar rats. Rats were killed and tissues were removed and frozen immediately in liquid nitrogen.

Treatment of mice with the β -cell toxin STZ

Four-week-old C57BL/6 mice received 40 mg STZ/kg body weight by i.p. injections on 5 consecutive days, according to a previously described low-dose STZ protocol (Lu *et al.* 1998). Blood glucose concentrations were measured weekly. Two weeks after STZ injection, mice became diabetic as indicated by a rise in blood glucose concentrations to ≥ 17 mM. Mice were killed at different time points 1–2 weeks after STZ injection, displaying blood glucose concentrations from 8.0 to 18.6 mM. Pancreata were removed and frozen in liquid nitrogen for histological staining.

Real-time RT-PCR

From INS-1 cells and rat tissues, total RNA was isolated using the RNeasy Mini Kit (Qiagen) and transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen). From mouse islets, total RNA was extracted using the RNeasy Plus Micro Kit (Qiagen) and transcribed with the High-Capacity cDNA Reverse Transcription Kit (Life Technologies Applied Biosystems). Analysis of mRNA expression was done in a LightCycler 2.0 qPCR system (Roche). PCR was performed with 40 ng cDNA in glass capillaries containing LightCycler FastStart DNA Master SYBR Green I Reaction Mix (Roche), 3 mM $MgCl_2$, and 1 μ M primers. PCR amplicons were quantified by the LightCycler software, employing the house-keeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT) HPRT1 for internal normalization as described (Speckmann *et al.* 2008). Primers were designed using the Universal ProbeLibrary Assay Design Center (Roche), and their specificity was confirmed by melting curve analysis and agarose gel electrophoresis of PCR products. The PCR product sizes and primer sequences were as follows: rat GPx1 (PCR product: 60 bp): F: CGACATCGAACCCGATATAGA, R: ATGCCTTAGGGGTTGCTAGG; rat HPRT1 (PCR product: 61 bp): F: GACCGGTTCTGTCATGTCG, R: ACCTGGTTCATCATCTAATCAC; rat Sepp1 (PCR product: 73 bp): F: GACAGTGGTTGCTCTTCTTCAA, R: TCGCAGGTCTTCCAATCTG; mouse HPRT1 (PCR product: 90 bp): F: TCCTCCTCAGACCGCTTTT, R:

CCTGGTTCATCATCGCTAATC; and mouse Sepp1 (PCR product: 125 bp): F: GGTGCGGAACTGCAATC, R: TTTGTTGTGGTGT-TTGTGGTG.

Immunoblotting

Whole-cell lysates were obtained by treatment of INS-1 cells with 1% SDS. The protein content of the lysates was determined by DC Protein Assay (Bio-Rad). Culture supernatants of INS-1 cells were concentrated 40-fold by ultrafiltration using Vivaspin 2 concentrator columns (Sartorius, Göttingen, Germany) as described (Steinbrenner *et al.* 2006). Immunoblotting was performed by standard techniques as described (Speckmann *et al.* 2008). Briefly, aliquots of 20–35 μ g protein or equal aliquots of INS-1 supernatants were separated on 10% SDS–PAGE gels followed by electroblotting onto PVDF membranes (GE Healthcare, Freiburg, Germany). After blocking unspecific binding sites with Tris-buffered saline Tween 20 (TBST)/5% milk powder (Roth), membranes were incubated with primary and secondary antibodies diluted in blocking buffer. Immunodetection was carried out with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce, Waltham, MA, USA) and X-ray film (GE Healthcare).

Plasmids and luciferase reporter gene assay

Sequence information on the rat *Sepp1* gene was obtained from the Rat Genome Database (Twigger *et al.* 2007). A 1500-bp genomic DNA fragment located immediately upstream of the translation start site of the predominant rat *Sepp1* transcript variant 1 (GenBank accession number: NM_019192) was cloned into the firefly luciferase reporter gene vector pGL3basic (Promega). Genomic DNA was isolated from INS-1 cells with PureLink Genomic DNA Mini Kit (Invitrogen) and subjected to PCR using *Pfu* DNA polymerase (Fermentas, St Leon-Rot, Germany) and the primers 5'-ATCAAGTCCGTTTACATTTC-3' (sense) and 5'-AACCCCTTCAGACTAGCTTG-3' (antisense). The PCR product served as template in a subsequent PCR with primers carrying recognition sites for KpnI and XhoI restriction enzymes. After restriction digest, the product of this second PCR was cloned into the KpnI and XhoI restriction sites of pGL3basic. The resulting plasmid construct was sequenced and termed ratSepp1-luc. The DNA sequence of the putative rat *Sepp1* promoter fragment (–1904/–16) was submitted to GenBank (accession number: JQ082498). For reporter gene assays, INS-1 cells grown in 24-well plates were co-transfected

with 0.5 µg ratSepp1-luc plasmid and 0.1 µg renilla luciferase pRL-TK plasmid (Promega) together with 3.2 µl nanofectamin (PAA) according to the manufacturer's protocol. The promoter/enhancer-less firefly luciferase vector pGL3basic was co-transfected with pRL-TK in control experiments for the determination of background promoter activity. Twenty-four hours after transfection, luciferase activities were determined by chemiluminescence detection in a Victor 1420 multi-label counter (Perkin Elmer Wallac, Waltham, MA, USA) using the Dual Luciferase Reporter Assay (Promega) as described (Schinner *et al.* 2008, Speckmann *et al.* 2008).

Immunohistochemistry and immunofluorescence

For immunohistochemistry analysis, pancreata of C57BL/6 mice were sliced and frozen. Cryosections were fixed with acetone at -20°C . Thereafter, the sections were washed thrice with TBST. Sections were blocked according to the secondary antibody used: for insulin and glucagon staining, sections were blocked with avidin (Dako, Hamburg, Germany) for 10 min and then with peroxidase blocking solution (Dako). For Sepp1 staining, sections were blocked with peroxidase blocking solution only. Thereafter, 5% normal swine serum (in *aqua dest.*) was applied. Primary antibodies against Sepp1 (C-14, sc-22639; Santa Cruz; 1:50), insulin (Dako; 1:50), or glucagon (GeneTex, Inc., Irvine, CA, USA; 1:10) were incubated overnight at 4°C . For negative controls, the primary antibody was omitted. Subsequently, sections were washed thrice with TBST for 5 min and incubated for 60 min at room temperature with the respective secondary antibodies (Sepp1: anti-goat IgG-HRP, Santa Cruz, 1:200; insulin: anti-guinea pig IgG-HRP, Dako, 1:150; glucagon: anti-rabbit IgG-biotin, AbD Serotec, 1:400). For staining, the VECTASTAIN Elite ABC kit (Vector, Burlingame, CA, USA) was applied together with AEC chromogenic substrate (Dako). Counterstaining was performed for 1 min in hematoxylin II.

For immunofluorescence analysis, cryosections were fixed in acetone, air dried, washed three times with TBST, and blocked with 5% normal swine serum for 60 min. Thereafter, primary antibodies against insulin (1:400), glucagon (1:10), or Sepp1 (1:50) were applied overnight at 4°C . The next day, sections were washed thrice with TBST for 5 min and incubated with Alexa Fluor-coupled secondary antibodies (Invitrogen) at a 1:1500 dilution. Digital images were produced using a Nikon Eclipse TE300 microscope equipped with a Nikon DXM1200 camera (Nikon, Tokyo, Japan).

Statistical analysis

Values are given as mean $\pm$ s.d. of three or more independent experiments. Differences between groups were tested for significance by Student's *t*-test with $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ as levels of significance.

Results

Pancreatic expression and localization of Sepp1

Pancreatic β -cells are relatively deficient in three major antioxidant enzymes: catalase, GPx, and SOD (Tiedge *et al.* 1997). We hypothesized that β -cells may possess an additional antioxidant protein, Sepp1, as the presence of *Sepp1* mRNA has been demonstrated before in the rodent pancreas and in β -cells (Niwa *et al.* 1997, Lee *et al.* 2008). As both *Sepp1* and *GPx1* mRNA have been detected in liver and kidney at high copy numbers (Hoffmann *et al.* 2007), we first compared their gene expression in liver, kidney, and pancreas taken from rats fed a Se-adequate standard diet. Gene expression of both selenoproteins was in the following order: liver > kidney > pancreas. In pancreas, Sepp1 was expressed relatively higher than GPx1. *Sepp1* mRNA levels in rat pancreas were 58%, whereas pancreatic *GPx1* mRNA levels were only 22% of the liver values (Fig. 1).

In order to examine the localization of Sepp1 in pancreas, we performed staining of mouse pancreas sections by means of fluorescence microscopy. This revealed that pancreatic Sepp1 expression is restricted to

Figure 1

Relative gene expression of Sepp1 and GPx1 in rat tissues. *Sepp1* and *GPx1* mRNA levels were analyzed by real-time RT-PCR and normalized against HPRT1. Data are given as mean $\pm$ s.e.m. from three animals. *Sepp1* and *GPx1* mRNA levels in pancreas and kidney were calculated in relation to the values in liver set as 100%.

the islets, where *Sepp1* appears to be co-localized with both insulin and glucagon (Fig. 2A, B and C). Consistently, immunohistochemistry analysis of mouse pancreas sections demonstrated *Sepp1* expression in both glucagon-producing α -cells and insulin-producing β -cells of the pancreatic islets, whereas no *Sepp1* staining was observed in the exocrine pancreas tissue (Supplementary Figure 1a, b and c, see section on supplementary data given at the end of this article).

Downregulation of *Sepp1* in pancreatic β -cells by high glucose

Next, we investigated the influence of dietary Se compounds and glucose on biosynthesis of *Sepp1* in β -cells *in vitro*, using the glucose-responsive rat insulinoma cell line INS-1 (Asfari *et al.* 1992). Biosynthesis of many selenoproteins depends on adequate Se supply (Steinbrenner & Sies 2009), and consequently, various Se compounds have been shown to stimulate *Sepp1* production in hepatocytes with sodium selenite being the most efficient Se donor (Hoefig *et al.* 2011). As high concentrations of Se compounds may decrease the viability of cultured cells, we first examined the cytotoxicity of three commonly ingested dietary Se compounds in INS-1 cells by MTS assay. Sodium selenate and selenomethionine did not impair the metabolic activity of INS-1 cells up to concentrations of 100 μ M. Sodium selenite was considerably more cytotoxic, as it lowered the metabolic activity to 50% when applied at a concentration of 10 μ M for 24 h (Fig. 3). Thus, we treated the INS-1 cells with 1 μ M of the Se compounds to exclude toxic effects while ensuring sufficient Se supply for biosynthesis of selenoproteins.

Each of the three applied Se compounds significantly upregulated *Sepp1* gene expression in INS-1 cells cultured under normoglycemic conditions (5 mM glucose). The strongest stimulatory effect was elicited by treatment with 1 μ M selenate, yielding a 2.8-fold increase in *Sepp1* mRNA levels compared with Se-deficient controls (Fig. 4A). In order to test for the influence of hyperglycemia, we compared *Sepp1* gene expression in INS-1 cells exposed to Se compounds in culture medium containing 5, 11, or 16.7 mM glucose. High glucose concentrations dose dependently suppressed the Se-induced elevation of *Sepp1* gene expression. Irrespective of the applied Se compound, a significant attenuation of *Sepp1* mRNA levels was observed at 16.7 mM glucose in comparison to 5 mM glucose (Fig. 4A).

We examined whether Se and glucose may influence *Sepp1* promoter activity. From isolated genomic DNA of

Figure 2

Localization of selenoprotein P in mouse pancreatic islets. Sections from pancreas of C57BL/6 mice were analyzed by immunofluorescence staining for *Sepp1* (green) (A), insulin (red) (B), and glucagon (red) (C).

Figure 3

Influence of selenium compounds on the metabolic activity of INS-1 rat insulinoma cells. INS-1 cells were treated for 24 h with sodium selenate, sodium selenite, or L-selenomethionine (SeMet) at the indicated concentrations, and a MTS assay was performed. The metabolic activity of mock-treated cells cultured in serum-free medium was set as 100%. Experiments were performed in triplicate; the data represent mean $\pm$ S.E.M.

INS-1 cells, a 1500 bp fragment located immediately upstream of the translation start site of the predominantly expressed transcript variant 1 of rat *Sepp1* was cloned into a luciferase reporter gene vector. The cloned rat *Sepp1* promoter region was chosen according to the sequence of the human *Sepp1* promoter region (GenBank accession number: Y12262) (Dreher *et al.* 1997). As previously described for the human *Sepp1* promoter (Dreher *et al.* 1997, Speckmann *et al.* 2008), the rat *Sepp1* promoter contains a TATA motif, suggesting *Sepp1* transcription by RNA polymerase II, and a combined binding site for hepatocyte nuclear factor 4 α (HNF-4 α) and forkhead box O1a (FoxO1a), required for high-level *Sepp1* expression in hepatocytes. *Sepp1* promoter activity in INS-1 cells was not significantly altered by Se, whereas high glucose concentrations (11 or 16.7 mM glucose) significantly inhibited *Sepp1* promoter activity when applied together with selenate (Fig. 4B).

As the strongest alterations of *Sepp1* mRNA levels and promoter activity in INS-1 cells were observed with selenate and high glucose (Fig. 4A and B), we investigated the influence of these stimuli on *Sepp1* protein expression. Intracellular *Sepp1* levels were elevated by selenate under normoglycemic conditions, and high glucose concentrations (11 and 16.7 mM glucose) suppressed the stimulatory effect of selenate. However, these changes of *Sepp1* protein expression did not reach statistical significance due to high variation between the experiments (Fig. 4C).

The molecular weight of *Sepp1* detected in INS-1 cell lysates corresponds to the 45 kDa-*Sepp1* isoform previously described in human astrocytes and mouse adipocytes (Steinbrenner *et al.* 2006, Zhang & Chen 2011). As *Sepp1* is primarily known as an extracellular protein (Burk & Hill 2009), we tested INS-1 culture supernatants for the presence of *Sepp1*. However, we did not detect any secreted *Sepp1* isoforms, even in 40-fold concentrated INS-1 supernatants (data not shown).

In order to verify the observed inhibitory effect of glucose on *Sepp1* expression in β -cells, we exposed primary islets isolated from mouse pancreas to increasing glucose concentrations within a range from 2.75 mM (hypoglycemic) to 22 mM (severely hyperglycemic) and analyzed *Sepp1* mRNA levels by real-time RT-PCR. In comparison with 5.5 mM glucose, *Sepp1* gene expression in islets was not altered at 2.75 mM glucose but significantly downregulated to 76% at 11 mM glucose and to 72% at 22 mM glucose respectively (Fig. 5).

High glucose is known to affect RNA stability in β -cells, including the stability of insulin mRNA (Welsh *et al.* 1985). To explore whether *Sepp1* mRNA is degraded faster at elevated glucose concentrations, we treated INS-1 cells with the transcription inhibitor actinomycin D. Addition of actinomycin D lowered *Sepp1* mRNA levels, and this was more pronounced under hyperglycemic conditions: after 20 h of treatment with actinomycin D, *Sepp1* mRNA levels were decreased to 36% at 5 mM glucose and to 12% at 16.7 mM glucose respectively (Fig. 6). Thus, high glucose appears to destabilize *Sepp1* mRNA in β -cells.

Alterations in pancreatic *Sepp1* expression in STZ-treated mice

STZ is frequently used to induce diabetes in animal models. STZ causes necrosis of the insulin-producing β -cells that is primarily mediated by DNA alkylation (Lenzen 2008). In addition, STZ has been shown to induce oxidative/nitrosative stress by generation of superoxide and NO (Nukatsuka *et al.* 1988, Turk *et al.* 1993). To examine the effect of this β -cell toxin on pancreatic *Sepp1* expression, C57BL/6 mice were treated with 40 mg STZ/kg body weight. Blood glucose concentrations were measured every second day. The animals were killed 1 or 2 weeks respectively after STZ treatment. Mice became prediabetic 1 week after STZ treatment, exhibiting fasting blood glucose concentrations from 8.0 to 14.7 mM. After 2 weeks, mice became diabetic with blood glucose concentrations ranging from 17.0 to 18.6 mM. *Sepp1*

expression in pancreatic islets was investigated by histological staining. Pancreatic *Sepp1* expression was elevated after short-term (7 days) exposure to STZ (blood glucose: 8.0 mM) (Fig. 7B and C). In the long term, pancreatic *Sepp1* expression decreased concomitantly with the progressive loss of β -cell mass and resulting increases in blood glucose concentrations after STZ treatment (Fig. 7D and E).

In order to explore whether STZ affects *Sepp1* expression in β -cells *in vitro*, we treated INS-1 cells with STZ (0.5 mM) for 24 h. However, *Sepp1* mRNA levels were not significantly altered by STZ, neither in Se-deficient nor in selenate-supplemented INS-1 cells (data not shown). On the other hand, selenate protected INS-1 cells to some extent from cell death induced by 1 mM STZ (Supplementary Figure 2, see section on supplementary data given

Figure 4

Modulation of *Sepp1* expression in INS-1 rat insulinoma cells by selenium compounds and glucose. INS-1 cells were cultured for 24 h in serum-free medium (-Se) or treated with 1 μ M of the indicated Se compound in combination with glucose at the indicated concentrations. (A) Relative *Sepp1* mRNA levels were determined by real-time RT-PCR, using HPRT1 for normalization. Data represent mean $\pm$ s.e.m. of three independent experiments (* P < 0.05, ** P < 0.01). (B) INS-1 cells were cultured as in Fig. 3 and co-transfected with a luciferase reporter gene construct containing the rat SeP promoter (rat*Sepp1*-luc) together with a renilla luciferase plasmid (pRL-TK). Twenty-four hour posttransfection, luciferase activity was

analyzed in cell lysates. Values are normalized to the activity of the promoter/enhancer-less luciferase construct pGL3. Data are given as mean $\pm$ s.e.m. of three to five independent experiments (** P < 0.01, *** P < 0.001). (C) INS-1 cells were cultured for 24 h in serum-free medium (-Se) or treated with 1 μ M sodium selenate in combination with glucose at the indicated concentrations. *Sepp1* protein levels were determined in immunoblots of cell lysates, using β -actin as loading control. A representative blot out of three experiments is shown (left part). Densitometric analysis of three independent experiments; data are given as mean $\pm$ s.e.m. (right part).

Figure 5

High glucose downregulates SeP mRNA expression in mouse islets. Isolated mouse islets were cultured in Krebs–Ringer buffer with the indicated concentrations of glucose for 24 h. *Sepp1* mRNA levels were analyzed by real-time RT-PCR, normalized against HPRT1, and calculated in relation to 5.5 mM glucose. Data are given as mean $\pm$ S.E.M. from three independent experiments (* $P < 0.05$).

at the end of this article). This suggests that other selenoproteins might be involved in selenate-mediated protection against STZ-induced β -cell death. For example, GPx1 has long been known to be induced by Se supplementation (Steinbrenner & Sies 2009) and GPx1 overexpression protected transgenic mice from STZ-induced β -cell destruction (Harmon *et al.* 2009).

Discussion

Superoxide and H_2O_2 fulfill a dual role in cellular metabolism: low levels serve as second messengers in intracellular signaling cascades but high levels may provoke dysfunction and cell death. In pancreatic β -cells, this notion is exemplified by the action of H_2O_2 on glucose-stimulated insulin secretion (GSIS). While a glucose-induced increase in intracellular H_2O_2 generation is required as a metabolic signal for GSIS (Pi *et al.* 2007), oxidative stress derived from exposure to high concentrations of H_2O_2 blunts GSIS in islets and INS-1 insulinoma cells (Maechler *et al.* 1999). Oxidative stress is also thought to be involved in the deleterious effects of chronic hyperglycemia on β -cell function and integrity, termed glucotoxicity (Poiutout & Robertson 2008, Kim & Yoon 2011). Thus, proper regulation of the cellular redox balance is of prime importance to maintain and adapt pancreatic insulin production and secretion. Low expression and activity of major antioxidant enzymes

such as catalase, SOD, and GPx may render β -cells particularly sensitive to signaling as well as to cytotoxic actions of ROS (Tiedge *et al.* 1997, Pi *et al.* 2007, Lei & Vatamaniuk 2011).

In this study, we demonstrate that β -cells express an additional antioxidant protein, Sepp1. Purified Sepp1 has been shown to reduce PH *in vitro*, although exhibiting lower reactivity than PH-GPx (Takebe *et al.* 2002). Endogenous Sepp1 in β -cells may thus participate in protection of components of the plasma membrane from oxidative damage. As Sepp1 contains up to ten Se atoms (Burk & Hill 2009), it might also serve as cellular Se pool that provides Se for the biosynthesis of selenoenzymes such as GPx. Sepp1 protein expression in pancreas appears to be restricted to islets, where Sepp1 was co-localized with both insulin (β -cells) and glucagon (α -cells) (Fig. 2). In INS-1 insulinoma cells, we found no evidence for secretion of Sepp1. Instead, INS-1 cells produce intracellularly localized Sepp1 that runs in immunoblots as a single band of ~ 45 kDa (Fig. 4C). We described previously a similar pattern of *Sepp1* expression in cultured astrocytes, containing Sepp1 in the form of a non-glycosylated membrane-associated protein (Steinbrenner *et al.* 2006). Two intracellular Sepp1 isoforms with molecular weights of ~ 40 and ~ 45 kDa have been detected in adipocytes, where Sepp1 appears to be required for adipogenic

Figure 6

High glucose destabilizes Sepp1 mRNA in INS-1 cells. INS-1 cells were cultured for 20 h in serum-free medium supplemented with glucose at the indicated concentrations and treated with actinomycin D (2 μ g/ml). *Sepp1* mRNA levels were determined by real-time RT-PCR, using HPRT1 for normalization. Values were calculated in relation to *Sepp1* mRNA levels in INS-1 cells cultured at 5 mM glucose without addition of actinomycin D. Data are given as mean $\pm$ S.E.M. from five independent experiments (* $P < 0.05$).

Figure 7

Modulation of pancreatic Sepp1 expression after STZ treatment of mice. Sections from pancreas of C57BL/6 mice treated with 40 mg STZ/kg body weight were analyzed by immunohistochemistry for the presence of Sepp1

(A) nontreated control (B) and (C) Sepp1 detection 1 week after STZ treatment (D) and (E) Sepp1 detection 2 weeks after STZ treatment. Blood glucose concentrations of the STZ-treated animals are given in parentheses.

differentiation (Zhang & Chen 2011). By contrast, hepatocytes and enterocytes secrete up to four highly glycosylated Sepp1 isoforms (Ma *et al.* 2002, Speckmann *et al.* 2010).

Biosynthesis of Sepp1 in hepatocytes depends on the availability of the micronutrient Se (Burk & Hill 2009). Consistently, we show here that dietary Se compounds increase expression of *Sepp1* in INS-1 cells (Fig. 4). Se has been reported to influence β -cell function: sodium selenite and selenate stimulated biosynthesis and secretion of insulin in Min6 insulinoma cells and isolated rat islets *in vitro*, probably by increasing intracellular GPx activity (Campbell *et al.* 2008). Paradoxically, elevated expression/activity of the selenoenzyme GPx1 in β -cells can elicit opposing metabolic outcomes *in vivo* (Lei & Vatamaniuk 2011). β -Cells of mice with global transgenic overexpression of GPx1 are hypertrophic, showing upregulated expression of pancreatic duodenal homeobox 1 (PDX1) transcription factor and elevated insulin production and secretion. These alterations were not beneficial but resulted in hyperinsulinemia, insulin resistance, and obesity in aged animals (McClung *et al.* 2004, Wang *et al.* 2008). On the other hand, β -cell-specific overexpression of

GPx1 ameliorated hyperglycemia in *db/db* mice and in STZ-treated mice (Harmon *et al.* 2009). Moreover, an adaptive increase in expression levels of antiapoptotic proteins and antioxidant enzymes including GPx1 has been proposed to contribute to survival of hypertrophic β -cells during chronic hyperglycemia (Laybutt *et al.* 2002).

As hepatic gene expression and secretion of Sepp1 is increased at high glucose concentrations (Speckmann *et al.* 2009, Misu *et al.* 2010), we hypothesized that glucose may affect Sepp1 levels in β -cells as well. In contrast to hepatocytes, high glucose (11–22 mM) downregulated *Sepp1* gene expression in isolated islets and decreased Sepp1 promoter activity, mRNA levels, and mRNA stability in INS-1 cells (Figs 4, 5 and 6). Consistently, exposure of isolated islets to high glucose has been shown to downregulate peroxisomal proliferator-activated receptor- γ coactivator 1 α (PGC-1 α ; Zhang *et al.* 2005), a transcriptional coactivator that is required for high-level gene expression of Sepp1 (Speckmann *et al.* 2008). Prolonged hyperglycemia causes dedifferentiation of β -cells, characterized by loss of GSIS, upregulated expression of glycolytic enzymes, and downregulated expression of insulin and β -cell-specific transcription factors such as

PDX1 (Weir & Bonner-Weir 2004, Kim & Yoon 2011). A differentiation-triggered increase in Sepp1 biosynthesis has been observed in intestinal epithelial cells and adipocytes (Speckmann *et al.* 2010, Zhang & Chen 2011). Conversely, the decrease in pancreatic Sepp1 expression at high glucose concentrations may derive from dedifferentiation of β -cells, induced by glucotoxicity. High glucose has been demonstrated to induce the biosynthesis of thioredoxin-interacting protein (Txnip) in pancreatic β -cells (Minn *et al.* 2005). Txnip modulates the cellular redox state by binding and inactivating thioredoxin (Nishiyama *et al.* 1999). Thioredoxin is the preferred electron donor for Sepp1, being 500-fold more effective than glutathione as cofactor for the GPx-like activity of Sepp1 (Takebe *et al.* 2002). Thus, both Sepp1 and its cofactor thioredoxin appear to be repressed in β -cells under hyperglycemic conditions.

To explore the effect of hyperglycemia on pancreatic Sepp1 expression *in vivo*, we applied an animal model of diabetes induction through the β -cell toxin STZ. Expression of the H₂O₂-degrading enzymes peroxiredoxin 1 and peroxiredoxin 2 has been reported to be elevated upon STZ treatment of INS-1 cells (Bast *et al.* 2002). We observed an STZ-induced increase in pancreatic Sepp1 protein levels that lasted as long as blood glucose concentrations remained within the normoglycemic range (Fig. 7). In the long term, progressive β -cell loss and elevated blood glucose concentrations were accompanied by a decrease in Sepp1 expression, suggesting that an adaptive antioxidant response is not sufficient to prevent STZ-induced β -cell death. As shown by other groups (Takeda *et al.* 2012), low-dose STZ leads to α -cell expansion and elevated glucagon levels in the pancreas. Therefore, it cannot be ruled out that Sepp1 expression after STZ treatment localizes to α -cells. However, the expression of Sepp1 in islets is enriched in β -cells compared with α -cells (Niwa *et al.* 1997), suggesting that the expression of Sepp1 in the remaining islet cells is most probably in β -cells.

Taken together, this study provides evidence for the presence of Sepp1 in pancreatic islets and β -cells. Our data suggest that Sepp1 may have a role in antioxidant protection of β -cells and that its downregulation under hyperglycemic conditions might contribute to β -cell failure and loss in the pathogenesis of T2DM.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-12-0105>.

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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