Impact of scavenging hydrogen peroxide in the endoplasmic reticulum for β cell function

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Abstract

Oxidative folding of nascent proteins in the endoplasmic reticulum (ER), catalysed by one or more members of the protein disulfide isomerase family and the sulfhydryl oxidase ER oxidoreductin 1 (ERO1), is accompanied by generation of hydrogen peroxide (H₂O₂). Because of the high rate of insulin biosynthesis and the low expression of H₂O₂-inactivating enzymes in pancreatic β cells, it has been proposed that the luminal H₂O₂ concentration might be very high. As the role of this H₂O₂ in ER stress and proinsulin processing is still unsolved, an ER-targeted and luminal-active catalase variant, ER-Catalase N244, was expressed in insulin-secreting INS-1E cells. In these cells, the influence of ER-specific H₂O₂ removal on cytokine-mediated cytotoxicity and ER stress, insulin gene expression, insulin content and secretion was analysed. The expression of ER-Catalase N244 reduced the toxicity of exogenously added H₂O₂ significantly with a threefold increase of the EC₅₀ value for H₂O₂. However, the expression of cytokine-induced ER stress genes and viability after incubation with β cell toxic cytokines (IL1 β alone or together with TNF α +IFN γ) was not affected by ER-Catalase N244. In control and ER-Catalase N244 expressing cells, insulin secretion and proinsulin content was identical, while removal of luminal H₂O₂ reduced insulin gene expression and insulin content in ER-Catalase N244 expressing cells. These data show that ER-Catalase N244 reduced H₂O₂ toxicity but did not provide protection against pro-inflammatory cytokine-mediated toxicity and ER stress. Insulin secretion was not affected by decreasing H₂O₂ in the ER in spite of a reduced insulin transcription and processing.

Key Words

- ▶ insulin-secreting cells
- ▶ endoplasmic reticulum
- ▶ catalase
- ▶ protein folding
- ▶ reactive oxygen species
- ▶ pro-inflammatory cytokines

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Introduction

Biosynthesis and secretion of insulin in response to glucose are the major tasks of pancreatic β cells. Endoplasmic reticulum (ER) is highly developed in these professional secretory cells to ensure oxidative protein folding and posttranslational modification of the synthesised proteins (Harding & Ron 2002). Since insulin, the main product of protein synthesis in the β cells, is not glycosylated, the most important function of the ER is the

formation of three disulfide bonds in each insulin molecule (Bulleid 2012). During this oxidative folding process the formation of each disulfide bond, thought to be catalysed by one or more members of the protein disulfide isomerase (PDI) family, and the sulfhydryl oxidase ER oxidoreductin 1 (ERO1), is associated with the generation of one molecule hydrogen peroxide (H₂O₂) as a by-product (Tu & Weissman 2004, Ramming &

Appenzeller-Herzog 2012). Through the large number of proinsulin molecules to be synthesised and processed in each β cell, an estimated one million molecules per minute (Scheuner & Kaufman 2008), the ER, along with the mitochondria and peroxisomes, represents a major site of H₂O₂ generation (Tu & Weissman 2004). However, the fate of the ER-generated H₂O₂ and its importance for the maintenance of ER homeostasis and oxidative folding efficacy is still unresolved. A number of studies indicate that this luminal H₂O₂ generation is mandatory for retaining and improving the oxidative protein folding capacity of the ER (Margittai *et al.* 2012, Ramming & Appenzeller-Herzog 2013). This H₂O₂ is required for the re-oxidation of the reduced PDI protein by ER-localised glutathione peroxidases (GPx7 and GPx8) and potentially also by peroxiredoxin 4 (Prdx4). On the other hand, the activation of the ER stress pathway, which may lead to apoptotic cell death, has also been associated to the fulminant ROS generation in the ER (Malhotra *et al.* 2008, Bhandary *et al.* 2012, Cao & Kaufman 2014).

To shed light on this contentious issue, we took advantage of a recently developed catalase variant, ER-Catalase N244, which shows full enzyme activity upon expression in the ER (Lortz *et al.* 2015). In insulin-secreting INS-1E tissue culture cells, we studied the influence of a high luminal H₂O₂ inactivating activity on insulin expression and secretion. Furthermore, the role of ER-derived H₂O₂ in the initiation of ER stress cascades and the reduction of cell viability through β cell toxic cytokines has been investigated.

Materials and methods

Tissue culture of insulin-producing cells

Insulin-secreting INS-1E tissue culture cells (kindly provided by C. Wollheim, University of Geneva Medical Center, Geneva, Switzerland) were cultured in RPMI 1640 medium supplemented with 10 mmol/l glucose, 10% (v/v) FCS, 10 mmol/l HEPES, 1 mmol/l sodium pyruvate, 50 μ mol/l 2-mercaptoethanol, penicillin, and streptomycin in a humidified atmosphere at 37 °C and 5% CO₂ as described previously (Asfari *et al.* 1992).

Lentiviral expression of ER-localised catalase in INS-1E cells

To express catalase specifically in the ER, the mutated catalase variant ER-Catalase N244 cDNA was used (Lortz *et al.* 2015). For this purpose, lentiviral particles were

prepared as described before in detail (Zufferey *et al.* 1998). 4 $\times$ 10⁶ 293T cells were transfected with the packaging plasmid pPAX2 (11.25 μ g), the envelope plasmid pcDNA-MDG (3.75 μ g), and the transfer plasmid pLenti6.3/V5-MCS-ER-Catalase N244 (15 μ g) by calcium phosphate precipitation. After 48 h the virus containing culture medium was collected and centrifuged for 5 min at 700 *g* to remove detached cells and cell debris; then the supernatant was filtered through 0.22 μ m filters (Merck Millipore, Darmstadt, Germany). The INS-1E cells were infected with the purified viral supernatant for 5–6 h and thereafter the viral supernatant was replaced by fresh medium. Transduced cells were selected for catalase expression by 1 μ M blasticidin (Life Technologies). These selected cells represented a mixed cell population. Thus, integration of the lentiviral construct happens randomly at various genomic locations and possible undesirable interactions with specific genes, altering β cell function, are less likely. Therefore the use of these cells, in contrast to single cell clones, can avoid unwanted clonal selection artefacts.

Quantification of catalase activity

Catalase activity was quantified as described (Tiedge *et al.* 1998). Briefly, whole cell extracts were prepared in 50 mmol/l potassium phosphate buffer (pH 7.8) through sonication on ice with a Braun-Sonic 125 sonifier (Braun, Melsungen, Germany). The homogenates were then centrifuged at 10 000 *g* and 4 °C for 10 min and the protein content of the supernatant was determined by the BCA assay (Thermo Fisher Scientific, Rockford, IL, USA). The catalase activity was measured by ultraviolet spectroscopy, monitoring the decomposition of H₂O₂ at 240 nm.

Immunofluorescence staining

INS-1E cells expressing ER-Catalase N244 or WT catalase without an ER-specific targeting signal were seeded at a density of 100 000 cells/well on LabTek chamber slides (Nunc, Roskilde, Denmark). After 48 h, the cells were washed twice with PBS and subsequently fixed with 4% paraformaldehyde at room temperature for 1 h. After washing, the cells were permeabilised and blocked with PBS containing 0.2% Triton X-100 and 1% BSA. The cells were incubated with primary antibodies (anti-PDI, ab5484, Abcam, Cambridge, UK, 1:100 dilution; anti-catalase, 100–4151, Rockland Immunochemicals, Inc., Limerick, PA, USA, 1:500 dilution) diluted in PBS containing 0.1% Triton X-100 and 0.1% BSA at room temperature for 1 h. Thereafter the cells were washed with

PBS and incubated with specific secondary antibodies (anti-mouse-Alexa Fluor 647, or anti-rabbit-Alexa Fluor 488, Jackson ImmunoResearch Laboratories, West Grove, PA, USA; 1:200 dilutions) for 1 h in the dark. Afterwards the cells were washed and the nuclei were counterstained with 300 nM DAPI for 5 min at room temperature. Finally, the cells were washed and mounted with Mowiol/DABCO anti-photobleaching mounting media (Sigma). Stained cells were examined with an Olympus IX81 inverted microscope (Olympus) and microscopic images were post-processed using AutoDeblur and AutoVisualize (Autoquant Imaging, New York, NY, USA).

Quantification of cell viability after H₂O₂ treatment

After 24 h seeding of 40 000 cells/well of a 96-well plate, cells were incubated with the indicated H₂O₂ concentrations (INS-1E: 0–125 µM, INS-1E ER-Catalase: 0–1250 µM) for 2 h in 20 mmol/l HEPES-supplemented Krebs–Ringer bicarbonate medium with 5 mmol/l glucose. After removal of the H₂O₂ containing medium the cells were incubated for another 22 h in fresh RPMI 1640 medium and thereafter cell viability was determined by a microplate-based MTT assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) (Mosmann 1983). Briefly, the incubation medium was carefully removed and replaced by MTT-solution (0.5 mg/ml). After 1 h incubation at 37 °C, the MTT solution was carefully removed and the intracellularly generated water-insoluble formazan crystals were dissolved in DMSO. The absorbance was quantified using a microplate photometer (Powerwave 340, BioTek Instruments, Winooski, VT, USA).

Quantification of cell viability after exposure to pro-inflammatory cytokines

Cells were seeded at a density of 25 000 cells/well in 100 µl culture medium onto 96-well plates and allowed to attach for 24 h before they were incubated for 72 h with 600 U/ml human IL1β or a combination of cytokines (cytokine mixture) consisting of 60 U/ml IL1β, 185 U/ml human TNFα, and 14 U/ml IFNγ (PromoCell, Heidelberg, Germany). Thereafter the cell viability was determined by using a microplate-based MTT assay.

Quantification of ER stress after exposure to pro-inflammatory cytokines

Cells were seeded on 6 cm tissue culture plates at a density of 1×10^6 cells and allowed to attach for a period of 24 h.

Thereafter the cells were exposed for 16 h to 600 U/ml human IL1β or a combination of cytokines (cytokine mixture) consisting of 60 U/ml IL1β, 185 U/ml human TNFα, and 14 U/ml IFNγ. After cytokine incubation total RNA was isolated as previously described (Tiedge *et al.* 1998). For cDNA synthesis, random hexamers were used to prime the reaction of the RevertAid H- M-MuLV reverse transcriptase (Life Technologies). The reactions were performed with GoTaq qPCR Master Mix (Promega) in a ViiA 7 real-time PCR system (Life Technologies). Samples were denatured at 94 °C for 3 min followed by 40 PCR cycles. Each cycle comprised a melting step at 94 °C for 30 s, an annealing step at 60 °C for 30 s, and an extension step at 72 °C for 30 s. Optimal parameters for the PCR reactions were empirically defined and the purity and specificity of the amplified PCR product in each experiment was verified by melting curve analysis. All transcripts showed *C_t*-values, which were at least ten *C_t*-values lower than the blank values. Each PCR amplification was performed in triplicate. Data are expressed as relative gene expression after normalisation against the geometric mean of the housekeeping genes *Tuba4a*, *Actb* and *Ppia* with qbasePLUS (Biogazelle, Zulte, Belgium). The primer sequences are listed in Table 1.

Quantification of glucose-induced insulin secretion, insulin and proinsulin content

INS-1E cells were seeded in six-well plates at a density of 0.5×10^6 cells and grown for 48 h. Then the cells were incubated for 1 h in bicarbonate-buffered Krebs–Ringer solution without glucose, supplemented with 0.1% albumin. Thereafter the cells were stimulated for 2 h either with 3, 10, or 30 mmol/l glucose. After incubation, medium was removed and gently centrifuged to remove detached cells. Secreted insulin in the supernatant and insulin content of the incubated cells were determined by RIA using rat insulin as standard and the resulting values were normalised to DNA content. Proinsulin content was quantified by a rat proinsulin specific ELISA according to the manufacturer's protocol (Mercodia, Uppsala, Sweden).

Statistical analyses

Data are expressed as means $\pm$ S.E.M. Statistical analyses were performed using ANOVA plus Bonferroni test for multiple comparisons or unpaired two-tailed Student's *t*-test. The EC₅₀ values for the H₂O₂-induced toxicity were calculated from the concentration-response curves fitted by the sigmoidal concentration-response logarithm.

Table 1 List of primers for the detection of ER stress-related genes by RT-qPCR

Gene	Gene symbol	Oligonucleotide sequence (5'–3')
ER stress specific genes		
Activating transcription factor 4	<i>Atf4</i>	Forward: 5'-AATGGCTGGCTATGGATGGG-3' Reverse: 5'-TCTGTCCCGGAAAAGGCATC-3'
Activating transcription factor 6	<i>Atf6</i>	Forward: 5'-GAGAGGTGTCTGTTTCGGGG-3' Reverse: 5'-CGTGGACTCCCAGTCTTCAC-3'
C/EBP homologous protein	<i>Chop</i>	Forward: 5'-CCAGCAGAGGTACAAGCAC-3' Reverse: 5'-CGCACTGACCACTCTGTTTC-3'
Glucose-regulated protein, 78 kDa	<i>Grp78/Bip</i>	Forward: 5'-CCACCAGGATGCAGACATTG-3' Reverse: 5'-AGGGCCTCCACTCCATAGA-3'
Housekeeping genes		
α -tubulin 4a	<i>Tuba4a</i>	Forward: 5'-ACGTGAGACGTACATCCCAAACTCA-3' Reverse: 5'-CTCCAGCAGGCATTGCCCA-3'
β -actin	<i>Actb</i>	Forward: 5'-GCGTCCACCCGCGAGTACAA-3' Reverse: 5'-TTGCAATGCCGAGCCGTT-3'
Peptidylprolyl isomerase A (cyclophilin A)	<i>Ppia</i>	Forward: 5'-TTGCAGACGCCGTGTCTCTT-3' Reverse: 5'-TGGAACCTTGTCTGCAACAGCTCG-3'

All analyses were performed with the GraphPad Prism 5.03 Software (Graphpad, San Diego, CA, USA).

Results

Subcellular localisation of ER-Catalase N244 and quantification of ER-Catalase N244 enzyme activity and its effect on H₂O₂ induced toxicity in INS-1E cells

To document the functional expression of ER-Catalase N244 in insulin-secreting INS-1E cells, the enzyme activity

of cell lysates was quantified after lentiviral transduction. ER-Catalase-N244 expressing INS-1E cells showed with 148.5 ± 4.9 U/mg protein a significantly higher enzyme activity than untransfected control cells (10.3 ± 3.2 U/mg protein, $P < 0.001$) (data not shown). As the ER-specific expression is crucial, an immunofluorescence staining was performed. The co-staining of catalase and PDI in Fig. 1 documents a colocalisation in the ER confirming the ER-specific expression of ER-Catalase N244 in the INS-1E cells. In contrast, INS-1E cells overexpressing the unmodified WT catalase showed no clear colocalisation

Figure 1

Subcellular localisation of ER-Catalase N244 and WT catalase in insulin-secreting INS-1E cells. Cells were seeded on LabTek chamber slides. After fixation and permeabilisation the cells were stained with specific antibodies

for catalase (green) and for the ER-specific protein disulfide isomerase (PDI, red), followed by counterstaining with DAPI (blue). A full colour version of this figure is available at <http://dx.doi.org/10.1530/JME-15-0132>.

Figure 2

Effects of ER-Catalase N244 expression on cytotoxicity after incubation of INS-1E cells with H₂O₂. INS-1E control and ER-Catalase N244 expressing cells were incubated for 2 h with the indicated H₂O₂ concentrations in Krebs–Ringer medium, and after removal of the H₂O₂-containing medium for another 22 h in fresh medium. Thereafter, the viability of the cells was determined by the MTT assay and expressed as % of untreated cells. Data are expressed as mean ± s.e.m. of six to eight independent experiments. ****P* < 0.001 compared with control cells (unpaired two-tailed Student's *t*-test).

of catalase and PDI (Fig. 1, lower panel). Thus, only the ER-targeted catalase variant ER-Catalase N244 was expressed in the ER, while the native catalase protein was localised outside the ER, presumably in the cytosol and the peroxisomes. To demonstrate the *in vivo* functionality of the expressed ER-Catalase N244 and to provide an estimate of its H₂O₂ inactivating capacity, INS-1E control and ER-Catalase N244 expressing cells were incubated with increasing H₂O₂ concentrations and the cell viability was determined thereafter. Both cell clones showed a concentration-dependent decrease of their cell viability with an EC₅₀ value of 45.6 μM H₂O₂ for the control cells (Fig. 2) and a significantly higher EC₅₀ value of 132 μM for

the ER-Catalase N244 expressing cells (*P* < 0.001, Fig. 2). These differences in the EC₅₀ values were especially obvious at concentrations up to 100 μM, which is the most relevant range for physiological and pathological processes. At 50 μM H₂O₂, the ER-Catalase expressing cells showed no decrease in cell viability and at 100 μM a residual cell viability of still 75% could be detected, whereas the control cells showed at the same H₂O₂ concentrations only a residual viability of 42 and 2%, respectively (Fig. 2).

Effects of ER-Catalase N244 on the ER stress response after exposure of INS-1E cells to pro-inflammatory cytokines

To determine the influence of ER-specific detoxification of H₂O₂ on the initiation of ER stress in response to β cell toxic cytokines, the gene expression of three major mediators of ER stress, namely *Chop*, *Atf4*, and *Atf6*, and of the ER-chaperon *Grp78/Bip* was quantified by RT-qPCR. As shown in Fig. 3A, a 16 h exposure to 600 U/ml IL1β alone resulted in a sixfold and incubation with a cytokine mixture (60 U/ml IL1β, 185 U/ml TNFα, and 14 U/ml IFNγ) in a 20-fold increase of *Chop* gene expression in INS-1E control cells. Both, *Atf4* and *Atf6*, were induced by the cytokine treatment, but to a much lower extent compared with *Chop*. However, *Atf4* gene expression was induced by IL1β (2.4-fold) and the cytokine mixture (4.2-fold), as well as *Atf6* (1.2- and 2.2-fold respectively) (Fig. 3B and C). At variance from the increased expression of the investigated ER stress mediators *Chop*, *Atf4*, and *Atf6*, the expression of the ER located chaperon *Grp78/Bip* was significantly reduced by IL1β alone and the cytokine mixture. After both incubations *Bip* expression was reduced approx. 40%.

Figure 3

Effects of ER-Catalase N244 expression on the induction of ER stress genes after incubation of INS-1E cells with cytokines. INS-1E control and ER-Catalase N244 expressing cells were incubated for 16 h under control conditions, with 600 U/ml IL1β or with a cytokine mixture, composed of 60 U/ml IL1β, 185 U/ml TNFα, and 14 U/ml IFNγ. After incubation, total RNA was isolated and the expression of *Chop* (A), *Atf4* (B), *Atf6* (C), and

Grp78/Bip (D) was analysed by RT-qPCR with specific primers. The expression levels were normalised to the housekeeping genes *Tuba4a*, *Actb*, and *Ppia*. The gene expression level of untreated control cells was set as 100%. Data are expressed as means ± s.e.m. of three independent experiments. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 compared with untreated cells (ANOVA plus Bonferroni test for multiple comparisons).

The expression level of all four investigated ER stress related genes in ER-Catalase N244 expressing INS-1E cells under control conditions and after cytokine exposure was comparable to that in control cells and no significant differences could be detected. These data indicate that expression of ER-Catalase N244 in INS-1E cells and thereby reduction of luminal H₂O₂ did not alter the expression of the ER stress-induced genes *Chop*, *Atf4*, and *Atf6* and of the ER located chaperon *Grp78/Bip* after cytokine exposure.

Effects of ER-Catalase N244 on cell viability after exposure of INS-1E cells to pro-inflammatory cytokines

After having shown that ER-specific inactivation of H₂O₂ had no influence on the cytokine-induced expression of ER stress genes, we also analysed the effect of ER-Catalase N244 expression on cytokine-mediated cellular toxicity.

Therefore transfected and untransfected insulin-secreting INS-1E cells were incubated again with 600 U/ml IL1 β or with the cytokine mixture (60 U/ml IL1 β , 185 U/ml TNF α , and 14 U/ml IFN γ). After a 72 h incubation with IL1 β a substantial 85% loss of viability could be detected for the INS-1E control cells ($P < 0.001$). With the cytokine mixture this loss of viability was only slightly augmented (Fig. 4). The viability of ER-Catalase N244 expressing INS-1E cells after cytokine treatment did not significantly differ from untransfected control cells.

Figure 4

Effects of ER-Catalase N244 expression on cytotoxicity after incubation of INS-1E cells with cytokines. INS-1E control and ER-Catalase N244 expressing cells were incubated for 72 h under control conditions, with 600 U/ml IL1 β or with a cytokine mixture, consisting of 60 U/ml IL1 β , 185 U/ml TNF α , and 14 U/ml IFN γ . The cell viability was determined thereafter by the MTT viability assay and expressed as % of untreated cells. Data are expressed as means $\pm$ s.e.m. of five independent experiments. *** $P < 0.001$ compared with untreated cells (ANOVA plus Bonferroni test for multiple comparisons).

Effects of ER-Catalase N244 on glucose-induced insulin secretion and insulin content of INS-1E cells

To study the influence of ER-specific H₂O₂ deprivation on glucose-induced insulin secretion, INS-1E cells were incubated for 2 h either with 3, 10, or 30 mM glucose and thereafter the amount of secreted insulin was quantified in the supernatant. Basal insulin secretion of INS-1E control cells at 3 mM glucose was 0.6 ng/(μ g/DNA per h) and was more than fourfold elevated by 10 and 30 mM glucose to ~ 2.8 ng/(μ g/DNA per h) (10 mM, $P < 0.001$; 30 mM, $P < 0.005$) (Fig. 5A). The insulin content of the INS-1E control cells was 28 ng/ μ g DNA and the proinsulin content was 5–6 ng/ μ g DNA, both without significant differences between the different glucose concentrations (Fig. 5B and C). The rate of glucose-induced insulin secretion from the ER-Catalase N244 expressing INS-1E cells was comparable to that of control INS-1E cells at all glucose concentrations, including the rate of basal insulin secretion at 3 mM (Fig. 5A). Although the proinsulin content in the ER-Catalase N244 transfected INS-1E cells remained unchanged at all three glucose concentrations, the insulin content was significantly lower and represented only two-thirds of that of untransfected control cells (Fig. 5B and C). Thus, the ratio between insulin and proinsulin content in control INS-1E cells was almost twice as high as in ER-Catalase N244 expressing cells (Fig. 5D).

Effects of ER-Catalase N244 on insulin gene expression in INS-1E cells

To determine whether the observed decreased insulin content might be a result of a lower insulin biosynthesis, the mRNA expression level of the insulin 1 and insulin 2 genes was quantified. Although the insulin 1 gene expression was the same in both cell clones, the expression of the insulin 2 gene was, in agreement with the reduced insulin content of ER-Catalase expressing INS-1E cells, 30% lower than in control cells (Fig. 6).

Discussion

H₂O₂ generation in the ER through PDI and ERO1, the main enzymes of the oxidative protein folding machinery, is a physiological process in all secretory cells including pancreatic β cells (Harding *et al.* 2003, Gross *et al.* 2006). Therefore, the question is how cells can cope with these high luminal H₂O₂ concentrations and if those huge amounts of H₂O₂ may support the folding of nascent

Figure 5

Effects of ER-Catalase N244 expression on glucose-induced insulin secretion, insulin content, and proinsulin content in insulin-secreting INS-1E cells. After 1 h incubation at 0 mmol/l glucose INS-1E control and ER-Catalase N244 expressing cells were incubated separately for 2 h with the indicated glucose concentrations. Thereafter insulin secretion (A) and insulin content (B) were determined by radioimmunoassay. The proinsulin content was quantified by a proinsulin-specific ELISA (C). The insulin

content to proinsulin content ratio was calculated from the presented content data (D). Data are expressed as means $\pm$ S.E.M. of 12 (insulin secretion and content) or nine (proinsulin content) independent experiments. ** $P < 0.01$ and *** $P < 0.001$ compared with cells incubated at 3 mmol/l glucose (ANOVA plus Bonferroni test for multiple comparisons). # $P < 0.05$ and ## $P < 0.01$ compared with control cells incubated with the same glucose concentration (unpaired two-tailed Student's *t*-test).

proteins. Both aspects are particularly relevant for insulin-secreting cells to satisfy the high insulin biosynthesis demand, which accounts for roughly 50% of protein synthesis under physiological conditions (Schuit *et al.* 1991). States of increased insulin demand, such as insulin resistance in type 2 diabetes, require a further increase of the insulin secretory capacity (DeFronzo *et al.* 1989). In addition, the expression of H₂O₂-degrading enzymes such as catalase and glutathione peroxidase and also of the ER-resident H₂O₂-metabolising enzyme Prdx4 is very low in insulin-secreting cells (Tiedge *et al.* 1998, Mehmeti *et al.* 2014). Thus, a possible effect of luminal H₂O₂ on oxidative protein folding is likely to be particularly prominent in insulin-secreting cells.

Moreover, there is increasing evidence that ER stress induced by β cell toxic cytokines may raise ROS production in the ER (Malhotra & Kaufman 2007). Although the involvement of nitric oxide and the disturbance of the calcium homeostasis in the ER have been identified as crucial mediators (Oyadomari *et al.* 2001, Cardozo *et al.* 2005), the relevance of luminal H₂O₂ for these processes is still unsolved.

We therefore investigated the importance of ER-specific H₂O₂ degradation in insulin-secreting INS-1E cells for insulin gene expression, insulin content and secretion, as well as for the activation of ER stress cascades through β cell toxic cytokines.

In the present study, we could show that insulin-secreting INS-1E tissue culture cells were successfully protected against H₂O₂-induced loss of viability through the expression of ER-Catalase N244. The threefold increase of the EC₅₀ value for H₂O₂ toxicity documents convincingly the high H₂O₂ inactivation capacity of ER-Catalase N244, also when compared with the overexpression of Prdx4 (Mehmeti *et al.* 2012, Mehmeti *et al.* 2014) or the

targeted expression of a WT catalase cDNA in the ER (Margittai *et al.* 2012).

This high luminal catalase activity did not change the expression of classical ER stress genes under control conditions and, more importantly, did not counteract the cytokine-induced initiation of the ER stress pathway. Furthermore, the expression of the luminally located protein *Grp78/Bip*, serving as a central sensor for ER stress due to its function as a molecular chaperone and the activation of the ER stress pathway through its dissociation from IRE1, PERK, and ATF6 (Bertolotti *et al.*

Figure 6

Effects of ER-Catalase N244 expression on insulin 1 and 2 gene expression in insulin-secreting INS-1E cells. INS-1E control (open bars) and ER-Catalase N244 (black bars) expressing cells were incubated for 24 h under control conditions and thereafter total RNA was isolated and gene expression was analysed by RT-qPCR with insulin 1 and insulin 2 specific primer sets. The expression levels were normalised to the housekeeping genes *Tuba4a*, *Actb*, and *Ppia*. Data are expressed as means $\pm$ S.E.M. of five independent experiments. * $P < 0.05$ compared with control cells (unpaired two-tailed Student's *t*-test).

2000, Schröder & Kaufman 2005), was downregulated by the used cytokines independently of the ER-Catalase N244 expression.

These observations indicate either that the luminal H₂O₂ concentration is not altered by pro-inflammatory cytokines or that the luminal H₂O₂ is not involved in the cytokine-mediated ER stress response. Additionally, ER-Catalase N244 could not antagonise the IL1 β - or cytokine mixture-induced cell death, indicating that generation of luminal H₂O₂ at variance to mitochondrial H₂O₂ is not involved in this process (Gurgul-Convey *et al.* 2011, Mehmeti *et al.* 2011).

Although a stimulatory function of H₂O₂ on insulin translation and secretion has been considered, the question of a direct influence of H₂O₂ on glucose-induced insulin secretion is yet unresolved. As an explanation, the unusually low expression of H₂O₂-inactivating enzymes in insulin-secreting cells (Tiedge *et al.* 1998) has been put forward (Pi *et al.* 2007, Leloup *et al.* 2009, Zito *et al.* 2010). On the other hand, it has been considered that stimulation of insulin biosynthesis in states of increased insulin demand such as insulin resistance in the early stage of type 2 diabetes is accompanied by increased H₂O₂ generation in the ER, causing defective insulin secretion, β cell dysfunction and ultimately glucose intolerance (Back & Kaufman 2012). However, an effective removal of H₂O₂ in the ER (present study) as well as in mitochondria by a mitochondrially located catalase (Lortz *et al.* 2013) did not affect glucose-induced insulin secretion. These results do not provide support for a significant role of H₂O₂ in glucose-stimulated insulin secretion.

Nevertheless, we could observe a reduction of insulin 2 mRNA expression, insulin content and a decrease of the insulin/proinsulin ratio due to an unchanged proinsulin content. This is in contrast to previous findings after Prdx4 overexpression, which increased insulin 1 and 2 mRNA expression, insulin content and glucose-induced insulin secretion (Mehmeti *et al.* 2014). These results indicate that efficient removal of H₂O₂ as such by ER-Catalase N244 does not favour proinsulin protein folding. ER-Catalase N244 expression could not replicate the positive effects of Prdx4 overexpression on gene expression, biosynthesis and secretion of insulin (Mehmeti *et al.* 2014).

In conclusion, the present results show that effective elimination of luminal H₂O₂ by ER-Catalase N244 left glucose-induced insulin secretion unaffected, even if insulin gene expression and insulin content were reduced. Cellular toxicity induced by pro-inflammatory cytokines and accompanying ER stress were also not abolished.

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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Author contribution statement

S Lo and I M contributed to the design, conduct, data analysis and manuscript preparation. S Le contributed to the planning, design, data analysis, and manuscript preparation. All authors approved the final manuscript.

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