Overexpression of the antioxidant enzyme catalase does not interfere with the glucose responsiveness of insulin-secreting INS-1E cells and rat islets

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Received: 7 June 2012 / Accepted: 12 December 2012 / Published online: 11 January 2013
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Abstract
Aims/hypothesis Hydrogen peroxide (H₂O₂)-inactivating enzymes such as catalase are produced in extraordinarily low levels in beta cells. Whether this low expression might be related to a signalling function of H₂O₂ within the beta cell is unknown. A high level of H₂O₂-inactivating enzymes could potentially be incompatible with glucose-induced insulin secretion. Therefore the effect of catalase overexpression on mitochondrial function and physiological insulin secretion was studied in insulin-secreting INS-1E and primary islet cells.

Methods INS-1E and rat islet cells were lentivirally transduced to overexpress catalase in the cytosol (CytoCat) or in mitochondria (MitoCat). Cell viability and caspase-3 activation were assessed after cytokine incubation and hypoxia. Insulin secretion was quantified and expression of the gene encoding the mitochondrial uncoupling protein 2 (Ucp2) was measured in parallel to mitochondrial membrane potential and reactive oxygen species (ROS) formation.

Results The ability to secrete insulin in a glucose-dependent manner was not suppressed by catalase overexpression, although the glucose-dependent increase in the mitochondrial membrane potential was attenuated in MitoCat cells along with an increased Ucp2 expression and reduced mitochondrial ROS formation. In addition, MitoCat overexpressing cells were significantly more resistant against pro-inflammatory cytokines and hypoxia than CytoCat and control cells.

Conclusions/interpretation The results demonstrate that an improved antioxidative defence status of insulin-secreting cells allowing efficient H₂O₂ inactivation is not incompatible with proper insulin secretory responsiveness to glucose stimulation and provide no support for a signalling role of H₂O₂ in insulin-secreting cells. Interestingly, the results also document for the first time that the decreased ROS formation with increasing glucose concentrations is of mitochondrial origin.

Keywords Catalase · Glucose-stimulated insulin secretion · Insulin-secreting cells · Pro-inflammatory cytokines · Reactive oxygen species

Abbreviations
C₉ Cycle threshold
CytoCat Cytosolic catalase
DCFDA-H₂ Dichlorodihydrofluorescein diacetate
DNP 2,4-Dinitrophenol
ECM Extracellular matrix
GSIS Glucose-stimulated insulin secretion
MitoCat Mitochondrial catalase
MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
ROS Reactive oxygen species
UCP2 Uncoupling protein 2

Introduction
Pancreatic beta cells are characterised by their very low levels of the hydrogen peroxide (H₂O₂)-inactivating enzymes catalase and glutathione peroxidase despite adequate expression of the superoxide (O₂⁻) -inactivating
superoxide dismutases [1, 2]. This imbalance results in beta cells having a high susceptibility to the toxicity of H$_2$O$_2$ and other reactive species (ROS) derived from it [3]. The extraordinary vulnerability of beta cells to ROS can explain a major part of pro-inflammatory cytokine toxicity resulting in the specific destruction of beta cells during type 1 diabetes [4]. The underlying reason for this lack of protection of beta cells against ROS toxicity is not yet clear. A possible reason might be that H$_2$O$_2$-inactivating enzymes interfere with physiological beta cell function. Genes associated with such an interfering effect have been termed ‘disallowed genes’ [5] and include $Hkl$ [6], the lactate dehydrogenase isofrom $Ldha$ and the monocarboxylate transporter $Mctl$ [7–10]. Expression of these genes in beta cells interferes with the normal control of insulin secretion by the physiological stimulus glucose, resulting in inappropriate insulin release (e.g. after physical exercise or in the fasted state) [11]. $Cat$ mRNA was also found to be selectively repressed in mouse islets as compared with a large tissue panel [8, 12]. A high expression level of H$_2$O$_2$-inactivating enzymes could potentially be detrimental for proper beta cell function. If so, a rapid dismutation of H$_2$O$_2$ through an efficient inactivating enzyme would interfere with beta cell metabolism, most likely manifesting as an impaired glucose-stimulated insulin secretion (GSIS).

This idea has been taken up by the hypothesis that H$_2$O$_2$, as a relatively stable and freely diffusible molecule, might serve as an intracellular second messenger for GSIS in the beta cell [13, 14]. Thus, high expression levels of the H$_2$O$_2$-inactivating enzymes catalase and glutathione peroxidase might interfere with the appropriate physiological insulin secretory response to rising postprandial glucose concentrations [13, 14]. Contrary to this idea it was hypothesised that further reduction of glutathione peroxidase enzyme activity at low glucose concentrations due to low cellular NADPH content can lead to H$_2$O$_2$ accumulation, which might prevent inappropriate insulin release [12].

It was therefore the aim of this study to elucidate whether an increase of the enzymatic H$_2$O$_2$ inactivation capacity through catalase overexpression in mitochondria or in the cytosol of insulin-secreting cells can protect against the toxicity of cytokines and hypoxia, without concomitantly disturbing insulin secretion in response to the physiological stimulus glucose.

**Methods**

*Tissue culture of INS-1E cells* Insulin-secreting INS-1E cells (kindly provided by C. Wollheim, University of Geneva Medical Center, Geneva, Switzerland) were cultured as previously described [15].

*Islet isolation and single-cell preparation* Pancreatic islets were isolated from 250–300 g adult male Lewis rats (Charles River, Sulzfeld, Germany) by collagenase digestion and handpicked under a stereo microscope. Thereafter, 70–100 uniformly sized isolated islets were cultured on extracellular matrix (ECM)-coated plates (35 mm) (Novamed, Jerusalem, Israel) in RPMI-1640 medium containing 5 mmol/l glucose, 10% FCS, penicillin and streptomycin at 37°C in a humidified atmosphere of 5% CO$_2$ as described earlier in detail [16]. Islets were cultured for 7–10 days on ECM plates to adhere and spread before they were lentivirally transduced and subsequently incubated with pro-inflammatory cytokines or used for insulin secretion studies. All animal procedures were conducted in accordance with the Principles of Laboratory Care.

*Preparation of lentiviruses* To overexpress the hydrogen peroxide-inactivating enzyme catalase in the cytosol (CytoCat) or in mitochondria (MitoCat) of INS-1E cells and rat islet cells, the cDNA of this enzyme [17, 18] was subcloned into the pLenti6/V5-MCS vector by standard molecular techniques. Lentiviral particles were prepared according to [19] and the virus titres were quantified as described elsewhere [20].

*Lentiviral transduction* INS-1E cells were separately infected with one of the constructs at a multiplicity of infection (MOI) of 10 for 2 h. Thereafter the culture medium was changed. After an additional 24 h the cells were selected for catalase expression by blasticidin (1 μmol/l). The selected cells represented a mixed cell population. In contrast to single-cell clones the use of these cells should avoid clonal selection artefacts. Successful catalase overexpression was verified by western blot analyses and catalase activity measurement as previously described [17]. Islet cells were transduced on ECM-coated plates in the same way as INS-1E cells. After transduction the cells were kept in culture under control conditions for an additional 72 h for appropriate catalase expression.

*Measurement of insulin secretion* 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, and thereafter stimulated for 1 h with 3, 10, or 30 mmol/l glucose. After the incubation, the medium was removed and gently centrifuged to remove detached cells. Secreted insulin in the supernatant fraction was determined by radioimmunoassay using rat insulin as standard and the resulting values were normalised to DNA content. Primary islets were pre-incubated on ECM-coated plates for 1 h in bicarbonate-buffered Krebs-Ringer solution without glucose, supplemented with 0.1% albumin. Thereafter
the cells were trypsinised, centrifuged, resuspended and incubated in Krebs-Ringer solution containing 3, 10 or 30 mmol/l glucose for 1 h. As for the INS-1E cells, the supernatant fraction was used for quantification of secreted insulin and the obtained values were normalised to DNA content.

**Incubation of INS-1E cells with 2,4-dinitrophenol** For mild uncoupling of the mitochondrial membrane potential, INS-1E cells were incubated with 25 μmol/1 2,4-dinitrophenol (DNP). Either the mitochondrial membrane potential was quantified by the use of rhodamine-123 or insulin secretion was quantified by RIA in the presence of DNP.

**Measurement of cell viability after cytokine exposure and hypoxia** For 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyterazolium bromide (MTT) assays 15,000 control and overexpressing INS-1E cells were seeded per well in 100 μl culture medium in 96-well microplates and allowed to attach for 24 h. Thereafter the cells were incubated for 72 h with 60 or 600 U/ml human IL-1β (PromoCell, Heidelberg, Germany) or a cytokine combination containing 60 U/ml IL-1β, 185 U/ml human TNF-α and 14 U/ml rat IFN-γ (PromoCell). For incubation under hypoxic conditions the cells were incubated with 1% O₂ balanced with N₂ for hypoxia. Hypoxia was generated in an oxygen-regulated incubator (CB210 incubator with O₂ control option; Binder, Tutlingen, Germany). After incubation, the viability of the cells was determined by the MTT assay as described earlier [17]. Viability was expressed as per cent of untreated cells. The viability under control conditions and the absolute optical density (OD₅₅₀) absorbance rates measured with the MTT assay were not significantly different between the control and overexpressing INS-1E cell clones, indicating that the cells showed no significant differences in metabolism, proliferation or basal viability.

**Flow cytometric quantification of caspase-3 activation** Caspase-3 activation was determined with the CaspGLOW fluorescent active caspase-3 staining kit (PromoCell). Control, CytoCat and MitoCat overexpressing INS-1E cells were seeded at a density of 1 × 10⁶ cells per well of a six-well-plate and allowed to attach for 48 h before incubation with the indicated cytokines. Islet cells were incubated after lentiviral infection on ECM-coated plates with the indicated cytokines for 24 h. After cytokine treatment INS-1E and islet cells were trypsinised and collected by centrifugation at 700 g for 5 min. Caspase-3 activation was quantified according to the manufacturer’s protocol as previously described [4].

**Real-time quantitative RT-PCR** Total RNA was isolated as previously described [21]. For cDNA synthesis, random hexamers were used to prime the reaction of the RevertAid H Minus M-MuLV reverse transcriptase (Fermentas, St Leon-Rot, Germany). QuantiTect SYBR Green technology (Qiagen, Hilden, Germany), which uses a fluorescent dye that binds only double-stranded DNA, was employed. The reactions were performed using the Opticon Realtime-PCR System (BioRad, Hercules, CA, USA). Samples were first denatured at 94°C for 3 min followed by 40 PCR cycles comprised of a melting step at 94°C for 30 s, an annealing step at 62°C for 30 s and an extension step at 72°C for 30 s. The optimal variables 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 cycle threshold (Cₜ)-values that were at least ten Cₜ-values lower than the blank values. Each PCR amplification was performed in triplicate. The primer sequences are listed in electronic supplementary material (ESM) Table 1. Data are expressed as relative gene expression after normalisation to the housekeeping gene β-actin using the Qgene96 and LineRegPCR 12.13 software (http://www.gene-quantification.de/download.html).

**Measurement of the mitochondrial membrane potential** Forty-thousand cells were seeded onto black 96-well plates and allowed to attach for 24 h. Thereafter cells were incubated with the indicated glucose concentrations followed by a 20 min incubation with 50 μmol/l rhodamine-123 (Life Technologies, Darmstadt, Germany) before the plates were washed and the rhodamine-123 fluorescence was quantified at 480/520 nm excitation/emission using the Victor2 1420 Multilabel Counter (Perkin Elmer, Fremont, CA, USA).

**Determination of oxidative stress using dichlorodihydrofluorescein diacetate** For the detection of the overall oxidative stress, 40,000 cells were seeded onto black 96-well plates and cultured for 24 h. Then the cells were pre-incubated with 10 μmol/l dichlorodihydrofluorescein diacetate (DCFDA-H₂;Life Technologies, Darmstadt, Germany) for 40 min at 37°C. The medium containing the chemical was discarded and fresh medium with the indicated glucose concentrations was added. After incubation for 24 h, the plates were analysed at 480/520 nm excitation/emission using the Victor² 1420 Multilabel Counter.

Detection of ROS by the use of DCFDA-H₂ is dependent on its oxidation to the stable fluorescent derivative dichlorofluorescein and its subsequent accumulation in the cell. To detect clear differences between the different glucose concentrations and the cell clones an incubation time of 24 h was necessary. The data were expressed as the percentage of ROS formation in INS-1E control cells at 3 mmol/l glucose.

**Statistical analyses** Data are expressed as mean values±SEM. Statistical analyses were performed with GraphPad Prism 5 software (GraphPad, San Diego, CA, USA) using ANOVA plus Bonferroni test for multiple comparisons.
Results

Effect of the hydrogen peroxide-inactivating enzyme catalase on glucose-induced insulin secretion in INS-1E and islet cells After validation of successful catalase overexpression and significant increase in catalase activity (ESM Fig. 1), the effects of lentivirus-induced overexpression of the hydrogen peroxide-inactivating enzyme catalase on glucose-induced insulin secretion were investigated. INS-1E control cells showed at 3 mmol/l glucose a basal insulin secretion rate of approximately 0.26 ng insulin (µg DNA)^-1h^-1 (Fig. 1a). At 10 and 30 mmol/l glucose, insulin secretion of INS-1E control cells was increased about three- and fivefold, respectively. Basal (3 mmol/l glucose) and glucose-induced (10 and 30 mmol/l glucose) insulin secretion in both the cytosolic and mitochondrial overexpressing catalase INS-1E cell clones, showed no significant difference when compared with control cells (Fig. 1a). Cytosolic or mitochondrial catalase overexpression in INS-1E cells also did not significantly affect insulin content at any glucose concentrations (data not shown).

Rat islet cells lentivirally transduced with the CytoCat or MitoCat construct exhibited a basal insulin secretion rate of 3.0–3.3 ng insulin (µg DNA)^-1h^-1 (Fig. 1b) at 3 mmol/l glucose. This insulin secretion was approximately increased threefold at 10 mmol/l and fivefold at 30 mmol/l glucose. At all three glucose concentrations, respectively, there was no significant difference between the catalase transduced and the control cells (Fig. 1b).

Effect of pro-inflammatory cytokines on cell viability and caspase-3 activation of catalase-overexpressing INS-1E cells Incubation of INS-1E control cells with 60 U/ml IL-1β for 72 h reduced the viability of INS-1E control cells by approximately 30% (Fig. 2a). Increasing the IL-1β concentration to 600 U/ml raised the loss of viability to approximately 50% (Fig. 2a) and exposure to the cytokine mixture, containing 60 U/ml IL-1β, 185 U/ml TNF-α and 14 U/ml IFN-γ caused a decrease of 90%.

In CytoCat and MitoCat overexpressing cells the treatment with 60 U/ml IL-1β alone caused a mild 20% viability reduction, which was less than in INS-1E control cells (Fig. 2a). In both catalase overexpressing cell clones treatment with a ten-times higher IL-1β concentration caused higher toxicity. In MitoCat INS-1E cells 600 U/ml IL-1β reduced the viability by 35%, which was significantly less than in equally treated INS-1E control cells (Fig. 2a), whereas cytosolic overexpression of catalase did not significantly protect against the toxicity of IL-1β. MitoCat overexpression also significantly reduced the toxicity induced by the cytokine mixture, while CytoCat overexpression again did not significantly increase viability after exposure to the cytokine mixture. To evaluate whether the observed viability reduction was caused by apoptosis, the activation of caspase-3 after cytokine treatment of INS-1E cells was quantified. Both control and CytoCat transfected cells showed a marked increase in caspase-3 activity (by 40%) after incubation with 60 U IL-1β (Fig. 2b), and this was further enhanced by 600 U IL-1β (80%) and the cytokine mixture (100%) (Fig. 2b). The caspase-3 activation was significantly lower in MitoCat transfected INS-1E cells (60 U IL-1β: 4%, 600 U IL-1β: 14% and cytokine mixture: 20%, Fig. 2b), indicating that the enhanced viability of MitoCat cells is caused by suppressed activation of apoptosis.

Effect of pro-inflammatory cytokines on caspase-3 activation of catalase-overexpressing islet cells Having demonstrated that MitoCat overexpression can provide protection against cytokine-induced apoptosis in insulin-secreting INS-1E cells, we next examined the impact of MitoCat overexpression on cytokine-activated apoptosis in primary rat islet cells.
islet cells. Incubation of islet cells with 600 U/ml IL-1β for 24 h induced caspase-3 activity in control and CytoCat transfectected cells by approximately 90% and the cytokine mixture further accelerated this increase up to 220% (Fig. 3). Consistent with the results in INS-1E cells, the increase in capase-3 activation was significantly attenuated in MitoCat overexpressing islet cells after incubation with pro-inflammatory cytokines (Fig. 3). IL-1β and the cytokine mixture elevated caspase-3 activity only by 15 and 30%, respectively, demonstrating that the protection of MitoCat transfected INS-1E cells could be reproduced in primary rat islet cells.

**Effect of hypoxia on the cell viability of catalase-overexpressing INS-1E cells** To investigate whether mitochondrially located catalase is able to prevent the deleterious effects of hypoxia, INS-1E control and MitoCat overexpressing cells were cultured for up to 72 h in the presence of 1% O2 (hypoxia) and at the physiological concentration of 21% O2 (normoxia). This hypoxic treatment of INS-1E control cells resulted in a time-dependent reduction in viability. After 24 and 48 h hypoxia viability was reduced by approximately 50% and after 72 h by 75% (Fig. 4). To mimic a transient hypoxic period, INS-1E cells were incubated for 24 h under hypoxia, followed by another 24 h incubation period under normoxic conditions. Under this experimental condition INS-1E cells showed a viability of 72%, indicating a partial recovery compared with the viability loss after a 24 h hypoxic incubation. In contrast to control cells, reduction in viability of MitoCat overexpressing INS-1E cells was negligible after 24 h and 48 h of hypoxia. After 72 h of hypoxia the reduction in viability of MitoCat cells was significant (30%) but threefold lower than in INS-1E control cells. Short-term hypoxia followed by normoxia (24 h hypoxia then 24 h normoxia) was without a significant effect on the viability of the MitoCat INS-1E cells (Fig. 4). Viability of control and transfected INS-1E cells was not significantly different under control conditions, indicating that catalase overexpression had no effect on basic viability (data not shown).
Effect of catalase overexpression on Ucp2 gene expression in INS-1E cells Uncoupling protein 2 (UCP2), the isoform expressed in insulin-secreting cells, has the ability to reduce the generation of mitochondrial ROS by uncoupling of the proton motive force. To test whether the overexpression of catalase in INS-1E cells affects the expression of UCP2 and thereby the mitochondrial membrane potential, Ucp2 gene expression in control and catalase-overexpressing INS-1E cells was analysed by real-time quantitative RT-PCR. Mitochondrial catalase overexpression induced Ucp2 gene expression significantly by 60%, while in CytoCat INS-1E cells Ucp2 expression was not significantly changed (120% Ucp2 expression of control cells) (Fig. 5).

Effect of catalase overexpression on mitochondrial membrane potential in INS-1E cells To study the influence of catalase overexpression on mitochondrial membrane potential in INS-1E cells, changes in the mitochondrial membrane potential were quantified by rhodamine-123 fluorescence at different glucose concentrations. The relative mitochondrial membrane potential of INS-1E control cells was not significantly increased by either 3 or 10 mmol/l glucose as compared with 0 mmol/l glucose (Fig. 6). Increasing the glucose concentration to 30 mmol/l significantly increased the mitochondrial membrane potential by more than 50% (Fig. 6). Catalase overexpression in INS-1E cells did not affect mitochondrial membrane potential at 3 or 10 mmol/l glucose when compared with 0 mmol/l. Increasing the glucose concentration to 30 mmol/l increased the membrane potential of CytoCat INS-1E cells slightly but not significantly (plus 20%) (Fig. 6). In MitoCat INS-1E cells this enhancing effect of increasing glucose concentrations on the mitochondrial membrane potential was not observed. Rather, at 30 mmol/l glucose, a significant reduction in the mitochondrial membrane potential was observed (compared with control cells at 30 mmol/l, Fig. 6).

Effect of catalase on glucose-induced oxidative stress in INS-1E cells ROS formation in INS-1E control cells exposed to 10 and 30 mmol/l glucose was significantly decreased by 30 and 45%, respectively, when compared with 3 mmol/l glucose (Fig. 7). Mitochondrial catalase overexpression reduced basal ROS formation at 3 mmol/l glucose significantly by 40% (Fig. 7); elevation of the glucose concentration to 10 and 30 mmol/l resulted in a further decrease in ROS formation. Compared with control cells the ROS concentrations were significantly lower in MitoCat cells at all three investigated glucose concentrations (Fig. 7). In CytoCat INS-1E cells, on the other hand, ROS formation was not significantly affected by 3 mmol/l glucose, although increasing glucose concentrations resulted in an analogous suppression of ROS generation in this cell clone (Fig. 7).

Discussion

Pro-inflammatory cytokines are important mediators of beta cell death during the development of type 1 diabetes mellitus [22, 23]. Likewise, after islet transplantation, when hypoxic conditions prevail at the site of implantation, pro-inflammatory mediators play an important role in the destruction of the transplanted beta cells [24, 25]. Pancreatic beta cells are extraordinarily vulnerable [3] and the reason for the weak protection against oxidative stress is their low antioxidative defence equipment [1, 2].
was measured by the DCFDA-H\textsubscript{2} oxidation assay after 24 h incubation incubated with different glucose concentrations. Cellular oxidative stress rially located catalase and control cells were seeded 24 h before cells were oxidative stress. INS-1E cells overexpressing cytosolically or mitochond-

Fig. 7 Effect of cytoplasmic (CytoCat) and mitochondrial (MitoCat) catalase overexpression in insulin-secreting INS-1E cells on cellular oxidative stress. INS-1E cells overexpressing cytosolically or mitochondrionally located catalase and control cells were seeded 24 h before cells were incubated with different glucose concentrations. Cellular oxidative stress was measured by the DCFDA-H\textsubscript{2} oxidation assay after 24 h incubation with 3 (white bars), 10 (grey bars) and 30 mmol/l (black bars) glucose. The ROS formation in INS-1E control cells at 3 mmol/l glucose was set as 100%. Data are means±SEM from 13–15 individual experiments. Each measurement was performed in triplicate. *p<0.05, **p<0.01 compared with cells of the same clone at 3 mmol/l glucose; †p<0.05, ††p<0.01 compared with control cells at the same glucose concentration.

A potential approach to protect beta cells against cytokine-mediated destruction by ROS is the beta cell-specific overexpression of the H\textsubscript{2}O\textsubscript{2}-inactivating enzyme catalase in mitochondria [4, 18]. However, since the ability of beta cells to secrete insulin in a glucose-dependent manner is indispensable for efficient regulation of glucose homeostasis, this mechanism should remain undisturbed by any protection strategy for insulin-secreting cells.

In an earlier study using the RINm5F cell line we successfully documented the protective potential of mitochondrial catalase overexpression [18]. Although we were not able to draw a conclusion about the impact of catalase overexpression on the insulin secretory potential of glucose, since insulin release from this cell line is not responsive to glucose stimulation at physiological concentrations, no deleterious effects on cell proliferation, glucose oxidation or insulin content were observed [18]. Likewise, in studies on transgenic animals, no negative impact of catalase or glutathione peroxidase overexpression on insulin secretion and beta cell phenotype has been reported [26, 27]. However, since these studies focused on the protective role of ROS-inactivating enzymes, interactions between ROS and GSIS were not analysed.

Using the glucose-responsive INS-1E insulin-secreting cell line [15] and primary rat islet cells, we were able to show in the present study that overexpression of H\textsubscript{2}O\textsubscript{2}-inactivating catalase provides protection against cytokine-induced toxicity through suppression of intramitochondrial ROS formation. Thus mitochondrial catalase overexpression is not incompatible with the signalling function of mitochondrial metabolism in pancreatic beta cells. Catalase overexpressing INS-1E cells were protected not only against cytokine-induced toxicity but also against hypoxic stress as is the case after islet transplantation [28–30], when hypoxic conditions prevail at the site of implantation and, in conjunction with pro-inflammatory cytokines, mediate the destruction of the transplant [24, 25].

Therefore the very low levels of H\textsubscript{2}O\textsubscript{2}-inactivating enzymes, which make the pancreatic beta cells so extraordinarily vulnerable and sensitive to oxidative stress [3], is not a necessity in order for beta cells to maintain a proper physiological insulin secretory responsiveness to glucose stimulation, and higher levels of these enzymes would not be incompatible with this function. Thus, expression of H\textsubscript{2}O\textsubscript{2}-inactivating enzymes, such as catalase and glutathione peroxidase, cannot be considered to be forbidden [5] with respect to a potential incompatibility with proper insulin secretory responsiveness to physiological glucose stimulation. This is at variance with other weakly expressed proteins in beta cells, such as lactate dehydrogenase and the monocarboxylate transporter, whose presence has been shown to be incompatible with undisturbed beta cell function [7–10, 12] and also even to be life threatening [11]. Therefore, mitochondrial catalase overexpression can be considered as a feasible therapeutic concept with the aim of protecting beta cells in islets isolated from the pancreas for subsequent transplantation to diabetic patients. This would protect against the particular stress to which the cells are exposed during the initial time after transplantation into liver and which can result in destruction of up to two-thirds of the transplanted cells [25] before proper supply of oxygen and nutrients is secured through restoration of full vascular supply in the implantation site [28–30]. Protection could also be provided against destruction by pro-inflammatory cytokines released from islet infiltrating immune cells during reoccurrence of the autoimmune attack against the transplanted islets in the recipient organism with type 1 diabetes, with its persisting autoimmunity [25, 31, 32].

In contrast to the overproduction of the B cell lymphoma (extra large) anti-apoptotic protein (Bcl\textsubscript{XL}) in islets [33], the overexpression of the H\textsubscript{2}O\textsubscript{2}-inactivating enzyme catalase in mitochondria did not blunt the physiological insulin secretory response to glucose. The intact secretory responsiveness was maintained although mitochondrial catalase overexpression caused mild uncoupling. This is likely to be the result of the increased Ucp2 expression found as a consequence of catalase overexpression in mitochondria.

It has been reported that a beta cell-specific knock out of Ucp2 was associated with increased mitochondrial membrane potential, ATP content and, subsequently, with elevated glucose-induced insulin secretion [34, 35]. Conversely, strong overexpression of UCP2 attenuated glucose-induced insulin secretion [36], thereby indicating that uncoupling of the proton flux by UCP2 has a negative effect on insulin secretory responsiveness. Data from studies investigating a common polymorphism in the Ucp2 promoter suggest that the level of Ucp2 expression correlates with the inhibition of glucose-
induced insulin secretion [37]. In catalase overexpressing INS-1E cells, however, the slight elevation of the Ucp2 expression level caused only a mild decrease in the mitochondrial membrane potential and thus did not negatively affect glucose-induced insulin secretion at physiological glucose concentrations. CytoCat cells represent a transition situation between the control situation and the significant decrease in the membrane potential in MitoCat cells. A significant reduction in the membrane potential was observed only at supraphysiological glucose concentrations (30 mmol/l). Also a mild uncoupling of INS-1E control cells by DNP was not able to diminish glucose-induced insulin secretion in these cells (ESM Fig. 2). Both findings indicate that Ucp2 expression may have to exceed a certain threshold level before a negative effect on insulin secretion is observed.

A signalling role for H$_2$O$_2$ is established in plants [38] and has also been considered in various mammalian cell types [39, 40], including insulin-secreting cells [13, 14]. However, in our study overall cellular ROS formation was lower at higher glucose concentrations as has been reported by other groups [41–43] and in addition it was suppressed by MitoCat overexpression at all three investigated glucose concentrations. Both findings, together with the insulin secretion data of overexpressing cells, indicate that there is no supporting role for H$_2$O$_2$, especially mitochondrial H$_2$O$_2$, as an amplifying signalling molecule in the regulation of physiological insulin secretion. Moreover, the data do not support the concept of beta cell glucose toxicity, mediated by an increasing ROS formation along with increasing glucose concentrations [44, 45]. Rather, an increased mitochondrial metabolic flux through the respiratory chain at higher glucose concentrations goes along with a more efficient electron flux so that less electrons leave the mitochondrial respiratory chain prematurely at complexes 1 and 3 to form O$_2$•– and subsequently H$_2$O$_2$.

In summary, mitochondrial catalase overexpression to prevent H$_2$O$_2$ formation and free radical species derived from it, such as the particularly toxic hydroxyl radical [4], does not blunt the physiological insulin secretory response to glucose.

Funding This work was supported by a grant from the Federal Ministry of Education and Research (Integrated Research and Treatment Center for Transplantation, reference No. 01EO0802) and by the European Union (IMI JU MIDIA IMI/115005 in the Framework Programme 7 [FP7] of the European-Community).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement SLe, EG-C and ON contributed to the design, conduct, data analysis and manuscript preparation. SLe contributed to the planning, design, data analysis, manuscript preparation and support for the study. All authors approved the final manuscript.

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