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ARTICLE

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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Abstract
Aims/hypothesis Hydrogen peroxide (H_2O_2)-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 H_2O_2 within the beta cell is unknown. A high level of H_2O_2-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 secret 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 H_2O_2 inactivation is not incompatible with proper insulin secretory responsiveness to glucose stimulation and provide no support for a signalling role of H_2O_2 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

C_t	Cycle threshold
CytoCat	Cytosolic catalase
DCFDA-H_2	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 (H_2O_2)-inactivating enzymes catalase and glutathione peroxidase despite adequate expression of the superoxide ($O_2^{\bullet-}$)-inactivating

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superoxide dismutases [1, 2]. This imbalance results in beta cells having a high susceptibility to the toxicity of H_2O_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_2O_2-inactivating enzymes interfere with physiological beta cell function. Genes associated with such an interfering effect have been termed 'disallowed genes' [5] and include *Hk1* [6], the lactate dehydrogenase isoform *Ldha* and the monocarboxylate transporter *Mct1* [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_2O_2-inactivating enzymes could potentially be detrimental for proper beta cell function. If so, a rapid dismutation of H_2O_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_2O_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_2O_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_2O_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_2O_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/l 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-diphenyltetrazolium 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% O_2 balanced with N_2 for hypoxia. Hypoxia was generated in an oxygen-regulated incubator (CB210 incubator with O_2 control option; Binder, Tuttlingen, 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 (OD_{550}) 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 fluorescein active caspase-3 staining kit (PromoCell). Control, CytoCat and MitoCat overexpressing INS-1E cells were seeded at a density of 1×10^6 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 (C_t)-values that were at least ten C_t-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 Victor^2 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-H_2;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 Victor^2 1420 Multilabel Counter.

Detection of ROS by the use of DCFDA-H_2 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 $(\mu g\ DNA)^{-1}h^{-1}$ (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 $(\mu g\ DNA)^{-1}h^{-1}$ (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

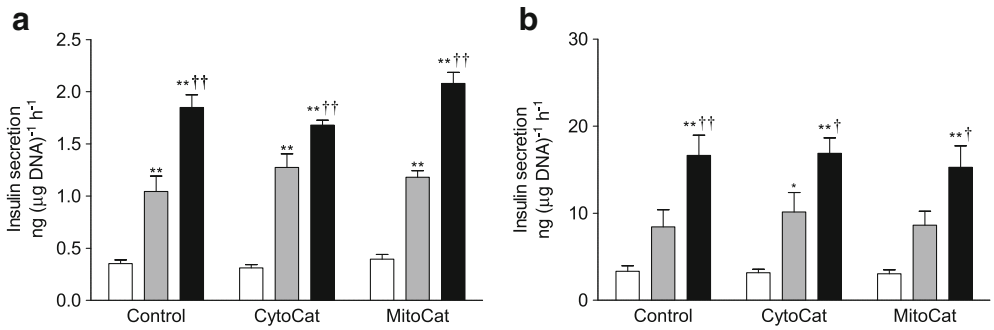

Fig. 1 Effect of cytoplasmic (CytoCat) and mitochondrial (MitoCat) catalase overexpression in insulin-secreting INS-1E and primary rat islet cells on insulin secretion. Insulin secretion was quantified after 1 h incubation with 3 (white bars), 10 (grey bars) and 30 mmol/l (black bars) glucose and normalised to the DNA content of the incubated cells. (**a**) INS-1E cells overexpressing cytosolically or mitochondrially located catalase and control cells were seeded 48 h before cells were incubated with different glucose concentrations. (**b**) Primary rat islet cells were lentivirally transduced to overexpress cytosolically or mitochondrially located catalase 72 h before the cells were incubated with different glucose concentrations. Data are means±SEM from six to nine (INS-1E cells) or seven to 12 (islet cells) individual experiments. $^{*}p<0.05$, $^{**}p<0.01$ compared with cells of the same clone at 3 mmol/l glucose; $^{\dagger}p<0.05$, $^{\dagger\dagger}p<0.01$ compared with cells of the same clone at 10 mmol/l glucose

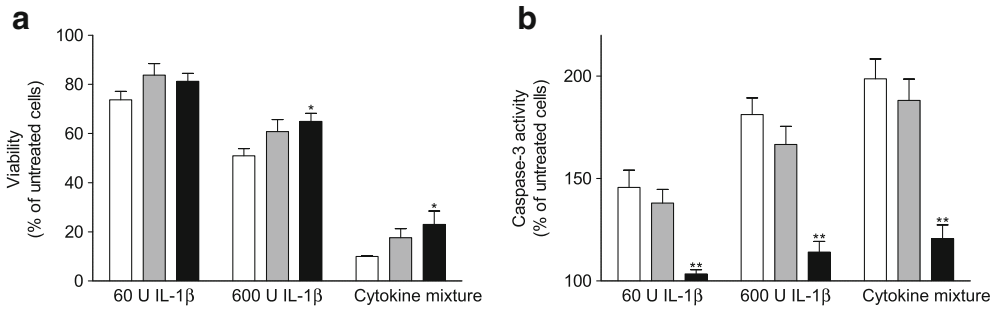

Fig. 2 Effect of cytoplasmic and mitochondrial catalase overexpression on the toxicity of pro-inflammatory cytokines in insulin-secreting INS-1E cells. INS-1E cells overexpressing cytosolically (grey bars) or mitochondrially (black bars) located catalase and control cells (white bars) were incubated with IL-1β (60 or 600 U/ml) alone or a cytokine mixture (60 U/ml IL-1β, 185 U/ml TNF-α, 14 U/ml IFN-γ). (**a**) Viability of the cells was determined after 72 h cytokine incubation by the MTT assay and expressed as % of the untreated cells. (**b**) Caspase-3 activation was determined after 24 h cytokine incubation by flow cytometry and expressed as % of untreated cells. Data are means±SEM from six to eight cytokine incubations. *$p<0.05$, **$p<0.01$ compared with control cells

islet cells. Incubation of islet cells with 600 U/ml IL-1β for 24 h induced caspase-3 activity in control and CytoCat transfected 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% O_2 (hypoxia) and at the physiological concentration of 21% O_2 (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).

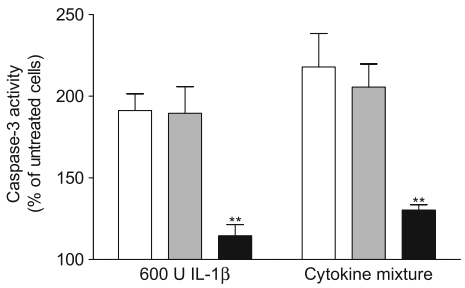

Fig. 3 Effect of cytoplasmic and mitochondrial catalase overexpression on cytokine-induced caspase-3 activation in primary rat islet cells. Islet cells overexpressing cytosolically (grey bars) or mitochondrially (black bars) located catalase and control cells (white bars) were incubated for 24 h with IL-1β (600 U/ml) alone or a cytokine mixture (60 U/ml IL-1β, 185 U/ml TNF-α, 14 U/ml IFN-γ). Caspase-3 activation was determined after 24 h cytokine incubation by flow cytometry and expressed as % of untreated cells. Data are means±SEM from four cytokine incubations. **$p<0.01$ compared with control cells

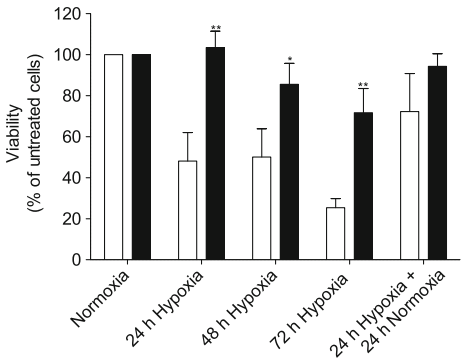

Fig. 4 Effect of mitochondrial catalase overexpression on the toxicity of hypoxia in insulin-secreting INS-1E cells. INS-1E cells overexpressing mitochondrially (black bars) located catalase and control cells (white bars) were incubated for up to 72 h with 1% O_2 or for 24 h under hypoxic conditions followed by 24 h incubation under control conditions. Viability of the cells was determined after hypoxic incubation by the MTT assay and expressed as % of untreated cells. Data are means±SEM from four or five individual experiments. *$p<0.05$, **$p<0.01$ compared with control cells

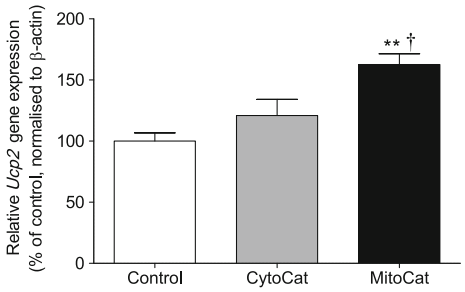

Fig. 5 Effect of cytoplasmic (CytoCat) and mitochondrial (MitoCat) catalase overexpression in insulin-secreting INS-1E cells on *Ucp2* gene expression. Total RNA was isolated 24 h after cell seeding from INS-1E cells overexpressing cytosolically (grey bar) or mitochondrially (black bar) located catalase and from control cells (white bar) and analysed by real-time quantitative RT-PCR with *Ucp2* specific primers. The *Upc2* expression levels were normalised to the housekeeping gene β-actin and the expression level of *Ucp2* in control cells was set as 100%. Data are means±SEM from five to 12 individual experiments. $^{**}p<0.01$ compared with control cells; $^{\dagger}p<0.05$ compared with CytoCat cells

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].

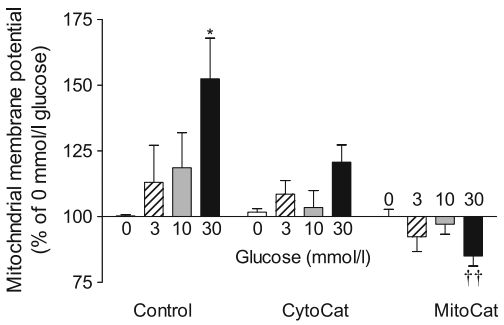

Fig. 6 Effect of cytoplasmic (CytoCat) and mitochondrial (MitoCat) catalase overexpression in insulin-secreting INS-1E cells on mitochondrial membrane potential. Changes in the rhodamine-123 fluorescence as an indicator for the mitochondrial membrane potential was measured in INS-1E cells overexpressing cytosolically or mitochondrially located catalase and in control cells by rhodamine-123 fluorescence. The cells were incubated with 0 (white bars), 3 (hatched bars), 10 (grey bars) and 30 mmol/l (black bars) glucose, labelled with rhodamine-123 and analysed by flow cytometry. The mitochondrial membrane potential of each cell clone at 0 mmol/l glucose was set as 100%. Data are means±SEM from seven to 15 individual experiments. $^{*}p<0.05$ compared with cells of the same clone at 0 mmol/l glucose; $^{\dagger\dagger}p<0.01$ compared with control cells at the same glucose concentration

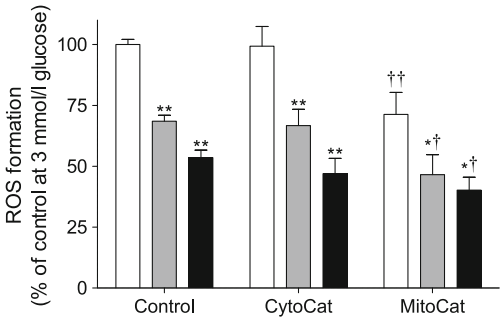

Fig. 7 Effect of cytoplasmic (CytoCat) and mitochondrial (MitoCat) catalase overexpression in insulin-secreting INS-1E cells on cellular oxidative stress. INS-1E cells overexpressing cytosolically or mitochondrially located catalase and control cells were seeded 24 h before cells were incubated with different glucose concentrations. Cellular oxidative stress was measured by the DCFDA-H_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_2O_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_2O_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_2O_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_2O_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_{XL}) in islets [33], the overexpression of the H_2O_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_2O_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_2O_2, especially mitochondrial H_2O_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 $O_2^{\bullet-}$ and subsequently H_2O_2.

In summary, mitochondrial catalase overexpression to prevent H_2O_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.

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