Mitochondrial Catalase Overexpression Protects Insulin-Producing Cells Against Toxicity of Reactive Oxygen Species and Proinflammatory Cytokines

Ewa Gurgul,1 Stephan Lortz,1 Markus Tiedge,1 Anne Jörns,2 and Sigurd Lenzen1

Insulin-producing cells are known for their extremely low antioxidant equipment with hydrogen peroxide (H2O2)-inactivating enzymes. Therefore, catalase was stably overexpressed in mitochondria and for comparison in the cytoplasmic compartment of insulin-producing RINm5F cells and analyzed for its protective effect against toxicity of reactive oxygen species (ROS) and proinflammatory cytokines. Only mitochondrial overexpression of catalase provided protection against menadione toxicity, a chemical agent that preferentially generates superoxide radicals intramitochondrially. On the other hand, the cytoplasmic catalase overexpression provided better protection against H2O2 toxicity. Mitochondrial catalase overexpression also preferentially protected against the toxicity of interleukin-1β (IL-1β) and a proinflammatory cytokine mixture (IL-1β, tumor necrosis factor-α [TNF-α], and γ-interferon [IFN-γ]) that is more toxic than IL-1β alone. Thus, it can be concluded that targeted overexpression of catalase in the mitochondria provides particularly effective protection against cell death in all situations in which ROS are generated intramitochondrially. The observed higher rate of cell death after exposure to a cytokine mixture in comparison with the weaker effect of IL-1β alone may be due to an additive toxicity of TNF-α through ROS formation in mitochondria. The results emphasize the central role of mitochondrially generated ROS in the cytokine-mediated cell destruction of insulin-producing cells. Diabetes 53:2271-2280, 2004

Pancreatic islets and insulin-producing tissue culture cells are known for their extremely low antioxidative defense status and their unusual susceptibility to reactive oxygen species (ROS) and nitric oxide (NO). In particular, the expression level of the H2O2-inactivating enzymes catalase and glutathione peroxidase is very low (1,2). Cytokine toxicity, as well as oxidative stress, is generally accepted to play an important role in the autoimmune destruction of pancreatic β-cells during the development of type 1 diabetes (3–5). It has been shown that overexpression of antioxidant enzymes provides protection against the toxicity of ROS and proinflammatory cytokines (6–10). However, overexpression of the H2O2-inactivating enzymes catalase and glutathione peroxidase in the cytoplasmic compartment only gave a limited protection of insulin-producing RINm5F cells against cytokine-mediated cell damage (9). Recent studies, however, have provided evidence for a crucial role of the mitochondrial antioxidative defense status for the susceptibility of insulin-producing cells to cytokine toxicity (11–13).

Superoxide radicals produced by the respiratory chain through the reduction of molecular oxygen are physiologically inactivated by the mitochondrial superoxide dismutase (MnSOD) to H2O2. This toxic compound can be further reduced by the thioredoxin-dependent peroxiredoxin III and H2O2-inactivating enzymes (14), which, however, are only weakly expressed in insulin-producing cells (1,2). As cytokines can enhance superoxide radical generation and induce MnSOD expression (15), thereby increasing the capacity for superoxide radical inactivation, the capacity for subsequent H2O2 inactivation may become rate limiting under these circumstances (16). Therefore, in this study, we have investigated whether it is possible to improve the cellular antioxidative defense status through targeted overexpression of the H2O2-inactivating enzyme catalase in the mitochondria.

To compare the importance of the intramitochondrial antioxidative defense capacity relative to that of the cytoplasm, we included in this investigation three additional chemical compounds that differ in their specific kinetic behavior, the radicals they release, and, in particular, the sides of their ROS generation: 1) H2O2, which mimics the accumulation of this compound from superoxide dismutation, 2) the hypoxanthine/xanthine oxidase (HX/XO) system (17), which releases superoxide radicals and H2O2 extracellularly and continuously in a long-term manner, and 3) menadione, which generates superoxide radicals in the mitochondrial compartment (18).

In this study, we show that mitochondrial catalase overexpression protects insulin-producing cells and is particularly efficient against toxic chemical compounds, which generate ROS preferentially in the mitochondria as well as against proinflammatory cytokines.

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ELISA, enzyme-linked immunoassay; HX/XO, hypoxanthine/xanthine oxidase; IFN-γ, interferon-γ; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; MnSOD, mitochondrial superoxide dismutase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; ROS, reactive oxygen species; SEAP, secreted alkaline phosphatase; TNF-α, tumor necrosis factor-α.

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MITOCHONDRIAL CATALASE OVEREXPRESSSION

RESEARCH DESIGN AND METHODS

Restriction enzymes and the Sp6/T7 transcription kit were obtained from Roche (Mannheim, Germany). Hybond N nylon membranes, the enhanced chemiluminescence detection system, and autoradiography films were from Amersham Biosciences (Freiburg, Germany). Immobilon-P polyvinylidone fluoride membranes were from Millipore (Bedford, MA). Horseradish peroxidase horse radish peroxidase was from Sigma (St. Louis, MO). 

Materials and methods

The vectors pcDNA3 and pcMyc/myc/mito were made by the use of standard techniques. The mitochondrial leader sequence were added (forward-primer: 5'-TACAGCGCGCCGCACATGTGGTCGCGACACGGGATCC-3') by PCR. The 3' end was equipped with an additional NotI restriction site for subcloning (reverse-primer: 5'-TACAGCGCGCCGCACATGTGGTCGCGACACGGGATCC-3'). This BssHII/NotI-cut catalase construct was subcloned in frame with the mitochondrial targeting signal into the precut vector by standard techniques. For the cytoplasmic catalase overexpression, cDNA for human catalase was subcloned into the pcDNA3 expression vector as described in detail earlier (7). RINm5F cells were transfected with the vector DNA by the use of lipofectamine (Invitrogen). Positive clones were selected through resistance against G 418 (250 µg/ml) (Invitrogen) and verified for catalase activity measurements. The sediment of cell debris was discarded. The mitochondrial fraction was obtained through centrifugation for 10 min at 8,000 × g at 4°C. The supernatant was then centrifuged for another 90 min at 100,000 × g at 4°C to separate the microsomal and the cytosolic fraction (21).

Tissue fractionation. The cells were homogenized in 500 µl of ice-cold H-medium (70 mmol/l sucrose, 210 mmol/l mannitol, 20 mmol/l HEPES, 0.5 mmol/l EGTA, pH 7.4) using a Potter-Elvehjem homogenizer with a teflon pestle and maintained on ice. Fractions were obtained through differential centrifugation. Supernatant after centrifugation for 10 min at 100,000 × g was taken as whole-cell extract and used for Western blot analyses and enzyme activity determinations. Mitochondrial, microsomal, and cytosolic fractions were separated by chemiluminescence detection.

Western blot analyses. RINm5F cell subcellular fractions (for catalase detection) or whole-cell extracts (for MnSOD detection) were sonicated in ice-cold PBS on ice for 15 s at 60 W with a Braun-Sonic 125 sonifier. Protein content was determined by the bicinchoninic acid assay (Pierce, Rockford, IL). SDS-PAGE was used to fractionate 10 µg (for catalase) or 20 µg (for MnSOD) of protein, which was then transferred to polyvinylidene fluoride membranes. Non-specific binding sites of the membranes were blocked by 5% BSA in 100 mmol/l PBS, pH 7.4, and the blots incubated with the polyclonal anti-bovine liver catalase antibody raised in rabbit or with a cytokine mixture for Western blot analyses, the measurement of accumulated nitrite, and the measurement of inducible NO synthase (iNOS) promoter activation or 72 h for ultrastructural analyses. Glucose oxidation in the different cell clones after a 72-h incubation with 600 µmol/l human IL-1β or with the 1 × cytokine mixture was determined at 10 mmol/l (L-1) glucose (1 U/mmol) as described earlier (20).

Catalase enzyme activity measurements. Catalase enzyme activity in homogenates and subcellular fractions was measured by ultraviolet spectroscopy, monitoring the decomposition of H₂O₂ at 240 nm as described previously (7). One unit of catalase activity was defined as 1 µmol H₂O₂/min at 25°C.

NITRITE MEASUREMENTS AND iNOS PROMOTER REPORTER GENE ASSAY

Nitrite accumulation after incubation with IL-1β or with a cytokine mixture was determined photometrically at 562 nm by the Griess reaction as described earlier (9). For the iNOS promoter reporter gene assay, 2 × 10⁴ cells/well were seeded in 96-well plates 24 h before transient transfection was performed and 48 h before the incubation with IL-1β or with a cytokine mixture. The pSEAP-iNOS construct was used as described in detail before (11).

Statistical analyses. Data are expressed as means ± SE. Unless stated otherwise, statistical analyses were performed using ANOVA plus Bonferroni test for multiple comparisons. Half-maximal concentration (EC₅₀) values were
RESULTS

Stable overexpression of catalase in insulin-producing RINm5F cells. Catalase with or without a mitochondrial leader sequence was successfully overexpressed in insulin-producing RINm5F cells through stable transfection. RINm5F cells stably transfected with the vector lacking a cDNA insert were used as control. Overexpression of catalase was verified by Northern and Western blot analyses (Fig. 1) and enzyme activity measurement (Table 1). In the RINm5F cell clone mito 1, catalase activity was 15 times higher than in control cells, and it was in the same range as the clone expressing catalase in the cytoplasmic (cyto) compartment (Fig. 1 and Table 1). In a second clone transfected with a plasmid containing catalase cDNA with a mitochondrial leader sequence (mito 2), the level of overexpression was increased 22-fold compared with controls and thus significantly higher than in the mito 1 clone (Fig. 1 and Table 1).

Studies on subcellular distribution of catalase after cell fractionation through homogenization and subsequent differential centrifugation revealed that catalase protein and enzyme activity was predominantly localized in the mitochondrial fraction in clones mito 1 and 2, while it was predominantly localized in the cytoplasmic fraction in the cyto clone (Fig. 1 and Table 1). Consequently, the ratio between catalase enzyme activity in mitochondria and in the cytoplasmic fraction was 0.8 in the cyto clone, while it was 1.8 in the mito 1 clone and 2.5 in the mito 2 clone (Table 1). While the ratio was <1.0 in the cells overexpressing catalase preferentially in the cytoplasm and >1.0 in the clones overexpressing catalase preferentially in the mitochondria, there was no such preference of catalase enzyme activity distribution in control cells (ratio 1.0) (Table 1). Control experiments revealed that >80% of catalase enzyme activity was localized in the mitochondrial matrix and thus not in the mitochondrial membrane (E.G., unpublished observations). In all RINm5F clones, the level of enzyme activity in the microsomal fraction was negligible (Table 1). In the clones overexpressing catalase, the enzyme activity was at least 20 times lower in the microsomal fraction than in either the mitochondrial or cytoplasmic fraction.

Effects of targeted catalase overexpression on the toxicity of chemical compounds in the MTT test

Toxicity of H₂O₂. H₂O₂ caused a concentration-dependent loss of cell viability in the MTT assay, in control cells, and in cell clones overexpressing catalase in the cytoplasmic (cyto) and mitochondrial (mito 1 and mito 2) compartment (Fig. 2A). In control RINm5F cells, the EC₅₀ value of H₂O₂, that is, the concentration of the toxin at which 50% of the viability of the cells was lost, was 52 μmol/l (Table 2). In the clones mito 1 and 2, which overexpressed catalase preferentially in the mitochondrial compartment, the EC₅₀ values were 129 and 231 μmol/l, respectively, and thus significantly (P < 0.01) higher than in control cells (Table 2). In the cyto clone, with preferential catalase overexpression in the cytoplasmic compartment, the EC₅₀ value was 346 μmol/l for the H₂O₂ toxicity, and thus even significantly (P < 0.01) higher than in the mito 1 and 2 clones (Table 2). Therefore, the protection against H₂O₂ toxicity was significantly better in the cyto clone than in the clones with a preferential mitochondrial catalase overexpression (Table 2).

Toxicity of menadione. Menadione caused a concentration-dependent loss of cell viability both in RINm5F control cells and cell clones overexpressing catalase in cytoplasm or mitochondria (Fig. 2B). Overexpression of catalase in the cytoplasmic compartment did not protect RINm5F cells against toxicity of menadione when compared with control cells (Table 2). However, a protection against menadione toxicity was observed in the clones with the preferential mitochondrial catalase overexpression, which was significant (P < 0.01) with an EC₅₀ value

### TABLE 1

<table>
<thead>
<tr>
<th>Cell fractions</th>
<th>Control</th>
<th>Cyto</th>
<th>Mito 1</th>
<th>Mito 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>12 ± 2 (8)</td>
<td>178 ± 21 (11)</td>
<td>172 ± 24 (8)</td>
<td>269 ± 30 (11)</td>
</tr>
<tr>
<td>Mitochondrial fraction</td>
<td>9 ± 1 (7)</td>
<td>155 ± 33 (10)</td>
<td>192 ± 43 (7)</td>
<td>300 ± 36 (10)</td>
</tr>
<tr>
<td>Microsomal fraction</td>
<td>3 ± 1 (7)</td>
<td>7 ± 1 (10)</td>
<td>6 ± 1 (7)</td>
<td>7 ± 1 (10)</td>
</tr>
<tr>
<td>Cytoplasmic fraction</td>
<td>11 ± 2 (7)</td>
<td>204 ± 37 (10)</td>
<td>129 ± 17 (7)</td>
<td>142 ± 15 (10)</td>
</tr>
<tr>
<td>Ratio mito/cytosol</td>
<td>1.0 ± 0.3 (7)</td>
<td>0.8 ± 0.1 (10)</td>
<td>1.8 ± 0.5 (7)*</td>
<td>2.5 ± 0.5 (10)†</td>
</tr>
</tbody>
</table>

Data are means ± SE (no. experiments). Catalase enzyme activities were determined in whole cells (total activity) and in mitochondrial, microsomal, and cytoplasmic fractions of insulin-producing RINm5F cells overexpressing catalase preferentially in the cytoplasm (cyto) or mitochondria (mito 1 and 2). In addition, the ratio between the enzyme activity in the mitochondrial and in the cytoplasmic fraction is given.

*P < 0.05; †P < 0.01 vs. the cyto clone and the control clone (Student’s t test).
of 20.0 μmol/l in the clone mito 2, as compared with an EC_{50} value of 13.1 μmol/l in control cells (Table 2). Thus only mitochondrial catalase overexpression provided protection against menadione toxicity (Table 2).

Toxicity of HXXO. The HXXO free radical–generating system caused a concentration-dependent loss of cell viability in the MTT assay (Fig. 2C). However, in comparison with control cells, protection against HXXO toxicity was significant only in the cells with the preferential cytoplasmic catalase overexpression and in the cells with a high level of preferential mitochondrial catalase overex-

<table>
<thead>
<tr>
<th>Cell clones</th>
<th>H_2O_2 (μmol/l)</th>
<th>Menadione (μmol/l)</th>
<th>HXXO (mU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>52 ± 8 (5)</td>
<td>13.1 ± 0.6 (4)</td>
<td>1.8 ± 0.2 (4)</td>
</tr>
<tr>
<td>Cyto</td>
<td>346 ± 27 (5)^†</td>
<td>13.6 ± 0.8 (4)^*</td>
<td>4.0 ± 0.3 (4)^*</td>
</tr>
<tr>
<td>Mito 1</td>
<td>129 ± 18 (5)^*</td>
<td>15.8 ± 1.1 (4)</td>
<td>3.0 ± 0.4 (4)</td>
</tr>
<tr>
<td>Mito 2</td>
<td>231 ± 18 (5)^*</td>
<td>20.0 ± 1.0 (4)^†</td>
<td>5.8 ± 0.5 (4)^†</td>
</tr>
</tbody>
</table>

Data are means ± SE (no. experiments). Insulin-producing RINm5F cell clones overexpressing catalase in the cytoplasm (cyto) or mitochondria (mito 1 and 2), and control cells were exposed to serial concentrations of H_2O_2, HXXO, and menadione, as indicated in Fig. 2. HXXO (expressed in mU/ml) is a mixture containing a fixed ratio of 20 parts xanthine oxidase (mU/ml) and 1 part hypoxanthine (mmol/l). Viability of the cells was determined by the MTT assay. The EC_{50} values were calculated by nonlinear regression analyses from the curves in Fig. 2A–C. ^*P < 0.01, ^†P < 0.001 vs. control cells.

**Effects of targeted catalase overexpression on the toxicity of cytokines in the MTT test**

**IL-1β.** Incubation of control RINm5F cells with IL-1β (600 units/ml) caused a loss of cell viability in the MTT assay of ~40% (Table 3). In the cyto clone, with a preferential cytoplasmic overexpression, and in the mito 1 clone, with a preferential mitochondrial overexpression of catalase, IL-1β (600 units/ml) caused a reduction in the cell viability of about one-third in both clones, which was not significantly different from the viability loss in the control clone (Table 3). In the mito 2 clone, with the highest catalase activity in the mitochondrial compartment, however, this protective effect of catalase overexpression was significantly (P < 0.01), as evidenced by a <20% reduction of the cell viability as compared with control cells (Table 3). This protection was also significantly better than that observed in the cyto clone (Table 3). Thus, the protection of RINm5F cells against IL-1β toxicity crucially depends on the level of catalase activity in the mitochondrial compartment.
**Cytokine mixture.** Exposure of RINm5F cells to a combination of cytokines consisting of IL-1β (60 units/ml), TNF-α (185 units/ml), and IFN-γ (14 units/ml) caused a loss of cell viability of 30–50% in RINm5F control cells in the MTT assay (Table 3). At the less toxic 0.5 × cytokine mixture, cells were excellently protected through the mitochondrial overexpression of catalase, as evidenced by minimal loss of cell viability of 13 and 6% in the cell clones mito 1 and 2, respectively (Table 3). This contrasts with the nonsignificant protective effect of preferential cytoplasmic catalase overexpression in the cyto clone (Table 3). At the higher 1.0 × concentration of the cytokine mixture, cells were protected by both the cytoplasmic and mitochondrial overexpression of catalase (Table 3). The mito 2 clone showed the best protection ($P < 0.01$) against the 1 × cytokine mixture; the protection of the mito 1 and cyto clones was weaker but also significant ($P < 0.05$) when compared with control cells (Table 3). Thus, the results of the experiments show that a higher concentration of the cytokine mixture also required a higher level of preferential mitochondrial catalase overexpression to achieve the best protection against cytokine toxicity.

Differences measured in the MTT assay between the different catalase-overexpressing cell clones were not caused by differences in the proliferation rate. Only the proliferation rate of nontransfected controls was significantly lower ($P < 0.05$), while all catalase-transfected clones did not differ in their proliferation rate. The proliferation rate (percentage of untreated cells) after a 72-h incubation was as follows in the control and catalase-overexpressing RINm5F clones. Incubation with 600 units/ml IL-1β: control cells, 85 ± 2%; cyto clone, 96 ± 2%; mito 1 clone, 91 ± 4%; mito 2 clone, 97 ± 2%. Incubation with a 1 × cytokine mixture: control cells, 59 ± 4%; cyto clone, 80 ± 2%; mito 1 clone, 91 ± 2%; mito 2 clone, 94 ± 2% (means ± SE of four experiments). The absolute proliferation values under control conditions did not differ among nontransfected, cyto, and mito RINm5F cell clones (data not shown). Therefore, the differences in the MTT assay do not represent different replication rates in the different catalase-overexpressing clones.

Catalase enzyme activity in the different catalase-overexpressing clones was not differentially affected by cytokine exposure. After incubation with 600 units/ml IL-1β or the 1 × cytokine mixture, all clones showed a reduction in catalase enzyme activity; however, there were no significant differences between the different clones. After an IL-1β incubation, the remaining enzyme activities were as follows: cyto clone, 78 ± 10%; mito 1 clone, 81 ± 17%; mito 2 clone, 77 ± 10% (means ± SE of four experiments). After a cytokine mixture incubation, the remaining enzyme activities were as follows: cyto clone, 75 ± 3%; mito 1 clone, 92 ± 8%; mito 2 clone, 85 ± 14% (means ± SE of three to four experiments).

**Effects of targeted catalase overexpression on glucose oxidation after cytokine exposure IL-1β.** Cytoplasmic but not mitochondrial catalase overexpression was accompanied by a slight reduction of glucose oxidation under control conditions (Table 4). Incubation of control RINm5F cells with IL-1β (600 units/ml) caused a decrease of the glucose oxidation rate by ~60% (Table 4). A comparable reduction of the glucose oxidation rate that was not significantly lower than in the control cell clone was observed in the cyto and the mito 1 clones (Table 4). No inhibitory effect of IL-1β, on the other hand, was seen in the mito 2 clone, with the highest catalase activity in the mitochondrial compartment. Rather, the glucose oxidation rate was found to be higher than under control conditions (Table 4).

**Cytokine mixture.** Comparable results were obtained when the cells were exposed to the 1 × cytokine mixture (60 units/ml IL-1β, 185 units/ml TNF-α, and 14 units/ml IFN-γ). Glucose oxidation rates at 10 mmol/l (U-14C glucose (1 Ci/mol)) were expressed as the percentage of untreated cells. Glucose oxidation rates under control conditions were the same in the control clone and in the mito 1 and 2 clone but significantly lower ($P < 0.01$) in the cytokine clone (glucose oxidation rate, expressed in mmol · g DNA $^{-1}$ · h $^{-1}$; control, 0.120 ± 0.006; cyto, 0.060 ± 0.004; mito 1, 0.108 ± 0.003; mito 2, 0.113 ± 0.005). *$P < 0.001$ vs. control clone.

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<tr>
<td>Mito 1</td>
<td>35 ± 4 (8)</td>
</tr>
<tr>
<td>Mito 2</td>
<td>128 ± 6 (12)*</td>
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Data are means ± SE (no. experiments). Insulin-producing RINm5F cell clones overexpressing catalase in the cyto and mito 1 and 2 and control cells were incubated for 72 h with IL-1β (600 units/ml) alone or with the 1 × cytokine mixture (60 units/ml IL-1β, 185 units/ml TNF-α, and 14 units/ml IFN-γ). Glucose oxidation rates at 10 mmol/l U-14C glucose (1 Ci/mol) were expressed as the percentage of untreated cells. Glucose oxidation rates under control conditions were the same in the control clone and in the mito 1 and 2 clone but significantly lower ($P < 0.01$) in the cytokine clone (glucose oxidation rate, expressed in mmol · g DNA $^{-1}$ · h $^{-1}$; control, 0.120 ± 0.006; cyto, 0.060 ± 0.004; mito 1, 0.108 ± 0.003; mito 2, 0.113 ± 0.005). *$P < 0.001$ vs. control clone.

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Effects of targeted catalase overexpression on the toxicity of cytokines in ultrastructural analysis

**IL-1β.** To compare the results obtained in the MTT assay and in the glucose oxidation experiments with morphological features of cell death, we performed a quantitative ultrastructural analysis through electron microscopy (Table 5). Effects of cytoplasmic (cyto) or mitochondrial (mito 1 and 2) catalase overexpression in insulin-producing RINm5F cells on the cell death rate after exposure to IL-1β alone or a cytokine mixture

<table>
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<th>Cell clones</th>
<th>Destroyed cells (%)</th>
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<tbody>
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<td>Control</td>
<td>19 ± 3 (4)</td>
</tr>
<tr>
<td>Cyto</td>
<td>10 ± 3 (4)*</td>
</tr>
<tr>
<td>Mito 1</td>
<td>8 ± 2 (4)*</td>
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<td>Mito 2</td>
<td>4 ± 1 (4)*</td>
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Data are means ± SE (no. experiments). Insulin-producing RINm5F cell clones overexpressing catalase in the cyto and mito 1 and 2 and control cells were incubated for 72 h with IL-1β (600 units/ml) alone or with a cytokine mixture (60 units/ml IL-1β, 185 units/ml TNF-α, and 14 units/ml IFN-γ). Cell pellets were fixed for ultrastructural analyses. Destroyed cells in the sections were identified by electron microscopy, counted, and expressed as the percentage of the total number of cells. A total of 100–200 cells were analyzed in each section. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ vs. control cells; §§$P < 0.01$ vs. mito 2 clone.

**TABLE 4** Effects of cytoplasmic (cyto) or mitochondrial (mito 1 and 2) catalase overexpression in insulin-producing RINm5F cells on the glucose oxidation rate after exposure to IL-1β alone or a cytokine mixture

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**TABLE 5** Effects of cytoplasmic (cyto) or mitochondrial (mito 1 and 2) catalase overexpression in insulin-producing RINm5F cells on the cell death rate after exposure to IL-1β alone or a cytokine mixture

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Data are means ± SE (no. experiments). Insulin-producing RINm5F cell clones overexpressing catalase in the cyto and mito 1 and 2 and control cells were incubated for 72 h with IL-1β (600 units/ml) alone or with a cytokine mixture (60 units/ml IL-1β, 185 units/ml TNF-α, and 14 units/ml IFN-γ). Cell pellets were fixed for ultrastructural analyses. Destroyed cells in the sections were identified by electron microscopy, counted, and expressed as the percentage of the total number of cells. A total of 100–200 cells were analyzed in each section. *$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$ vs. control cells; §§$P < 0.01$ vs. mito 2 clone.
Overexpression of catalase resulted in a significant protection against IL-1β toxicity in the cyto and mito clones. The greatest protection was evident in the mito 2 clone (Table 5).

**Cytokine mixture.** A 72-h incubation with a cytokine mixture (60 units/ml IL-1β, 185 units/ml TNF-α, and 14 units/ml IFN-γ) caused a drastic loss of cell viability and integrity with >80% destroyed RINm5F control cells (Table 5). Catalase overexpression significantly protected cells against the cytokine mixture toxicity. In the cyto clone, with a preferential cytoplasmic catalase overexpression, only 58% of cells were destroyed after exposure to the cytokine mixture. In the mito 1 clone, the protection against cytokine mixture toxicity was better (44% destroyed cells). In the mito 2 clone, with a high preferential mitochondrial catalase overexpression, an excellent protection was found, as evident from the greatest reduction of the cell death rate found in control cells (15% destroyed cells) (Table 5). Thus, the results obtained in the electron microscope cell death analyses confirmed the results from the MTT cell viability assays. Ultrastructurally, the cytokine toxicity presented itself in the cyto clone by a destruction of the cells, as characterized by rupture of the plasma membrane and damage to the cell organelles, including mitochondria (Fig. 3A). A special analysis of the damage caused to the mitochondria revealed a complete loss of the integrity of all mitochondrial membranes in one-third of the mitochondria. The other two-thirds of the mitochondria showed a severe swelling with or without loss of the cristae structure of the inner membrane. The loss of structure was due to the rupture of the inner mitochondrial membrane. A comparable morphology was also observed in cytokine-damaged control cells (not shown in Fig. 3), however, with a higher cell death rate (Table 5) than in the cyto clone. Some of the less severely damaged mitochondria that were presented ultrastructurally as destroyed may still retain dehydrogenase enzyme activity, which will contribute to the estimation of viability in the MTT assay, thus resulting in the somewhat higher viability values measured after exposure to the cytokine mixture in the MTT assay than the extent of destruction detected on the ultrastructural level. In the mito 2 clone with a high preferential catalase overexpression, cytokine toxicity damaged only a few cells. These cells were characterized by a disrupted integrity of the plasma membrane and other cell organelles, but importantly all mitochondria remained well preserved (Fig. 3B). In the mito 1 clone with the lower mitochondrial catalase activity, some of the mitochondria were damaged while the others were still intact in the cytokine-treated cells (not shown in Fig. 3).

**Effects of targeted catalase overexpression on NO production and iNOS promoter activity after exposure to IL-1β alone or a cytokine mixture.** To elucidate the influence of catalase overexpression in different subcellular compartments on the NO-induced toxicity, the activity of the iNOS promoter was measured with an iNOS-secreted alkaline phosphatase (SEAP) reporter gene construct, and the amount of accumulated nitrite was quantified using the Griess assay (9).

The induction of the iNOS promoter after a 24-h incubation with IL-1β (600 units/ml) or with the 1 × cytokine mixture is expressed as the percentage induction of the iNOS promoter activity under nonstimulated control conditions. The data are given as means ± SE from at least six individual experiments (control, IL-1β: 850 ± 53%; 1 × cytokine mixture: 805 ± 70%; cyto, IL-1β: 945 ± 69%; 1 × cytokine mixture: 938 ± 115%; mito 1, IL-1β: 700 ± 102%; 1 × cytokine mixture: 689 ± 71%; mito 2, IL-1β: 1,063 ± 70%; 1 × cytokine mixture: 922 ± 67%).

Accumulated nitrite (in pmol/μg protein) after a 24-h incubation was as follows: control, nonstimulated: 0.28 ± 0.05, IL-1β: 1.70 ± 0.54, 1 × cytokine mixture: 1.61 ± 0.28; cyto, nonstimulated: 0.28 ± 0.17, IL-1β: 1.71 ± 0.24, 1 × cytokine mixture: 1.61 ± 0.29; mito 1, nonstimulated: 0.25 ± 0.10, IL-1β: 1.90 ± 0.36, 1 × cytokine mixture: 1.38 ± 0.50; mito 2, nonstimulated: 0.26 ± 0.16, IL-1β: 1.28 ± 0.51, 1 × cytokine mixture: 1.11 ± 0.31. The data
Effects of targeted catalase overexpression on MnSOD protein expression after exposure to IL-1β alone or a cytokine mixture

Incubation of RINm5F cells, both with IL-1β (600 units/ml) alone or with the cytokine mixture (600 units/ml) resulted in a significant increase of MnSOD protein expression (Fig. 4). This increase of MnSOD expression was not significantly affected by catalase overexpression in the cyto and the mito 1 and 2 clones (Fig. 4).

DISCUSSION

Pancreatic β-cells are equipped with superoxide radical inactivating superoxide dismutase isoenzymes in the cytoplasmic (Cu/Zn isoform) and mitochondrial (Mn isoform) compartment (~50% of liver expression level) (1,2). The catalytic capacities are moderate but physiologically sufficient for conversion of superoxide radicals into H₂O₂ in the cytoplasm and mitochondria.

H₂O₂-inactivating enzymes are typically considered to reside in the cytoplasmic compartment of the cell (24). In the present study, we also observed a minor fraction of catalase expression in the mitochondrial compartment of insulin-producing cells, thus confirming corresponding observations in other cell types (25–27). However, the expression level of the H₂O₂-inactivating enzymes glutathione peroxidase and catalase, both of which convert H₂O₂ into H₂O, is extremely low in insulin-producing cells, comprising only 1% of the expression level in liver (1,2). This represents a particular challenge for the antioxidative defense situation of the mitochondria in insulin-producing cells because these organelles are physiologically the major site of superoxide radical formation in the cell (28,29). The imbalance between superoxide radical and H₂O₂-inactivating enzymes in the mitochondrial compartment makes pancreatic β-cells particularly vulnerable to oxidative stress.

To determine the importance of the intramitochondrial antioxidative defense status for cellular survival, we bio-engineered, through targeted expression, insulin-producing RINm5F cells that stably overexpressed catalase preferentially in the mitochondria or in the cytoplasm. In these cell clones, we studied the protective effects of catalase overexpression against the toxic action of a number of ROS-generating chemical compounds as well as cytokines.

Catalase overexpression in the cytoplasmic or in the mitochondrial compartment protected insulin-producing RINm5F cells against the toxicity of ROS, which is in agreement with observations made in HepG2 hepatoma cells (30). Interestingly, however, it was only the high mitochondrial overexpression of catalase that provided significant protection against cell death in the case of menadione toxicity; a compound that preferentially generates superoxide radicals intramitochondrially (31,32). In contrast, catalase overexpression in the cytoplasmic cell compartment was not effective, thus confirming an earlier observation (7).

On the other hand, the cell clones with mitochondrial catalase overexpression were less well protected against cell damage than cells with catalase overexpression in the cytoplasmic compartment when exposed to H₂O₂. This is not surprising because catalase in the cytoplasmic compartment is better suited to inactivate H₂O₂ entering the cell from the extracellular space, thus inactivating H₂O₂ before causing damage to the cytoplasmic matrix and before reaching the mitochondria. This confirms the results of earlier studies (7), and it is also in agreement with the observation that exogenous addition of catalase to the incubation medium provided complete protection against H₂O₂ toxicity (7).

When cells are incubated with HX/XO, H₂O₂ will arise extracellularly through an enzymatic reaction at a relatively lower concentration than in the case of the incubation with H₂O₂ (7,17), but it will be generated continuously over a prolonged period. Comparing the cell clones cyto and mito 1 with the same level of total catalase activity, the better protection was observed in the cyto clone with the preferential cytoplasmic catalase overexpression rather than mitochondrial catalase overexpression.
than in the mito 1 clone with the preferential mitochondrial catalase overexpression. This is not surprising, as in analogy to the situation in the experiments with H$_2$O$_2$ incubation, catalase in the cytoplasmic compartment is better suited to inactivate H$_2$O$_2$ entering the cell from the extracellular space. Nevertheless, a further improvement of the protection was obtained through an additional increase of the catalase activity in the mitochondrial compartment, as is evident from the comparison of the defense situation in the mito 2 clone, which showed an even better protection with a comparable level of cytoplasmic catalase overexpression but a higher level of catalase activity in the mitochondria than the cyto clone.

Thus, it can be concluded from the experiments with ROS-generating compounds that targeted overexpression of catalase in the mitochondria provide particularly effective protection against cell death when ROS are generated intramitochondrially.

Using these catalase-overexpressing cell clones, we addressed the question of whether an enhanced intramitochondrial H$_2$O$_2$-inactivating capacity might also improve the resistance against cytokine-mediated toxicity, since ROS have also been considered to be mediators of the proinflammatory action of cytokines (3,4,33,34).

Both IL-1β and mixtures of proinflammatory cytokines favor the intramitochondrial generation of H$_2$O$_2$ through induction of MnSOD expression (35–38), an observation that we confirmed in the present study for insulin-producing RINm5F cells. Though there was no difference in the degree of MnSOD expression among the different catalase-overexpressing clones, the protection against cytokine-induced cell death, as analyzed in the MTT assay, was significantly better in the clones overexpressing catalase preferentially in the mitochondria. In line with previous observations, cytoplasmic overexpression of catalase provided less protection (9,39).

The observed high rate of cell death after exposure to a cytokine mixture composed of IL-1β, TNF-α, and IFN-γ when compared with the weaker effect of IL-1β alone may be due to TNF-α-induced ROS formation in the mitochondria. A number of recent reports have shown direct ROS generation after exposure of cells to TNF-α (40–43), emphasizing a major role of ceramide as a mediator of superoxide radical formation at the ubisemiquinone site (41,44,45). An enhancement of the toxicity of IL-1β by an additional induction of MnSOD gene expression by TNF-α (E.g., unpublished observations) provides additional support for increased H$_2$O$_2$ generation (16). IL-1β toxicity is enhanced in the presence of TNF-α and IFN-γ probably by a signaling pathways cross talk between these cytokines (46–48). In addition, the induction of iNOS and the subsequent production of NO through cytokines is able to reduce catalase enzyme activity by binding to the iron of the catalase haem groups (49). Although the mitochondrial localization of catalase could theoretically decrease this inactivation reaction, this was not the case. Thus, differences in NO-mediated inhibition of catalase enzyme activity cannot account for the better protection of mitochondrial catalase overexpression against cytokine toxicity.

The iNOS promoter activity and nitrite accumulation after exposure to IL-1β alone or to a cytokine mixture were not significantly affected both in the clones overexpressing catalase in the cytoplasm and in the mitochondria. This is in contrast to MnSOD suppression, which significantly increases iNOS promoter activity and confirms earlier observations made with RINm5F cells overexpressing catalase in the cytoplasm (9,13).

Both IL-1β alone and the cytokine mixture significantly inhibited glucose oxidation and overexpression of catalase in the cyto clone and, in the mito 1 clone, did not prevent this inhibition, thus confirming earlier observations (50). Only the high catalase expression level in the mitochondria in the mito 2 clone was able to prevent the cytokine-induced inhibition of glucose oxidation. These results indicate that a high mitochondrial capacity for H$_2$O$_2$ inactivation is obligatory to maintain mitochondrial function, as documented by both preservation of dehydrogenase enzyme activity and glucose oxidation capacity.

The observations made in the present study in the MTT assay and the glucose oxidation studies that targeted overexpression of catalase in the mitochondria, providing particularly effective protection, were confirmed in the present study on the ultrastructural level. Nearly all cells of the mito 2 clone remained intact after cytokine exposure. Ultrastructural analyses additionally revealed well-preserved mitochondria, even in the few cells of the mito 2 clone in which the plasma membrane and the membranes of intracellular organelles other than mitochondria had been damaged through the cytokine action. This was different from the situation observed in the damaged cells of the cyto clone, in which virtually no intact mitochondria could be detected.

While the toxic effect of IL-1β alone was somewhat more pronounced in the MTT assay, in the case of the incubation with the cytokine mixture the proportion of destroyed cells was larger on the ultrastructural level. This indicates that a combination of IL-1β, TNF-α, and IFN-γ compared with IL-1β alone decreased the cellular membrane integrity more than IL-1β alone, which had a proportionally larger effect on mitochondrial function. This may be related to an activation of additional pathways of cytokine signaling by the cytokine mixture (42,56).

The results clearly indicate that mitochondrial overexpression of catalase provides superior protection against cytokine-mediated cell destruction and death. The protection achieved through catalase overexpression in the mitochondria is apparently a result of the inactivation of the toxic H$_2$O$_2$, which is produced in increased amounts (16,42) through cytokine-induced MnSOD upregulation (13,36,52) in conjunction with cytokine-induced superoxide radical production (44,45). A high level of catalase expression in the mitochondria guarantees a fast and efficient inactivation of the toxic H$_2$O$_2$ and at the same time prevents formation of highly toxic hydroxyl radicals in the Haber-Weiss reaction (53). While the initial mitochondrial damage is likely to be caused by H$_2$O$_2$ itself, progressive destruction of the proteins in the mitochondria may facilitate the release of Fe$^{2+}$ and other trace metals, which subsequently amplify the destructive potential through hydroxyl radical formation via the Haber-Weiss reaction (53). Thus, it can be concluded that increased intramitochondrial H$_2$O$_2$ generation contributes significantly to cytokine-induced cell death. This is partic-
ularly unfavorable in insulin-producing cells because of the extremely low constitutive level of the H$_2$O$_2$-inactivating enzymes and thus provides a plausible explanation for the particular sensitivity of pancreatic β-cells against ROS-mediated cytokine toxicity (54,55).

A further aggravation can be anticipated in situations of increased and prolonged hyperglycemia when, during autoimmune attack in a developing diabetic metabolic state, the remaining pancreatic β-cells are under particular functional workload (56,57). The resulting increased rate of glucose metabolism and oxygen consumption in insulin-producing cells (51,58) will foster the generation of superoxide radicals in the respiratory chain (59). This vicious cycle will ultimately reduce the chances of survival of the remaining β-cells under autoimmune attack (60–64). This scenario may also help to explain the causes underlying glucotoxicity in situations of prolonged hyperglycemia (65–67).

Our results provide evidence that the mitochondrial targeting of catalase is an attractive strategy to protect insulin-producing cells against oxidative injury and cytokine toxicity. This may also open new perspectives for antioxidative gene therapy of type I diabetes.

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