Protection of Insulin-Producing RINm5F Cells Against Cytokine-Mediated Toxicity Through Overexpression of Antioxidant Enzymes

Stephan Lortz, Markus Tiedge, Thomas Nachtwey, Allan E. Karlsen, Jørn Nerup, and Sigurd Lenzen

Nitric oxide (NO) and reactive oxygen species (ROS) are crucial elements in cytokine-mediated β-cell destruction. In insulin-producing RINm5F cells, overexpression of cytoprotective enzymes provides significant protection against the synergistic toxicity of NO and ROS. We therefore examined whether overexpression of catalase (Cat), glutathione peroxidase (Gpx), and Cu/Zn superoxide dismutase (SOD) can provide protection for bioengineered RINm5F cells against cytokine-mediated toxicity. A 72-h exposure of RINm5F control cells to interleukin-1β (IL-1β) alone or a combination of IL-1β, tumor necrosis factor-α, and γ-interferon resulted in a time- and concentration-dependent decrease of cell viability in the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) cytotoxicity assay. Although IL-1β alone caused only a moderate reduction of viability in the range of 25%, the cytokine mixture induced a significant loss of viability of >75%. This increased toxicity of the cytokine mixture compared with that of IL-1β alone could be explained by a higher rate of NO generation within the early 24–48 h incubation period that would favor the toxic synergism of NO and oxygen free radicals. Overexpression of Cat, Gpx, and Cu/Zn SOD protected against toxicity of the cytokine mixture but not against that of IL-1β alone. The reduction of cytokine-mediated toxicity was evident also because of an increased proliferation rate and a drastic decrease in the cell death rate. The improved antioxidant defense status did not prevent the activation of iNOS after cytokine exposure. However, RINm5F cells overexpressing cytoprotective enzymes showed a significantly lower level of ROS-damaged protein residues. Thus, protection through Cat, Gpx, and Cu/Zn SOD overexpression was apparently because of an inactivation of ROS generated in the signal cascades of the cytokines. Overexpression of cytoprotective enzymes thus represents a feasible strategy to protect insulin-producing cells against cytokine-mediated cytotoxicity. ***Diabetes* 49: 1123–1130, 2000**

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ANOVA, analysis of variance; BrdU, 5-bromo-2′-deoxyuridine; Cat, catalase; DNP, dinitrophenyl; DNPH, dinitrophenylhydrazine; ELISA, enzyme-linked immunosorbent assay; Gpx, glutathione peroxidase; IFN-γ, γ-interferon; IL-1β, interleukin-1β; iNOS, inducible nitric oxide synthase; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; OD, optical density; RIN-Cat, RINm5F tissue culture cells overexpressing catalase; RIN-Gpx, RINm5F tissue culture cells overexpressing glutathione peroxidase; RIN-SOD, RINm5F tissue culture cells overexpressing superoxide dismutase; ROS, reactive oxygen species; SIN-1, 3-morpholinosynonimine; SOD, superoxide dismutase; TNF-α, tumor necrosis factor-α.

Autoimmune destruction of pancreatic β-cells during the development of type 1 diabetes is a complex process involving both cellular and humoral elements of cytotoxicity (1–4). Inflammatory cytokines initiate signal pathways in β-cells, resulting in a broad spectrum of events ranging from a functional disturbance to cell death through necrosis and apoptosis (3–6). The generation of nitric oxide (NO) through activation of the inducible NO synthase (iNOS) gene is a well-known characteristic of cytokine action that emphasizes the importance of free radical attack in β-cell destruction (3,7–10). In addition, experimental evidence indicates that oxygen free radicals are generated in cytokine-stimulated β-cells by an as-yet-unknown mechanism (11–17). In contrast with other tissues, pancreatic β-cells show a low enzymatic antioxidant defense status, particularly regarding the hydrogen peroxide–inactivating enzymes catalase (Cat) and glutathione peroxidase (Gpx) (18–21). Overexpression of these antioxidant enzymes protected insulin-producing cells against chemical generators of oxygen free radicals and NO (21–24). Cytoprotective enzymes did not directly inactivate NO but efficiently prevented the synergistic toxicity of oxygen free radicals and NO (17,24–27). However, whether these antioxidant enzymes confer protection against the complex scenario of cytokine-induced toxicity is not known. Recently, a study showed that stable overexpression of the mitochondrial Mn superoxide dismutase (MnSOD) protected INS-1 insulin-producing cells against interleukin-1β (IL-1β) toxicity apparently through a decrease in iNOS activation (28). In the present study, we evaluated the protective potential of Cu/Zn SOD, Cat, and Gpx overexpression in RINm5F insulin-producing cells (23,24) against the toxicity of IL-1β alone and a combination of cytokines (13,29). Our data provide evidence that these antioxidant enzymes significantly increase the resistance of RINm5F insulin-producing cells against the toxic action of cytokines, apparently through inactivation of oxygen free radicals rather than through effects on NO generation. Thus, the antioxidant enzymes Cat, Gpx, and Cu/Zn SOD apparently represent an efficient principle of β-cell protection.

RESEARCH DESIGN AND METHODS

Tissue culture of RINm5F cells. RINm5F tissue culture cells overexpressing Cat (RIN-Cat), Gpx (RIN-Gpx), or Cu/Zn SOD (RIN-SOD) were generated through stable transfection of the cDNAs in the pcDNA3 vector (23). The antioxidant enzyme activities were in the following different control and overexpressing cell clones, respectively (23).

- Gpx: 0.2 (control), 3.9 (Gpx), 2.6 (Gpx-2), and 2.3 (Gpx-3) U/mg protein
- Cat: 6 (control), 564 (Cat), 466 (Cat-2), and 398 (Cat-3) U/mg protein
- Cu/Zn SOD: 70 (control), 154 (SOD), 134 (SOD-2), and 142 (SOD-3) U mg/protein

Control experiments confirmed that transfection with the pcDNA3 vector–lacking insert did not affect the expression of the cytoprotective enzymes. Cell proliferation and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) absorbance rate as well as insulin secretion and content of RINm5F cells remained unchanged after overexpression of cytoprotective enzymes (23). The cells were cultured as described (23) in RPMI-1640 medium supplemented with 10 mmol/l glucose, 10% (vol/vol) fetal calf serum, penicillin, and streptomycin in a humidified atmosphere at 37°C in 5% CO_2. All tissue culture equipment was from Gibco (Gaithersburg, MD). Selenium (10 nmol/l) as an essential cofactor for Gpx was added to the tissue culture medium of Gpx-transfected cells. Selenium did not affect enzyme expression (23) or protection against the tested cytokines (data not shown).

Cytokine exposure and induction of apoptosis. Control and transfected RINm5F cells were seeded at a concentration of 6,000 cells/well in 100 µl culture medium in 96-well microplates and were allowed to attach for 24 h before 60, 300, or 600 U/ml human IL-1β (PromoCell, Heidelberg, Germany) or a combination of cytokines (1× cytokine mixture) consisting of 60 U/ml IL-1β, 185 U/ml human tumor necrosis factor-α (TNF-α), and 14 U/ml rat γ-interferon (IFN-γ) (PromoCell, Heidelberg, Germany) was added. Apoptosis of RINm5F control cells was induced through exposure to the topoisomerase inhibitor camptothecin (5 µmol/l for 24 h) (Sigma, St. Louis, MO).

MTT cell viability assay. In all sets of experiments, the viability of the cells was determined after the 72-h incubation period using a microplate-based MTT assay (30). Viability was expressed in the percentage of the MTT absorbance in the absence of cytokines. The absolute optical density $(OD)_{550}$ absorbance rates of the MTT assay were as follows in the control and overexpressing RINm5F cell clones, respectively. (Numbers in parentheses indicate number of experiments.)

- Control: 1,314 ± 46 (4) $mOD_{550}/6 \times 10^3$ cells/well (96 h after seeding)
- Gpx: 1,097 ± 17 (4), Gpx-1: 963 ± 15 (3), Gpx-2: 1,057 ± 16 (3) $mOD_{550}/6 \times 10^3$ cells/well (96 h after seeding)
- Cat: 1,759 ± 31 (4), Cat-1: 1,688 ± 55 (3), Cat-2: 1,664 ± 35 (3) $mOD_{550}/6 \times 10^3$ cells/well (96 h after seeding)
- SOD: 1,099 ± 20 (4), SOD-1: 1,237 ± 34 (3), SOD-2: 1,001 ± 33 (3) $mOD_{550}/6 \times 10^3$ cells/well (96 h after seeding)

Nitrite determination. A total of 50 µl culture medium was incubated with an equal volume of Griess reagent (0.1% naphtyl ethylenediamine and 1% sulfanilamide in 0.1 mol/l HCl, 1:1 vol/vol) for 10 min at room temperature. Nitrite production was determined spectrophotometrically at an absorbance of 562 nm in a microplate using sodium nitrite solutions (0–0.6 µmol/l) as standards (31).

Western blot analyses. RINm5F cells were homogenized in ice-cold homogenization medium (20 mmol/l HEPES, 210 mmol/l mannitol, 70 mmol/l sucrose, pH 7.4). The tissue homogenates were centrifuged at 1,000*g* and 4°C for 10 min to pellet insoluble material. The supernatant was used for Western blot analyses. Protein was determined by the bicinchoninic acid assay (Pierce, Rockford, IL). For visualization of reactive carbonyl side groups, protein fractions (10–15 mg/ml) were treated with 10 mmol/l dinitrophenylhydrazine (2,4-DNPH in 2 mol/l HCl) (Sigma) for 30 min at room temperature (32,33). A total of 10 µg protein was fractionated by reducing 10% SDS-PAGE and was transferred to polyvinylidene fluoride membranes. Nonspecific binding sites of the membranes were blocked by nonfat dry milk for 1 h at 37°C, and then the blots were incubated with specific primary antibodies against rat iNOS (Santa Cruz Biotechnology, Santa Cruz, CA) or dinitrophenyl (DNP) (Sigma) (32,33) at a dilution of 1:5,000 to 1:10,000 for 4 h at room temperature followed by a 2-h incubation period with peroxidase-labeled secondary antibody at a dilution of 1:15,000 at room temperature. The protein bands were visualized using the enhanced chemiluminescence detection system. For comparison, total protein expression of the samples (10 µg/lane) was shown by a reducing 10% SDS-PAGE stained by Coomassie blue. Autoradiograms were quantified by computer-assisted densitometry using the Image 1.52 program (National Institutes of Health, Bethesda, MD).

Measurement of BrdU incorporation. The incorporation of 5-bromo-2′-deoxyuridine (BrdU) in RINm5F cells was quantified by the colorimetric cell proliferation enzyme-linked immunosorbent assay (ELISA) specific for BrdU (Roche Molecular Biochemicals, Mannheim, Germany) according to the protocol of the supplier. Data are percentages of the untreated cells after different periods of cytokine exposure.

Morphological characterization of cell viability and integrity. RINm5F cells were stained using the DNA-binding dyes Hoechst 33342 (Molecular Probes, Eugene, OR) and propidium iodide (Sigma) as indices for the number of viable, necrotic, and apoptotic cells, as described previously (5).

DNA fragmentation assay. Low-molecular-weight DNA was extracted from RINm5F cells as described previously (34). A total of 30–50 µg DNA was separated in a 0.8% tris-borate-EDTA agarose gel and stained with ethidium bromide.

Statistical analyses. Data are means ± SE. Statistical analyses were performed using analysis of variance (ANOVA) plus Dunnett's test for multiple comparisons.

RESULTS

Toxicity of cytokines

IL-1β. Incubation of nontransfected control RINm5F cells with IL-1β alone for 72 h resulted in a significant ($P < 0.05$) concentration-dependent decrease in viability of up to 25% at 600 U/ml (Fig. 1*A*, Table 1). Overexpression of Gpx in the RIN-Gpx cells increased resistance against IL-1β marginally (Fig. 1*A*; Table 1). Cat or Cu/Zn SOD overexpression did not provide protection against IL-1β compared with controls (Fig. 1*A*, Table 1). IL-1β induced a significant ($P < 0.05$) concentration-dependent accumulation of nitrite as an indicator of NO production (Fig. 1*A*). Although nitrite accumulation was somewhat lower in RINm5F-Cat and RINm5F-Gpx cells at a concentration of 60 U/ml IL-1β, no significant difference was evident both at 60 and 600 U/ml IL-1β compared with nontransfected control cells (Fig. 1*A*).

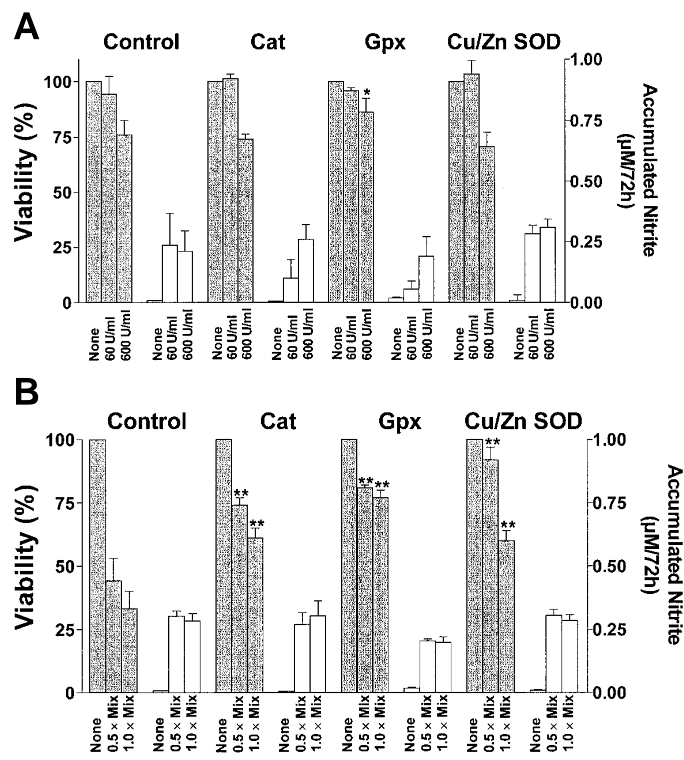

FIG. 1. Effects of overexpression of antioxidant enzymes in RINm5F cells on the toxicity of (*A*) IL-1β alone and (*B*) the 1× cytokine mixture (60 U/ml IL-1β, 185 U/ml TNF-α, and 14 U/ml IFN-γ). Cells were incubated with the cytokines for 72 h. Thereafter, viability of the cells was determined by the MTT assay and expressed as a percentage of the untreated cells (■). Nitrite accumulation was determined by the Griess assay (□). Data are means ± SE from 4–6 individual experiments. *$P < 0.05$; **$P < 0.01$ vs. nontransfected control cells (ANOVA/ Dunnett's test).

TABLE 1
Remaining viability of 3 different RINm5F clones overexpressing Cat, Gpx, or Cu/Zn SOD after 72 h of treatment with IL-1β alone or the IL-1β, TNF-α, and INF-γ cytokine mixture

RINm5F	600 U/ml IL-1β	0.5 × cytokine mixture	1 × cytokine mixture
Control	76 ± 5 (6)	44 ± 9 (6)	33 ± 7 (6)
Gpx	92 ± 2 (4)*	81 ± 1 (4)†	77 ± 3 (4)†
Gpx-2	86 ± 6 (3)	76 ± 5 (3)†	71 ± 6 (3)†
Gpx-3	88 ± 4 (3)	75 ± 7 (3)†	68 ± 8 (3)†
Cat	74 ± 2 (4)	74 ± 3 (4)†	61 ± 4 (4)†
Cat-2	75 ± 2 (3)	74 ± 3 (3)*	64 ± 4 (3)†
Cat-3	73 ± 2 (3)	72 ± 2 (3)*	58 ± 4 (3)*
SOD	77 ± 4 (4)	92 ± 5 (4)†	60 ± 4 (4)†
SOD-2	67 ± 8 (3)	85 ± 7 (3)†	60 ± 6 (3)*
SOD-3	71 ± 7 (3)	87 ± 6 (3)†	59 ± 6 (3)*

Data are means ± SE (*n*). $^*P < 0.05$ for cells vs. nontransfected controls; $^\dagger P < 0.01$ cells vs. nontransfected controls (ANOVA/Dunnett's test). Cells were exposed for 72 h to IL-1β (600 U/ml) alone or to the cytokine mixtures (1 × cytokine mixture: 60 U/ml IL-1β, 185 U/ml TNF-α, 14 U/ml IFN-γ; 0.5× cytokine mixture: 30 U/ml IL-1β, 92.5 U/ml TNF-α, 7 U/ml IFN-γ). Viability of the cells was determined by the MTT assay and expressed as a percentage of the untreated cells.

Cytokine mixture. In contrast with IL-1β alone, a combination of cytokines consisting of IL-1β, TNF-α, and IFN-γ (60 U/ml IL-1β, 185 U/ml TNF-α, and 14 U/ml IFN-γ) induced a significant ($P < 0.05$) concentration-dependent loss of viability of up to 65% in RINm5F control cells (Fig. 1*B*; Table 1). Overexpression of Cat, Gpx, and Cu/Zn SOD provided significant protection with a 30–80% higher viability after exposure to the cytokine mixture (Fig. 1*B*, Table 1). At the lower 0.5× concentration of the cytokine mixture, Cu/Zn SOD overexpressing cells showed the highest viability, whereas Gpx overexpressing cells showed the highest viability after exposure to the 1× cytokine mixture concentration (Fig. 1*B*; Table 1). In the presence of the NOS inhibitor N^ω-nitro-L-arginine (5 mmol/l), the cytokine mixture did not affect the viability of RINm5F control cells and RINm5F cells overexpressing antioxidant enzymes (data not shown). Thus, the toxicity in RINm5F cells resulting from this cytokine combination depends on the generation of NO. The resistance of RINm5F cells overexpressing antioxidant enzymes was not a phenomenon of clonal selection because 3 independent clones of each enzyme have comparable results (Table 1). The addition of TNF-α and IFN-γ together with IL-1β (cytokine mixture) resulted in a significant ($P < 0.05$) increase in NO production after 24 and 48 h compared with that of IL-1β alone (Fig. 2). Thus, the higher vulnerability of RINm5F control cells in the presence of the cytokine mixture compared with that of IL-1β alone can be explained by the toxic effect of an increased NO production rate during the early events of cytokine toxicity. The amount of accumulated nitrite after a 72-h incubation period was, however, comparable in supernatants from RINm5F cells exposed to IL-1β alone and to the cytokine combination (Fig. 1). Conclusively, the protective effects of the antioxidant enzymes correlate with the time course of NO production, which determines the potential toxic synergism with oxygen free radicals.

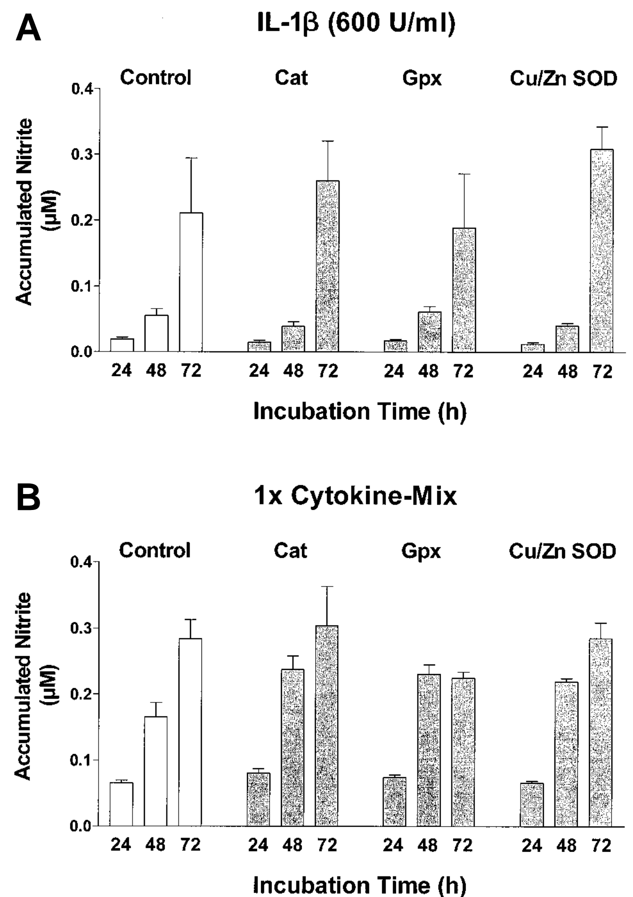

FIG. 2. Effects of overexpression of antioxidant enzymes in RINm5F cells on nitrite accumulation after 24 and 48 h of incubation with IL-1β alone (*A*) (600 U/ml) or the 1× cytokine mixture (*B*) (60 U/ml IL-1β, 185 U/ml TNF-α, and 14 U/ml IFN-γ). Cells were incubated with the cytokines for 24, 48, or 72 h. Thereafter, nitrite accumulation was determined by the Griess assay. Data are means ± SE from 6 individual experiments.

Induction of iNOS by cytokines. To evaluate the effects of antioxidant enzyme overexpression on cytokine-mediated induction of iNOS protein, the expression of this enzyme was quantified by Western blot analyses. In nontransfected RINm5F cells, a concentration-dependent increase of iNOS protein was detectable after a 6-h incubation with IL-1β (Fig. 3). Overexpression of Gpx, Cat, or Cu/Zn SOD did not suppress the activation of iNOS by IL-1β (Fig. 3). The iNOS protein expression levels after a 6-h incubation with 60 U/ml IL-1β were consistently higher in RINm5F cells overexpressing Gpx, Cat, or Cu/Zn SOD than in control cells (Fig. 3). Exposure of control RINm5F cells and transfected RINm5F cells to the 1× cytokine mixture gave results that were comparable with the induction of iNOS by IL-1β alone (data not shown). Conclusively, overexpression of antioxidant enzymes in RINm5F cells did not affect the iNOS signal pathway of cytokine toxicity.

Protection against cytostatic and cytotoxic effects of cytokines. Antioxidant enzymes may protect RINm5F cells against cytokine-mediated toxicity either by ameliorating the cytostatic effects of cytokines or by preventing cell death. The incorporation of BrdU shows the combined effects of cytokines on cell proliferation and cell death. The average

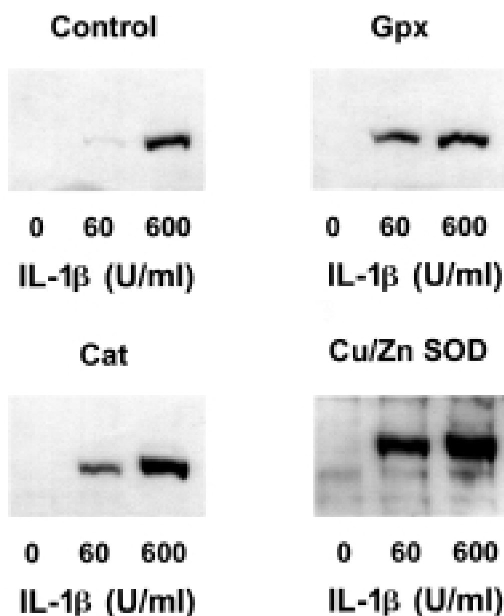

FIG. 3. Effects of overexpression of antioxidant enzymes in RINm5F cells on iNOS activation by IL-1β. Cells were exposed to 0, 60, or 600 U/ml IL-1β for 6 h. Thereafter, iNOS activation was quantified in cellular extracts (50 µg protein/lane) by Western blot analyses. Data are representative blots from 4 individual experiments.

absolute OD_{492} absorbance rates (562 nm) in the BrdU incorporation assay were 1,429 ± 72 (4) mOD/6 × 10^3 cells for RINm5F control cells, 1,150 ± 63 (4) mOD/6 × 10^3 for RINm5F-Gpx cells, 1,323 ± 40 (4) mOD/6 × 10^3 for RINm5F-Cat cells, and 1,029 ± 126 (4) mOD/6 × 10^3 for RINm5F-SOD cells (96 h after seeding). Thus, the absolute absorbance rates of the RINm5F control cells and the transfected cells did not differ significantly, which indicates comparable rates of proliferation. Incubation of RINm5F control cells with the 1× cytokine mixture revealed a time-dependent decrease of the proliferation rate of 35–40% after 48 and 72 h (Fig. 4). Overexpression of Gpx, Cat, or Cu/Zn SOD significantly reduced the cytostatic effects of cytokines (Fig. 4). The 1× cytokine mixture induced a significant increase in the cell death rate from 0 to 50% of the RINm5F control cells monitored by differential staining with Hoechst dye and propidium iodide (Fig. 5). Based on the nuclear morphology, the mode of cell death was dominantly necrosis (>95%). Only 3% of the nuclei showed an apoptotic morphology. These results were confirmed by electron microscopy (data not shown). Overexpression of antioxidant enzymes drastically reduced the cell death rate in RINm5F cells 24, 48, and 72 h after cytokine exposure to values <5% (Fig. 5).

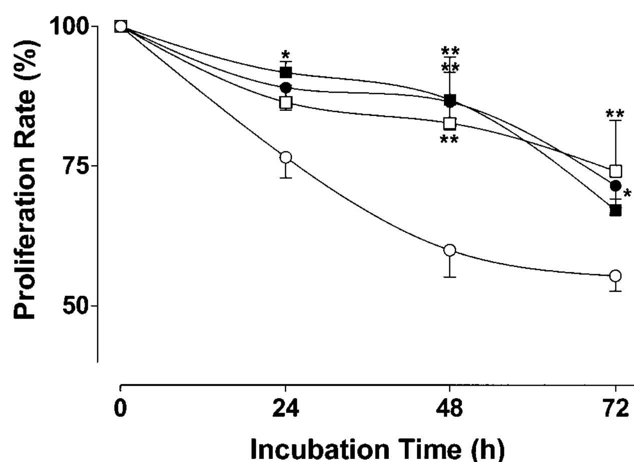

FIG. 4. Effects of overexpression of antioxidant enzymes in RINm5F cells on the proliferation rate after cytokine treatment. Cells were exposed to the 1× cytokine mixture for 24, 48, or 72 h. Proliferation was quantified by a BrdU-specific ELISA and is a percentage of the proliferation rate of cells without cytokine treatment. Data are means ± SE from 4 individual experiments. ■, RINm5F-Cat cells; ○, RINm5F control cells; □, RINm5F-Cu/Zn SOD cells; ●, RINm5F-Gpx cells. *P < 0.05; **P < 0.01 vs. nontransfected control cells (ANOVA/ Dunnett's test).

DNA and protein damage by cytokines. A characteristic apoptotic DNA fragmentation can be efficiently induced in RINm5F control cells through exposure to the topoisomerase inhibitor camptothecin (5 µmol/l) for 24 h (Fig. 6, lane 1). In contrast, incubation of RINm5F control cells with 1× cytokine mixture for 48 h (Fig. 6, lane 2) or 72 h (Fig. 6, lane 3) did not result in the formation of low-molecular-weight DNA laddering. The DNA pattern corresponded with that observed in nontreated RINm5F cells (Fig. 6, lane 4) with a slight smear of low-molecular-weight DNA. Thus, apoptotic DNA fragmentation is apparently not the predominant cellular mechanism in cytokine-mediated destruction of RINm5F cells.

The significant protection of RINm5F cells against cell death by antioxidant enzymes prompted us to evaluate the oxidative damage of cellular protein after exposure of the cells to the cytokine mixture. Free carbonyl side groups, which are considered to be an indicator for oxidatively damaged proteins, were visualized through reaction with DNPH and immunodetection with an anti-DNP antibody. The number and the intensities of the protein bands corresponded with the

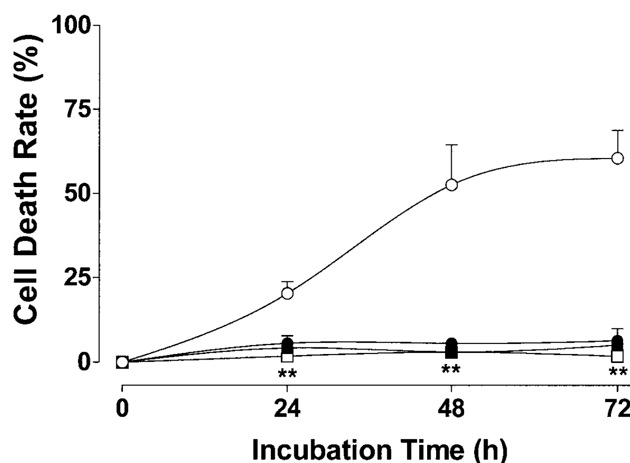

FIG. 5. Effects of overexpression of antioxidant enzymes in RINm5F cells on cell death rate after cytokine treatment. 10^5 cells were seeded on chamber slides and exposed to the 1× cytokine mixture for 24, 48, or 72 h. Cells were characterized morphologically for cell viability through fluorescence microscopy using Hoechst dye and propidium iodide. Data are a percentage of dead cells from the total cell population. Dead cells showed a necrotic morphology (>95%). Data are means ± SE from 4 individual experiments. ■, RINm5F-Cat cells; ○, RINm5F control cells; □, RINm5F-Cu/Zn SOD cells; ●, RINm5F-Gpx cells. **P < 0.01 vs. nontransfected control cells (ANOVA/Dunnett's test).

level of protein damage by oxygen free radicals. This can be demonstrated by the oxidative damage of proteins from RINm5F control cells when they are exposed to 3-morpholinosynonimine (SIN-1) (Fig. 7*B*), a chemical compound that generates NO and oxygen free radicals. The total protein stain of the cell extracts (Fig. 7*A*) did not provide evidence that the increased DNP-modified protein bands after cytokine exposure resulted from altered levels of the respective proteins. Incubation of RINm5F control cells with IL-1β or the cytokine mixture for 72 h resulted in an increase of oxidatively damaged proteins (Fig. 7*B*). Notably, the DNP-protein band pattern differed between cells exposed to IL-1β alone and to the cytokine mixture, providing evidence of different target proteins (Fig. 7*B*). RINm5F cells overexpressing Cat, Gpx, or Cu/Zn SOD, however, showed virtually no such differences in the intensities of oxidatively damaged proteins between the control condition and after cytokine exposure (Fig. 7*B*). This protection was verified through quantification of 2 prominent protein bands that were susceptible to oxidative damage. RINm5F control cells showed a 13-fold increase of band 1 and a 3.6-fold increase of band 2 in the DNP-band intensities after a 72-h exposure to the cytokine mixture (Figs. 7 and 8). In contrast, RINm5F cells overexpressing Cat, Gpx, or Cu/Zn SOD did not reveal any significant increases in the DNP-band intensities after incubation with IL-1β alone or with the cytokine mixture (Figs. 7 and 8). Thus, the improved enzymatic antioxidant defense status efficiently protected RINm5F cells against oxidation of cellular proteins during cytokine toxicity.

DISCUSSION

Cytokines as humoral mediators of inflammation confer destruction to pancreatic β-cells of various species, including rats and humans (1,3,4,35). In this study, we showed that antioxidant enzymes efficiently protected RINm5F insulin-producing cells against the toxicity of the cytokine combination of IL-1β, TNF-α, and IFN-γ. Previous reports on rodent islets provide evidence that oxygen free radicals are potent executors of cell death in the cytokine signal cascade (12,13,36). In a synergism with NO, these reactive oxygen species form various toxic radicals that attack cellular structures such as proteins, lipids, and nucleic acids in a more or less nonspecific manner (26,37). This does not devalue the role of NO as an essential effector of cytokine toxicity. Several investigators have shown that chemical inhibitors of NO generation protected insulin-secreting cells against cytokine-mediated toxicity but with variable efficacy depending on the species and the combination of the cytokines (7,38–44). Notably, RINm5F cells overexpressing Cat, Gpx, or Cu/Zn SOD showed an increased iNOS protein expression after a 6-h incubation with 60 U/ml IL-1β. This phenomenon may be explained by the inactivation of oxygen free radicals, which favors the translational induction of iNOS in the early phase of cytokine signaling. In the present study, NO proved to be an indispensable component of cytokine toxicity in RINm5F cells as demonstrated by a complete protection of the NOS inhibitor N^{ω}-nitro-L-arginine both in control cells and in overexpressing cells. The increased toxicity of the cytokine combination compared with that of IL-1β alone is because of a

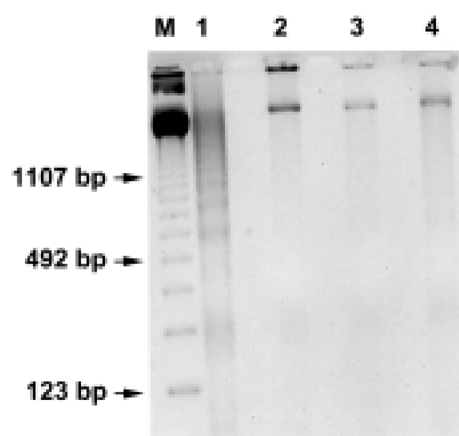

FIG. 6. DNA fragmentation in RINm5F cells after exposure to cytokines and the topoisomerase inhibitor camptothecin. Nontransfected RINm5F cells were exposed to the 1× cytokine mixture for 48 or 72 h. For induction of apoptosis, cells were incubated with 5 µmol/l camptothecin for 24 h. A total of 20–30 µg DNA/lane isolated from floating and adherent cells was separated in a 0.8% agarose gel and stained with ethidium bromide. Apoptotic DNA fragmentation was detectable only after exposure to camptothecin in lane 1. Data are representative gels from 4 individual experiments. M, DNA molecular weight marker (123-bp DNA ladder); 1, camptothecin 24 h; 2, 1× cytokine mixture for 48 h; 3, 1× cytokine mixture for 72 h; 4, untreated control.

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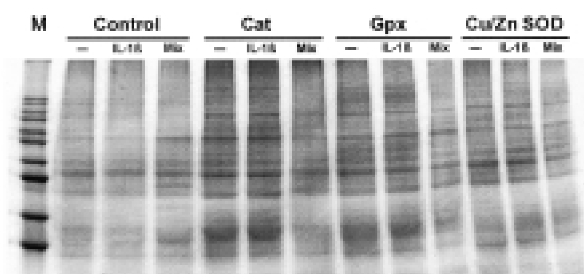

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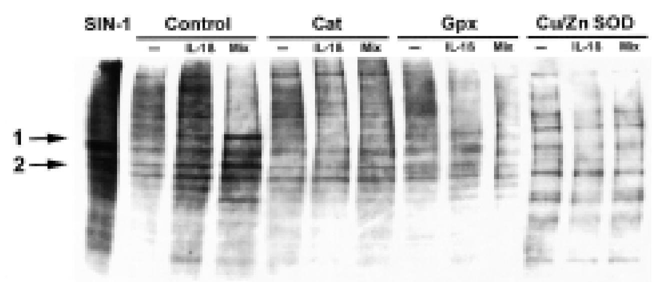

FIG. 7. Effects of overexpression of antioxidant enzymes in RINm5F cells on oxidatively modified cellular proteins after exposure to cytokines. *A*: Coomassie stain; *B*: DNP Western blot. Cells were exposed to 600 U/ml IL-1β or the 1× cytokine mixture for 72 h. Nontransfected RINm5F cells that were incubated with SIN-1 (5 mmol/l for 2 h) served as positive control cells for oxidative damage of proteins by chemically generated free radicals. Thereafter, cells were homogenized, and the protein was reacted with the carbonyl reagent DNPH followed by Western blot analyses with an anti-DNP antibody. Data are representative blots from 4 individual experiments. Protein bands 1 and 2 are the bands quantified in Fig. 8. M, molecular weight marker (10-kDa ladder).

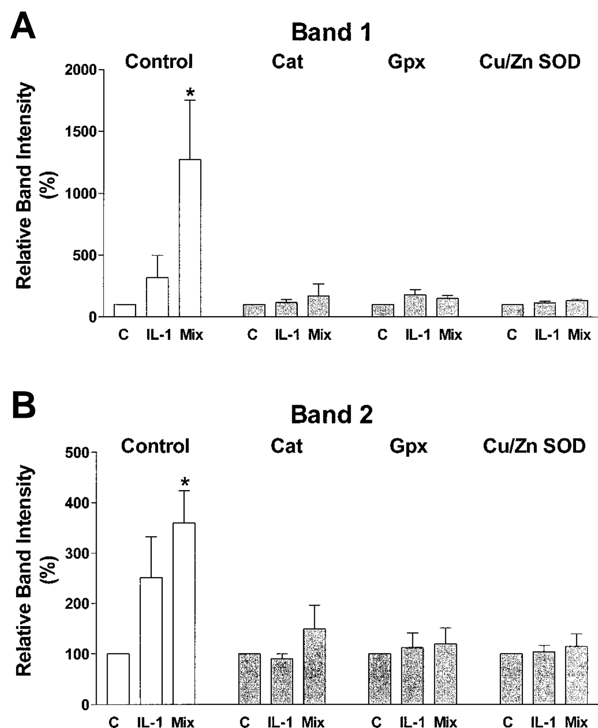

FIG. 8. Quantification of 2 different oxidatively modified cellular proteins after exposure to cytokines. *A*: protein band 1; *B*: protein band 2. Cells were exposed to 600 U/ml IL-1β or the 1× cytokine mixture for 72 h. Autoradiograms were quantified by computer-assisted densitometry. Data are means ± SE from 4 individual experiments. **P* < 0.05 for the cytokine mixture vs. control cells (ANOVA/Dunnett's test).

significantly higher rate of NO generation during the initial 48-h incubation period. This is an observation that has also been made in previous studies of mouse pancreatic islets (29). Our present results thus support the concept that cytokine-induced pancreatic β-cell destruction is ultimately mediated by highly toxic radical species formed by NO and oxygen free radicals (17,43). The low enzymatic antioxidant defense status of pancreatic β-cells, which could be demonstrated on the level of gene expression and catalytic activity (18–21), favors the formation of the oxygen free radical component of cytokine-mediated toxicity. Thus, not surprisingly, overexpression of antioxidant enzymes protected insulin-producing cells not only against chemical generators of oxygen free radicals and NO (23,24) but also against cytokine-mediated toxicity. Notable in particular is the fact that an efficient protection was provided both by Cat and Gpx overexpression. Although both are hydrogen peroxide–inactivating enzymes, they differ in their enzymatic characteristics. The high affinity of Gpx for its substrate particularly enables this cytoprotective enzyme to provide protection against a continuous rate of oxygen free radical production, whereas Cat inactivates hydrogen peroxide with a higher rate constant but with a lower affinity (24). The better protection through Gpx overexpression therefore indicates that a persistent generation of moderate amounts of oxygen free radicals is a specific characteristic of cytokine-mediated toxicity. The results of the DNP Western blot analyses of cytokine-exposed RINm5F cells show that the protection against oxidative protein damage through overexpression of cytosolic antioxidant enzymes as performed in the present study is an efficient way to protect the whole cell against damage induced by these inflammatory mediators. Notably, the cytokine-induced DNP protein band pattern did not result from absolute changes in protein expression within the signal pathways (45–47), as demonstrated by the total protein stain of cellular extracts that remained unaffected by cytokines (Fig. 7*A*).

None of the antioxidant enzymes that were overexpressed in RINm5F cells could prevent the generation of NO via the iNOS pathway, as shown by the induction of the iNOS protein and the accumulation of nitrite, both of which were not different from that in nontransfected control cells. In our study, exposure of RINm5F cells to cytokines induced cell death by necrosis within 72 h, although apoptosis could be initiated in these cells through exposure to the topoisomerase inhibitor camptothecin. Our observation of cytokine-mediated necrosis of insulin-producing cells agrees with earlier reports on functional impairment of insulin secretion, free radical production, and lipid peroxidation, observations that cannot be regarded as signs of an apoptotic destruction of β-cells (12,13,16,36,48). Undoubtedly, however, apoptosis of β-cells can occur in animal models of diabetes as well as in rodent insulin-producing cell lines and purified human β-cells incubated with cytokines (5,49–51). Remember that apoptosis is a complex process that depends on several signal cascades that trigger initiation as well as prevention of apoptosis (52,53). Thus, insulin-producing cells show a variant susceptibility to cytokines, depending on the combination of cytokines, concentration, incubation time, and animal species (4,54). This may explain differences among results obtained with immortalized rodent insulin-producing cell lines, rodent islet cells, and human purified β-cells, respectively (4,54). Conclusively, determining whether cytokines induce β-cell death by necrosis or apoptosis is not as important as defining the exact conditions under which the different modes of cell death occur and, even more importantly, how these forms of cell death could be prevented efficiently.

The signal function of free radicals is another important aspect of cytokine-mediated toxicity. Studies have demonstrated that induction of oxidative stress by hydrogen peroxide leads to an activation of the transcription factor NF-κB and apoptosis in nonislet cells (55,56), which also triggers the induction of the iNOS gene in pancreatic β-cells (3). However, overexpression of Cat or Gpx, which protected RINm5F cells efficiently against hydrogen peroxide (23), did not block the iNOS signal pathway, as shown in this study by the drastic induction of iNOS protein by cytokines and by the accumulated nitrite. We cannot exclude the possibility that oxygen free radicals are involved in cytokine-mediated signal pathways that activate genes coding for free radical–generating enzymes, chemotactic factors, or proteins of programmed cell death. A detailed analysis of the differential gene expression pattern in β-cells after cytokine exposure may in the future provide an answer to this question.

In an alternative approach, Hohmeier et al. (28) overexpressed Mn SOD in the mitochondria of INS-1 and RIN1046-38 insulin-producing cell lines. The overexpression of this mitochondrial antioxidant enzyme also provided protection against IL-1β–mediated toxicity and supernatants from activated peripheral blood mononuclear cells (28). Interestingly, the resistance to cytokines was accompanied by a reduced activation of the iNOS enzyme on the transcriptional level,

resulting in a significantly lower accumulation of nitrite. Mn SOD, which is also activated by cytokines as a late-response gene (3,57), apparently interacts with various stress-regulated transcription factors by an as-yet-unknown mechanism and may thereby provide protection. Thus, the overexpression of cytoplasmic as well as mitochondrial antioxidant enzymes can provide pancreatic β-cell protection.

Altogether, the data convincingly support the assumption that an improved antioxidant defense status protects pancreatic β-cells effectively against cytokine-mediated autoimmune attack. Thus, antioxidant enzymes are an important element of β-cell protection within a complex defense system that also consists of antiapoptotic proteins (6,50), heat shock proteins (58,59), and the prevention of secondary suicide pathways such as the activation of the poly(ADP-ribose) polymerase (60,61).

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