

Improvement of the Mitochondrial Antioxidant Defense Status Prevents Cytokine-Induced Nuclear Factor- κ B Activation in Insulin-Producing Cells

Anna Karenina Azevedo-Martins,¹ Stephan Lortz,¹ Sigurd Lenzen,¹ Rui Curi,² Decio L. Eizirik,³ and Markus Tiedge¹

Proinflammatory cytokines (interleukin-1 β [IL-1 β], tumor necrosis factor- α [TNF- α], and γ -interferon [IFN- γ]) initiate a variety of signal cascades in pancreatic β -cells that affect the expression level of genes involved in both the destruction and the protection of the β -cell. The generation of nitric oxide (NO) via the inducible NO synthase (iNOS) and oxygen free radicals play a key role in cytokine-mediated β -cell destruction. Within these signal cascades, the activation of the transcription factor nuclear factor- κ B (NF- κ B) is crucial, and many cytokine-sensitive genes contain binding sites for this transcription factor in their promoter regions. The aim of this study was to characterize the cytokine-mediated activation of NF- κ B and the subsequent expression of iNOS protein in insulin-producing RINm5F cells with an improved antioxidant defense status by overexpression of the cytoprotective enzymes catalase (Cat), glutathione peroxidase (Gpx), and the cytoplasmic Cu/Zn superoxide dismutase (Cu/ZnSOD). RINm5F cells with diverse mitochondrial antioxidative defense status were generated by stable overexpression of MnSOD constructs in sense (MnSOD *sense*) and antisense orientation (MnSOD *antisense*). Cytokine-induced (IL-1 β or cytokine mix consisting of IL-1 β + TNF- α + IFN- γ) activation of NF- κ B in RINm5F cells was reduced by >80% through overexpression of MnSOD. The activity of the iNOS promoter remained at basal levels in cytokine-stimulated MnSOD *sense* cells. In contrast, the suppression of MnSOD gene expression in cytokine-stimulated MnSOD *antisense* cells resulted in a threefold higher activation of NF- κ B and a twofold higher activation of the iNOS promoter as compared with control cells. The iNOS protein expression was significantly reduced after a 6- and 8-h cytokine incubation of MnSOD *sense* cells. The low activity level of MnSOD in RINm5F MnSOD *antisense* cells increased the iNOS protein expression in particular during the early phase of cytokine-mediated toxicity. Cat, Gpx, and the cytoplasmic Cu/ZnSOD did not affect the activation of NF- κ B

and the iNOS promoter. In conclusion, the overexpression of MnSOD, which inactivates specifically mitochondrially derived oxygen free radicals, significantly reduced the activation of NF- κ B in insulin-producing cells. As a consequence of this protective effect in the early cytokine signaling pathways, the induction of iNOS, an important event in the β -cell destruction process, was also significantly reduced. The results provide evidence that mitochondrially derived reactive oxygen species (ROS) play a critical role in the activation of the cytokine-sensitive transcription factor NF- κ B. Overexpression of MnSOD may thus be beneficial for β -cell survival through suppression of oxygen free radical formation, prevention of NF- κ B activation, and iNOS expression. *Diabetes* 52:93–101, 2003

The destruction of β -cells leading to type 1 diabetes is a multifactorial process, mediated at least in part by the interaction of cytokines, in particular interleukin-1 β (IL-1 β), nitric oxide (NO), and oxygen free radicals with the target β -cells (1,2). IL-1 β alone is able to provoke dysfunction of β -cells finally leading to cell death, an effect that is potentiated in combination with the cytokines interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α) (3). These IL-1 β effects are primarily associated with activation of the transcription factor nuclear factor- κ B (NF- κ B) and consequent changes in the gene expression profile (4,5). Although the expression of protective proteins such as mitochondrial Mn superoxide dismutase (MnSOD), heat shock protein 70, and heme oxygenase is induced by the cytokine, up-regulation of the inducible NO synthase (iNOS) and the consequent intracellular production of NO, together with changes in the expression of other NF- κ B-regulated genes, eventually lead to β -cell death (1,5,6). The effects of NF- κ B on gene expression are conferred by specific binding motifs in the promoter region of target genes (7,8). NF- κ B activation is regulated by a variety of mechanisms, including the redox status of the cell, posttranscriptional modifications, the activity level of I- κ B kinase and the subsequent release from the I- κ B inhibitor, the selective transport into the nucleus, and the formation of dimeric complexes (7–10). Oxidative stress by oxygen free radicals is associated with NF- κ B activation, an effect preceded by the rapid degradation of I- κ B (7). However, once inside the nucleus, a more reducing environment favors NF- κ B binding to its DNA consensus motif (9). Pancreatic β -cells

From the ¹Institute of Clinical Biochemistry, Hannover Medical School, Hannover, Germany; ²Department of Physiology and Biophysics, Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil; and ³Laboratory of Experimental Medicine, Free University Brussels, Brussels, Belgium.

Address correspondence and reprint requests to Dr. Markus Tiedge, Institute of Clinical Biochemistry, Hannover Medical School, D-30623 Hannover, Germany. E-mail: tiedge.markus@mh-hannover.de.

Received for publication 12 June 2002 and accepted in revised form 30 September 2002.

Cat, catalase; EMSA, electrophoretic mobility shift assay; Gpx, glutathione peroxidase; IFN- γ , γ -interferon; IL-1 β , interleukin-1 β ; iNOS, inducible nitric oxide synthase; NF- κ B, nuclear factor- κ B; ROS, reactive oxygen species; SEAP, secreted alkaline phosphatase; SOD, superoxide dismutase; TNF- α , tumor necrosis factor- α .

show remarkably low antioxidant enzyme activities. Gene expression and activity of the main antioxidant enzymes including Cu/ZnSOD, MnSOD, glutathione peroxidase (Gpx), and catalase (Cat) are significantly lower than in liver or kidney (11,12). This low antioxidant defense capacity of β -cells and the resultant enhanced sensitivity toward oxidative stress have been considered to be an important aspect of oxygen free radical-induced damage leading to β -cell death and insulin-dependent diabetes (13).

Overexpression of cytoprotective enzymes in insulin-producing RINm5F cells resulted in a significantly higher resistance against oxygen free radical-mediated toxicity (14) as well as against NO generated by different chemical NO donors (15). Stable overexpression of MnSOD in INS-1 cells provided complete protection against IL-1 β -mediated cytotoxicity (16), and IL-1 β had no effect on another insulinoma cell line, RIN1046-38, with a high level of MnSOD expression (16). In RINm5F insulin-producing cells, the overexpression of Cat, Gpx, and Cu/ZnSOD protected the cells against oxidation of cellular proteins and prevented viability loss after a cytokine mix treatment. This protective effect was due to direct inactivation of radicals by the antioxidant enzymes, as suggested by a reduction of ROS-damaged protein residues (17). In the present study, we evaluated the effects of an improved or diminished enzymatic antioxidative defense status on the activation of NF- κ B and iNOS gene regulation in insulin-producing cells in the early phase of cytokine toxicity. Importantly, the overexpression of MnSOD, which inactivates selectively mitochondrially derived superoxide radicals, significantly decreased the activation of both the NF- κ B and iNOS promoter. In line with these results, a reduction of MnSOD expression by antisense technique enhanced the activation of both the NF- κ B and the iNOS promoter after cytokine induction. Our results provide evidence that mitochondrially derived oxygen free radicals play an important role within the NF- κ B signaling pathways involved in cytokine toxicity. Overexpression of MnSOD in β -cells thus may be a feasible strategy to protect insulin-producing cells against cytokine-induced toxicity.

RESEARCH DESIGN AND METHODS

Tissue culture of RINm5F cells. RINm5F tissue culture cells overexpressing Cat, Gpx, and Cu/ZnSOD were generated through stable transfection as described elsewhere (14). The full-length MnSOD cDNA fragment (18) was subcloned into the *EcoRI* site of the pcDNA3 plasmid, resulting in MnSOD sense and antisense expression constructs. The vector inserts were verified by sequence analysis. RINm5F cells were stably transfected with the pcDNA MnSOD sense and antisense vector by Lipofectamine (GIBCO Life Technologies, Gaithersburg, MD). Cell clones overexpressing MnSOD (MnSOD *sense*) or cell clones with a reduced expression level of MnSOD (MnSOD *antisense*) were selected in the presence of 250 μ g/ml geneticin (G418). Nontransfected RINm5F cells served as internal control in the cytokine experiments (RINm5F control cells). It was verified by control experiments that transfection with the pcDNA3 vector lacking insert did not affect the expression of the cytoprotective enzymes (data not shown). The level of antioxidant enzyme expression was regularly controlled by specific enzyme activity assays. The cells were cultured as described in RPMI-1640 medium, supplemented with 10 mmol/l glucose, 10% (v/v) FCS, penicillin, and streptomycin in a humidified atmosphere at 37°C and 5% CO₂ (14). All tissue culture equipment was from GIBCO Life Technologies. Selenium (10 mmol/l), an essential cofactor for Gpx, was added to the tissue culture medium of Gpx-transfected cells. Selenium did not affect enzyme expression (14) or protection against the tested cytokines (data not shown).

Cytokine exposure and nitrite determination. Control and transfected RINm5F cells were seeded at different concentrations depending on the further experimentation and allowed to attach for a period of 24 h before 60, 300, or 600 units/ml human IL-1 β (PromoCell, Heidelberg, Germany), or a combination of cytokines (1 \times cytokine mixture) consisting of 60 units/ml IL-1 β , 185 units/ml human TNF- α , and 14 units/ml rat IFN- γ (PromoCell) was added. Accumulated nitrite was determined in the culture supernatant by the Griess reaction as described elsewhere (17).

Northern and Western blot analyses. Total RNA from RINm5F cells was isolated by a combined water-saturated phenol-chloroform-isoamyl alcohol extraction according to Chomczynski and Sacchi (19). Twenty micrograms of RNA was subjected to electrophoresis on denaturing formamide/formaldehyde 1% agarose gels and transferred to nylon membranes. Hybridization was performed as described before (14) using a digoxigenin-labeled cRNA probe coding for human MnSOD.

For Western blot analyses, RINm5F control cells and transfected RINm5F cells were homogenized in ice-cold PBS. The cell homogenates were centrifuged at 10,000g and 4°C for 10 min to pellet insoluble material. The supernatant was used for Western blot analyses. Protein was determined by the BCA assay (Pierce, Rockford, IL). Fifty micrograms of protein was fractionated by reducing 10% SDS-PAGE and transferred to polyvinylidene fluoride membranes. Nonspecific binding sites of the membranes were blocked by nonfat dry milk for a period of 1 h at 37°C. Then the blots were incubated with specific primary antibody against rat iNOS at a dilution of 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA) or with specific primary antibody against rat MnSOD at a dilution of 1:10,000 (a gift of Dr. M.D. Asayama, Yamanashi, Japan) for 3 h at room temperature, followed by a 1-h incubation period with peroxidase-labeled secondary antibody at a dilution of 1:15,000 at room temperature. The protein bands were visualized by chemiluminescence using the ECL detection system (Amersham Bioscience, Freiburg, Germany).

Reporter gene assays. For the reporter gene assays, we used the Mercury Pathway Profiling System (Clontech, Palo Alto, CA). The vector pSEAP2-Control served as a positive control, containing the secreted alkaline phosphatase (SEAP) gene under the control of the SV40 early promoter and the SV40 enhancer element. As a negative control, the pTAL-SEAP vector was used to determine the background of the SEAP gene. The background expression of SEAP was quantified by transfection of the RINm5F cells with the pTAL-SEAP vector in which transcription is driven by the thymidine kinase minimal promoter without enhancer elements. These background levels were 18% of the reporter activities with the NF- κ B enhancer and 29% of the reporter activities with the iNOS promoter (mean values from the time points 2, 4, 6, 8, and 24 h under normal culture conditions in the absence of cytokines). The SEAP background values at the different time points were always subtracted from the reporter data resulting from the activation of the NF- κ B and iNOS enhancer elements. The thymidine kinase minimal constitutive promoter element did not affect the level of SEAP background activities after exposure to cytokines (data not shown).

The constitutive expression under the SV40 promoter as well as the background expression was similar in all investigated clones (data not shown). The pSEAP-NF- κ B vector contains the SEAP reporter gene under the control of four NF- κ B response elements, namely [GGGAATTTCC]₄. The pSEAP-iNOS vector was constructed by subcloning the *KpnI/BglII* fragment from the piNOS-1002luc construct (20), containing the rat iNOS promoter region (-1,514 to 132) into the *KpnI/BglII* sites of the pSEAP2-control vector. Twenty-four hours before transient transfection, 20,000 cells/well were seeded in 96-well plates. Cells were transfected with Effectin (Qiagen, Hilden, Germany) in fully supplemented RPMI-1640 medium according to the manufacturer's protocol. The cells were incubated with cytokines 24 h after transient transfection as described above. For quantification of SEAP expression, 50 μ l of medium was collected from the 100- μ l supernatant 2, 4, 6, 8, and 24 h after cytokine exposure. The samples were kept at -20°C until the assay was performed. Quantification of SEAP expression was done with the AURORA chemiluminescent detection kit (ICN, Costa Mesa, CA) through the dephosphorylation of the chemiluminescent substrate disodium 3-(4-methoxy-spiro [1,2-dioxetane-3,2-(5-chloro) tricyclo [3.3.1.1^{3,7}] decan]-4-yl)phenyl phosphate (CSPD) (Roche Diagnostics, Mannheim, Germany), by the expressed SEAP. Collected samples of culture medium were thawed and transferred into white opaque 96-well plates. Samples were diluted, then incubated at 65°C and treated with phosphatase inhibitors to abolish the endogenous alkaline phosphatase activity. CSPD substrate, diluted in enhancer buffer, was added to each sample. After a 20-min incubation, the intensity of the chemiluminescent signal was measured by the Wallac Victor² 1420 multilabel counter (Perkin Elmer Life Sciences, Boston, MA).

Electrophoretic mobility shift assays. Nuclear extracts were prepared from RINm5F cells as described (21). Nuclear proteins (4 μ g) were preincubated with 1 μ g poly(dIdC) in 20 μ l containing 10 mmol/l HEPES (pH 7.9), 50

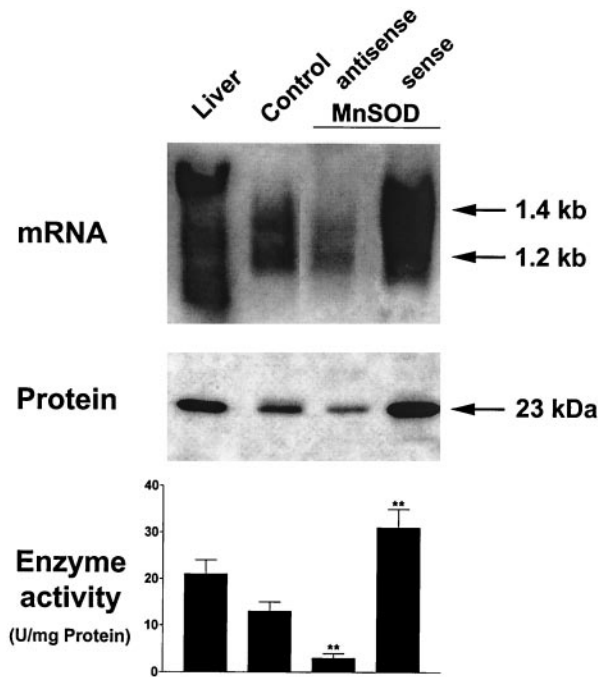


FIG. 1. Stable overexpression of MnSOD (MnSOD *sense*) and suppression of MnSOD (MnSOD *antisense*) in RINm5F cells. Northern and Western blot analyses and enzyme activity measurements of rat liver, RINm5F control cells, RINm5F-MnSOD *sense*, and RINm5F-MnSOD *antisense* cells. The Northern and Western blots shown are representative of three independent experiments. Enzyme activity data are means \pm SE from four individual experiments. $**P < 0.01$ versus control cells.

mmol/l KCl, 5 mmol/l MgCl₂, 0.05 mmol/l EDTA, 0.5 mmol/l dithiothreitol, and 10% glycerol for 10 min at 0°C before addition of probe end-labeled by ³²P- γ -dATP (15,000 cpm). Specificity was verified by 50–100 molar excess of competing oligonucleotide and by an anti-p65 antibody (Santa Cruz Biotechnology) (data not shown). Nuclear protein extracts and labeled oligonucleotides were incubated for 30 min at 0°C. The samples were electrophoresed on 5% polyacrylamide gels in 25 mmol/l Tris, 25 mmol/l boric acid, and 0.5 mmol/l EDTA. Oligonucleotide consensus sequence for NF- κ B was 5'-agCTTCA-GAGGGGACTTTCGAGA (coding strand).

TABLE 1

Effects of the antioxidative defense status on the activation of NF- κ B in the intermediate phase (8 and 24 h) after exposure to IL-1 β alone or a combination of cytokines

RINm5F cell clones	Incubation times	Groups of treatment			
		IL-1 β (60 units/ml)	IL-1 β (600 units/ml)	Mix 0.5 \times	Mix 1 \times
Control	8 h	868 \pm 95	1005 \pm 114	1139 \pm 246	874 \pm 245
	24 h	1019 \pm 174	757 \pm 160	467 \pm 88	474 \pm 103
Cu/ZnSOD	8 h	670 \pm 141	1038 \pm 212	811 \pm 142	900 \pm 169
	24 h	648 \pm 3	671 \pm 144	576 \pm 91	516 \pm 103
MnSOD <i>sense</i>	8 h	282 \pm 43*	269 \pm 49†	206 \pm 33†	239 \pm 57
	24 h	115 \pm 15†	207 \pm 75*	77 \pm 20†	78 \pm 24*
MnSOD <i>antisense</i>	8 h	663 \pm 287	1581 \pm 210*	2279 \pm 282†	1370 \pm 342
	24 h	1720 \pm 45†	1667 \pm 171†	1412 \pm 214†	1240 \pm 225†
Gpx	8 h	506 \pm 102	504 \pm 83	656 \pm 75	603 \pm 155
	24 h	739 \pm 121	843 \pm 136	491 \pm 80	956 \pm 134*
Cat	8 h	551 \pm 93	826 \pm 191	619 \pm 102	628 \pm 161
	24 h	964 \pm 208	1057 \pm 227	597 \pm 60	713 \pm 91

Cells were transiently transfected with a SEAP reporter gene vector under transcriptional control of the NF- κ B enhancer element. Twenty four hours after transfection, the cells were incubated for different periods of time (8, 24 h) with 60 or 600 units/ml IL-1 β and alternatively with mixtures of cytokines consisting of 30 units/ml IL-1 β , 92.5 units/ml TNF- α , and 7 units/ml IFN- γ (0.5 \times mix) or 60 units/ml IL-1 β , 185 units/ml TNF- α , and 14 units/ml IFN- γ (1 \times mix). Thereafter, alkaline phosphatase activity was determined in the culture medium. Results are expressed as relative chemiluminescence values normalized to nonstimulated cells that were set as 100%. Data are means \pm SEM from five individual experiments. * $P < 0.05$; † $P < 0.01$ for stably transfected cells versus RINm5F control cells (ANOVA/Dunnett's test).

Statistical analyses. Data are expressed as mean values \pm SE. Statistical analyses were performed using ANOVA plus Dunnett's test for multiple comparisons.

RESULTS

MnSOD overexpression and suppression in RINm5F cells. Stable transfection of RINm5F cells with the MnSOD sense construct resulted in a significant overexpression of MnSOD in comparison with RINm5F control cells, which was evident at the level of MnSOD mRNA and MnSOD protein and a 2.4-fold increase of enzyme activity (Fig. 1). In contrast, MnSOD antisense cells showed decreased expression levels of MnSOD mRNA and MnSOD protein and a 77% reduction of enzyme activity (Fig. 1). Thus, the effects of the MnSOD expression vectors on the mRNA expression correlated well with the enzyme activities. The enzyme activities of Cat, Gpx, and CuZnSOD in RINm5F MnSOD sense and MnSOD antisense cells were not different from those observed in RINm5F control cells (data not shown).

Activation of the NF- κ B enhancer element.

IL-1 β . Incubation of insulin-producing RINm5F control cells with IL-1 β alone resulted in a significant ($P < 0.05$) time-dependent activation of the NF- κ B enhancer element at both 60 and 600 units/ml IL-1 β in the early (0–8 h) as well as in the intermediate period (8 and 24 h) after cytokine exposure (Fig. 2; Table 1). Overexpression of MnSOD (MnSOD *sense*), which resulted in a 2.4-fold higher enzyme activity in RINm5F cells, virtually abolished the time-dependent activation of the NF- κ B enhancer element by IL-1 β in the early phase (Fig. 1) as well as in the intermediate phase (Table 1). Conversely, suppression of MnSOD expression through the antisense technique resulted in a time-dependent activation of the NF- κ B enhancer element that was significantly ($P < 0.05$) higher even when compared with control cells 8 and 24 h after incubation with 60 or 600 units/ml IL-1 β (Fig. 2, Table 1).

The activation of the NF- κ B enhancer element was also verified through analysis of DNA binding of NF- κ B by

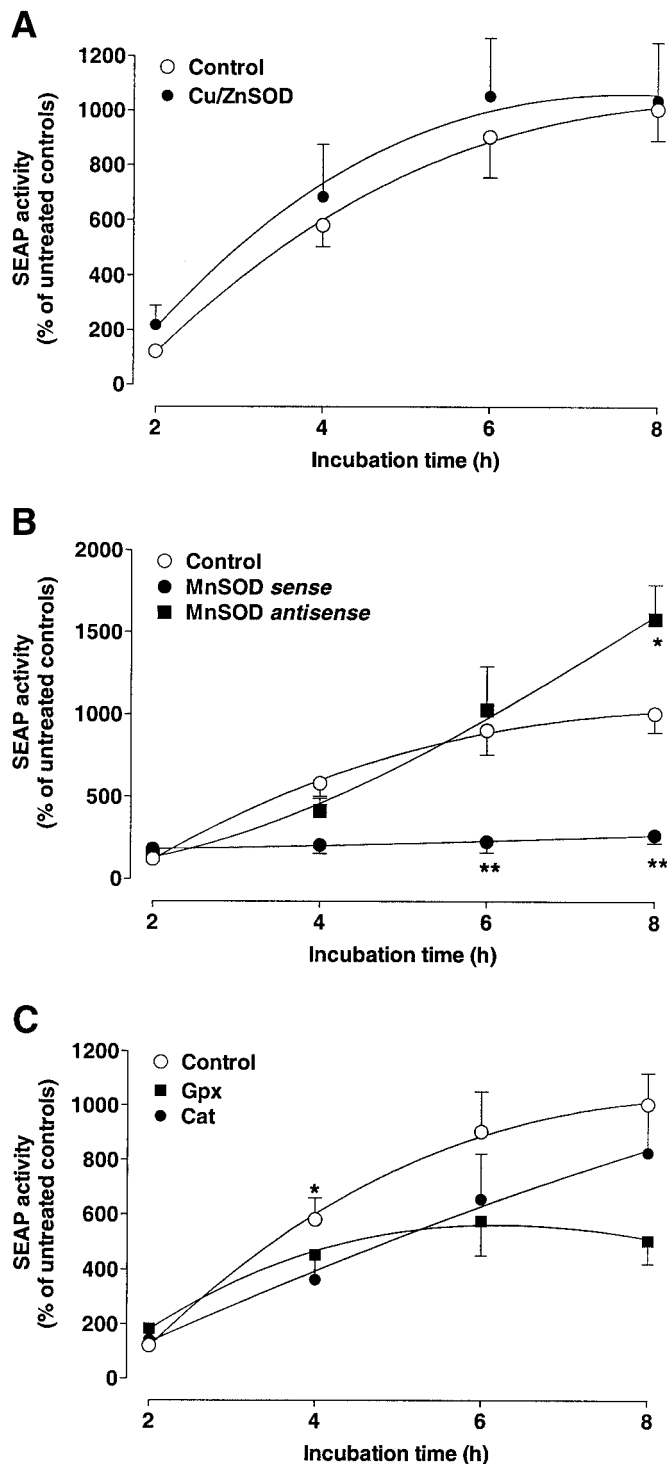


FIG. 2. Time-dependent effects of IL-1 β on the activation of the NF- κ B enhancer element in RINm5F cells in dependence on the antioxidative defense status in the early phase of cytokine signaling (0–8 h). **A:** Cu/Zn SOD overexpression. **B:** MnSOD *sense* and MnSOD *antisense*. **C:** Gpx and Cat overexpression. Cells were transiently transfected with a SEAP reporter gene vector under transcriptional control of the NF- κ B enhancer element. Twenty-four hours after transfection, the cells were incubated for different periods of time (2, 4, 6, 8 h) with 600 units/ml IL-1 β . Thereafter, alkaline phosphatase activity was determined in the culture medium. Results are expressed as relative chemiluminescence values normalized to nonstimulated cells that were set as 100%. Data are means \pm SE from five individual experiments. * P < 0.05; ** P < 0.01 for stably transfected cells versus RINm5F control cells (ANOVA/Dunnnett's test).

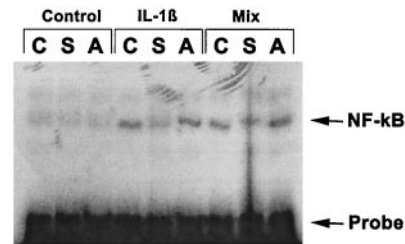


FIG. 3. Effects of MnSOD expression on DNA binding of NF- κ B after cytokine exposure. Nuclear protein extracts were prepared from RINm5F control cells (C), RINm5F-MnSOD *sense* cells (S), and RINm5F-MnSOD *antisense* cells (A) 1 h after exposure to 600 units/ml IL-1 β or a cytokine mix consisting of IL-1 β (60 units/ml), TNF- α (185 units/ml), and IFN- γ (14 units/ml). Five micrograms of nuclear protein extracts were incubated with 32 P-labeled oligoprobe representing the NF- κ B consensus binding sequence. Shown is a representative autoradiogram from four independent experiments.

electrophoretic mobility shift assay (EMSA; Fig. 3). IL-1 β resulted in a significant binding of nuclear protein extracts from RINm5 control cells to the NF- κ B oligoprobe. Nuclear protein extracts from RINm5F-MnSOD *sense* cells showed a lower level of NF- κ B binding, whereas the intensity of the NF- κ B gel retardation band by nuclear extracts from RINm5F-MnSOD *antisense* cells was comparable to that of RINm5F control cells (Fig. 3). Thus, the transcriptional activation in the NF- κ B SEAP reporter gene assays correlates well with the recruitment of NF- κ B to the nuclear compartment. In contrast, the overexpression of the cytoplasmic isoform of SOD (Cu/ZnSOD) did not significantly affect the activation pattern of the NF- κ B enhancer element at both IL-1 β concentrations tested (Fig. 2, Table 1).

In the case of the hydrogen peroxide-inactivating enzymes, overexpression of both Gpx and Cat had at most marginal effects on the activation of the NF- κ B enhancer element at both IL-1 β concentrations. Only at early incubation times (4 and 8 h) was there a slight significant decrease of the activation in particular with Gpx-transfected cells (Fig. 2).

Cytokine mixture. The incubation of RINm5F control cells with a cytokine mixture (60 units/ml IL-1 β , 185 units/ml TNF- α , and 14 units/ml IFN- γ) elicited a significant activation of the NF- κ B enhancer element at both concentrations (0.5 \times and 1 \times) also in a time-dependent manner (curve not shown). Maximal activations after incubation with 0.5 \times mix were observed at 8 h (~10-fold), decreasing to 5-fold after 24 h (Table 1). Almost the same profile was observed after incubation with the full mix, an eight- to nine-fold increase after 8 h, falling to five-fold after 24 h (Table 1).

Again, MnSOD overexpression (MnSOD *sense*) resulted in a significant decrease of the NF- κ B activation after 8 and 24 h at both cytokine mixture concentrations (Table 1). The activity of NF- κ B remained at the basal level of nontreated control cells (Table 1). In agreement, the incubation of MnSOD *antisense* cells with the cytokine mixtures resulted in a greater NF- κ B activation than that in RINm5F control cells after 8 and 24 h (P < 0.01; Fig. 2, Table 1). After 8 h of incubation with the 0.5 \times mix, the activation level reached a maximum (23-fold increase) and declined thereafter to a 14-fold upregulation after 24 h probably as a result of negative feedback regulations, e.g.,

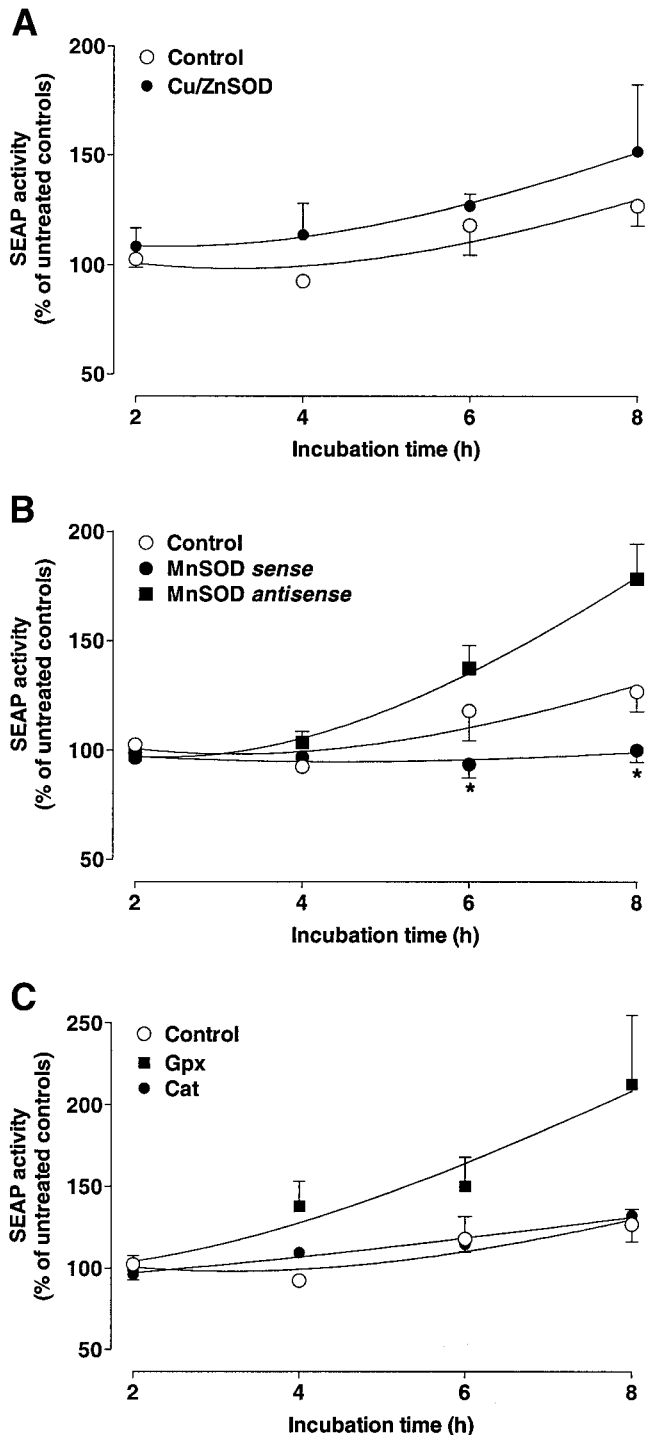


FIG. 4. Time-dependent effects of IL-1 β on the activation of the iNOS promoter in RINm5F cells in dependence on the antioxidative defense status in the early phase of cytokine signaling. **A:** Cu/Zn SOD overexpression. Cells were transiently transfected with a SEAP reporter gene vector under transcriptional control of the iNOS promoter. Twenty-four hours after transfection, the cells were incubated for different periods of time (2, 4, 6 and 8 h) with 600 units/ml IL-1 β . Thereafter, alkaline phosphatase activity was determined in the culture medium. Results are expressed as relative chemiluminescence values normalized to nonstimulated cells that were set as 100%. Data are means \pm SE from five individual experiments. * $P < 0.05$; ** $P < 0.01$ for stably transfected cells versus RINm5F control cells (ANOVA/Dunnnett's test).

by NO (22), which were operative at this lower cytokine concentration (Table 1).

This contention is supported by the accumulation of nitrite in the culture medium of MnSOD *antisense* cells, which was significantly higher after a 24-h incubation with the 1 \times cytokine mix compared with the incubation with the 0.5 \times cytokine mix (117 ± 20 vs. 66 ± 13 nmol/l, $n = 4$; $P < 0.05$), explaining the lower activation level of the NF- κ B promoter element after 8 h of cytokine exposure to 1 \times cytokine mix (Table 1).

In case of incubation with the full mix, the maximal activation after 8 h was ~ 14 times higher and remained at this level until the last measurement at 24 h (Table 1). Notably the cytokine mix (both 0.5 \times and 1 \times) did not induce significant cytotoxic effects within the first 24 h of cytokine exposure (viability $>85\%$ by vital dye staining and MTT assay).

Comparable to the situation with IL-1 β alone, the exposure to a 1 \times cytokine mix resulted in a significant binding of nuclear protein extracts from RINm5F control cells to the NF- κ B oligo probe in the gel retardation assay (Fig. 3). In contrast, nuclear protein extracts from RINm5F-MnSOD *sense* cells showed a lower level of NF- κ B binding, whereas the intensity of the NF- κ B gel retardation band by nuclear extracts from RINm5F-MnSOD *antisense* cells was slightly higher than in RINm5F control cells (Fig. 3). Again, the overexpression of Cu/ZnSOD did not modify the NF- κ B activation profile after incubation with the 1 \times cytokine mix as it was also the case with IL-1 β alone (Table 1). The overexpression of the hydrogen peroxide-inactivating enzymes Cat and Gpx was able to decrease the NF- κ B activation only in the early phase (6 and 8 h) and when the cells were exposed to the lower 0.5 \times concentration of the cytokine mixture (Table 1).

Activation of the iNOS promoter. To investigate further the effects of overexpressing the mitochondrial isoform of the SOD, we also studied the influence of this enzyme on the promoter activation of iNOS. The rat iNOS promoter region contains two NF- κ B binding sites, and activation of this transcription factor is required for iNOS expression in insulin-producing cells (20).

IL-1 β . In RINm5F control cells, the iNOS promoter was activated after 6–8 h by IL-1 β (60 and 600 units/ml) and remained activated until 24 h (Fig. 4, Table 2). The overexpression of MnSOD in these cells abolished the time-dependent significant activation of the iNOS promoter at both IL-1 β concentrations (Fig. 4, Table 2). In line with the NF- κ B results, the overexpression of an antisense construct for the mitochondrial MnSOD caused a significant activation of the iNOS promoter, which at the higher concentration of 600 units/ml IL-1 β was even greater than in RINm5F control cells after 24 h of incubation (Table 2). Again, no effect of the cytoplasmic Cu/ZnSOD overexpression on the activation of the iNOS promoter was observed, as shown by an activation profile identical to the control (Fig. 4, Table 2). At both IL-1 β concentrations, the overexpression of Cat did not interfere with the activation pattern of the iNOS promoter. However, with the Gpx overexpressing RINm5F cells, the degree of iNOS promoter activation was higher within incubation periods of up to 8 h (Fig. 4).

Cytokine mixture. The incubation of RINm5F control

TABLE 2

Effects of the antioxidative defense status on the activation of the iNOS promoter in the intermediate phase (8 and 24 h) after exposure to IL-1 β alone or a combination of cytokines

RINm5F cell clones	Incubation times	Groups of treatment			
		IL-1 β (60 units/ml)	IL-1 β (600 units/ml)	Mix 0.5 \times	Mix 1 \times
Control	8 h	151 \pm 13	127 \pm 9	214 \pm 39	252 \pm 59
	24 h	166 \pm 26	148 \pm 9	195 \pm 20	207 \pm 25
Cu/ZnSOD	8 h	122 \pm 16	152 \pm 31	296 \pm 22	250 \pm 65
	24 h	136 \pm 23	140 \pm 23	206 \pm 46	206 \pm 35
MnSOD <i>sense</i>	8 h	105 \pm 5	100 \pm 5*	108 \pm 9*	110 \pm 10
	24 h	104 \pm 4*	104 \pm 8*	107 \pm 6*	108 \pm 4*
MnSOD <i>antisense</i>	8 h	134 \pm 13	178 \pm 16	269 \pm 73	394 \pm 56*
	24 h	148 \pm 12	217 \pm 29*	352 \pm 75*	475 \pm 75 \dagger
Gpx	8 h	224 \pm 34*	212 \pm 42	279 \pm 54	322 \pm 59
	24 h	240 \pm 48	203 \pm 40	206 \pm 33	203 \pm 24
Cat	8 h	105 \pm 4	132 \pm 16	177 \pm 30	246 \pm 54
	24 h	154 \pm 18	161 \pm 27	228 \pm 18	290 \pm 49

Cells were transiently transfected with a SEAP reporter gene vector under transcriptional control of the iNOS promoter. Twenty-four hours after transfection, the cells were incubated for different periods of time (8, 24 h) with 60 or 600 units/ml IL-1 β and alternatively with mixtures of cytokines consisting of 30 units/ml IL-1 β , 92.5 units/ml TNF- α , and 7 units/ml IFN- γ (0.5 \times mix) or 60 units/ml IL-1 β , 185 units/ml TNF- α , and 14 units/ml IFN- γ (1 \times mix). Thereafter, alkaline phosphatase activity was determined in the culture medium. Results are expressed as relative chemiluminescence values normalized to non-stimulated cells that were set as 100%. Data are means \pm SEM from five individual experiments. * P < 0.05; $\dagger P$ < 0.01 for stably transfected cells versus RINm5F control cells (ANOVA/Dunnett's test).

cells with the 1 \times cytokine mix was more effective in activating the iNOS promoter than IL-1 β alone (Table 2). The maximal response appeared at 8 h and was approximately two- to threefold increased compared with non-stimulated cells (Table 2). Again, the overexpression of MnSOD *sense* was highly effective in suppressing the iNOS promoter activation at both cytokine mixture concentrations (0.5 \times and 1 \times), significant in particular at 24 h (Table 2).

With MnSOD *antisense* cells, a stronger activation of the iNOS promoter was observed when compared with control cells. The strongest activation was elicited after 24 h of incubation with the highest concentration of the cytokine mixture (Table 2). In *antisense* cells, this significant (P < 0.01) fivefold activation was greater than the twofold increase in RINm5F control cells (Table 2). In Cu/ZnSOD overexpressing cells, the activation profile of iNOS promoter again was identical with that of RINm5F control cells (Fig. 4, Table 2). In Cat and Gpx overexpressing cells, the iNOS promoter activation level followed almost the same pattern as was seen in RINm5F control cells, with no significant differences of the iNOS activation (Fig. 4, Table 2). Although iNOS promoter activities in Gpx overexpressing cells seemed higher 8 h after exposure to the cytokine mix, this increase did not reach statistical significance and was not observed at other time points during the 24-h incubation period (Fig. 4, Table 2).

iNOS protein expression. To investigate the importance of MnSOD for the protein expression of the iNOS gene, we performed immunoblots for iNOS after different periods of incubation of control RINm5F as well as for MnSOD *sense* and MnSOD *antisense* cells with a cytokine mixture (60 units/ml IL-1 β , 185 units/ml human TNF- α , and 14 units/ml rat IFN- γ). RINm5F control cells as well as MnSOD *sense* and MnSOD *antisense* cells showed no detectable iNOS protein expression in the absence of cytokines (time point 0 h, Fig. 5). In accordance with the reporter gene expression data (Table 2, Fig. 4), there was a time-dependent increase of iNOS protein expression at 4, 6, and 8 h of

incubation in RINm5F control cells with the cytokine mix (Fig. 5, lanes 4, 7, and 10). In comparison with these controls, the level of iNOS protein expression was significantly reduced in MnSOD *sense* overexpressing RINm5F cells, in particular after 6 and 8 h of incubation (Fig. 5, lanes 5, 8, and 11), whereas iNOS expression was significantly increased in MnSOD *antisense* RINm5F cells, in particular after 4 h of incubation (Fig. 5, lanes 6, 9, and 12).

DISCUSSION

In insulin-dependent diabetes, pancreatic β -cells are destroyed through an autoimmune process involving cytokines, generation of oxygen free radicals, and activation of β -cell-specific transcription factors (rev. in 1).

β -Cells express low levels of antioxidant enzyme activities, making them particularly susceptible to attacks of radicals after exposure to cytokines or activated immune cells (11,12). Overexpression of antioxidant enzymes protects insulin-producing cells against cytokine-mediated toxicity through prevention of oxidative damage of cellular compounds such as proteins, DNA, or the plasma membrane (17,23). However, ROS may affect signal pathways of cytokines through modulation of transcription factors and gene expression profiles that initiate the process of β -cell destruction by programmed cell death (1).

Cytokines activate transcription factors, including the STAT and NF- κ B pathways (1). In particular, NF- κ B proved to be a key transcriptional regulator of the β -cell, controlling the expression of >100 cytokine-induced genes, including iNOS, the enzyme responsible for NO production, and C/EBP homologous protein (CHOP), a transcription factor involved in endoplasmic reticulum stress-mediated β -cell apoptosis (5). Inhibition of cytokine-induced NF- κ B activation prevents cytokine-induced cell death in human islets (24), purified rat β -cells (6), and mouse insulin-producing MIN6 cells (25), suggesting that

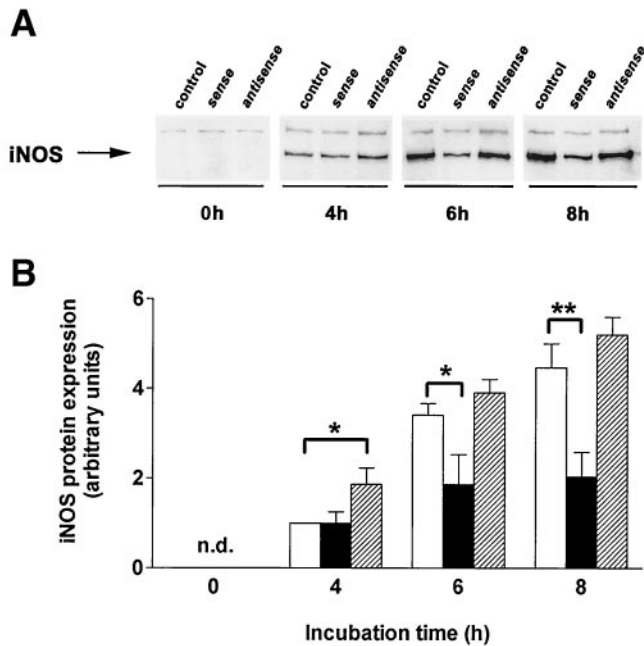


FIG. 5. Time-dependent effect of a cytokine combination on the iNOS protein expression in RINm5F cells in dependence on the overexpression of MnSOD *sense* and MnSOD *antisense*. Cells were seeded 24 h before the incubation with a cytokine mix consisting of IL-1 β (60 units/ml), TNF- α (185 units/ml), and IFN- γ (14 units/ml). The cells were lysed and used for Western blot analyses 0, 4, 6, and 8 h after incubation. **A:** Lanes 1, 4, 7, and 10 show RINm5F control cells incubated for different periods of time (0, 4, 6, and 8 h, respectively). Lanes 2, 5, 8, and 11 represent the decreased iNOS expression in MnSOD *sense* cells (0, 4, 6, and 8 h, respectively). Lanes 3, 6, 9, and 12 represent the increased iNOS expression in MnSOD *antisense* cells (0, 4, 6, and 8 h, respectively). Shown is a representative blot from four individual experiments. **B:** Quantitative densitometric analysis of three independent experiments. White bars, RINm5F controls; black bars, RINm5F-MnSOD *sense*; hatched bars, RINm5F-MnSOD *antisense*. Data are means \pm SE. * $P < 0.05$; ** $P < 0.01$ for stably transfected cells versus RINm5F control cells (ANOVA/Dunnett's test).

cytokine-induced NF- κ B activation has a proapoptotic effect in pancreatic β -cells.

Under normal conditions, NF- κ B is sequestered in the cytoplasm in an inactive state through binding to the inhibitory protein I- κ B. Exposure of cells to an appropriate stimulus, such as cytokines, causes phosphorylation, ubiquitination, and degradation of I- κ B and liberates the activated NF- κ B dimer allowing it to migrate into the nucleus and to bind to its consensus sequence of various promoters (7). Studies in different cell types provide evidence that NF- κ B activation is modulated by oxidative stress (26). Thus, ROS may play a specific role in the cytokine signaling pathways at the level of this redox-sensitive "master regulator," directing the expression of clusters of genes toward destruction. Moreover, antioxidants can block I- κ B phosphorylation and thus prevent the activation of NF- κ B, suggesting that I- κ B kinase is a target for antioxidants (7). Conversely, increased levels of ROS are associated with an activation of NF- κ B (8,27).

In the present study, we provide evidence that the cytokine-induced activation of NF- κ B is suppressed through an overexpression of the mitochondrial isoform of SOD (MnSOD). With the use of RINm5F insulin-producing model cells with different levels of MnSOD enzyme activities, the cytokine-mediated activation of NF- κ B proved to

be dependent on the intracellular antioxidant defense status. In these cells, a quadruplicate NF- κ B enhancer element was activated both by IL-1 β and by a mixture of three cytokines, thus confirming its role in the regulation of cytokine-triggered signal cascades. These reporter gene data correlated well with the EMSA assays, indicating that the transcriptional activation of the NF- κ B enhancer element is the result of translocation of NF- κ B to the nuclear compartment of RINm5F cells.

Inactivation of mitochondrially derived radicals, through overexpression of MnSOD, effectively prevented a 10-fold increase of NF- κ B activation in cytokine-stimulated RINm5F cells. Vice versa, the NF- κ B activation levels after cytokine exposure were significantly higher in MnSOD *antisense* cells (15- to 25-fold). In these MnSOD *antisense* cells, the enzyme activity was reduced by 50% compared with controls. Cytokines do not induce an oxidative burst in β -cells, as they do in macrophages, but they induce a protracted production of ROS, as evidenced by lipid peroxidation, contributing to β -cell death (23).

The reporter gene data indicate that the activation level of NF- κ B is inversely related to SOD activity at the mitochondrial level. Increased MnSOD expression may probably affect intramitochondrial generation of superoxide radicals. Oxygen free radicals are generated during respiratory chain electron transfer (28). At high concentrations, they disturb the mitochondrial membrane potential, through affecting the transition pore and the permeability of the inner membrane of this organelle, ultimately resulting in a release of cytochrome *c* (29). This leakage can affect the activation/inhibition of stress-sensitive transcription factors and trigger apoptotic pathways (30).

Another mechanism by which cytokines contribute to β -cell death is via formation of peroxynitrite. Peroxynitrite is a highly reactive oxidant species, produced by the reaction between superoxide and NO (31). The development of autoimmune diabetes in NOD mice is associated with peroxynitrite formation at the β -cell level (32), and the iNOS inhibitor and peroxynitrite scavenger guanidinoethylidysulphide prevents diabetes in these animals (33). Furthermore, human pancreatic islets are sensitive to peroxynitrite-induced cell dysfunction and cell death (34), and this radical mediates some of the deleterious effects of cytokines in human pancreatic β -cells (35). Thus, protection against peroxynitrite may be important to prevent β -cell death. Overexpression of MnSOD may prevent peroxynitrite formation by two mechanisms, by directly scavenging superoxide radicals and by inhibiting NF- κ B activation thereby suppressing iNOS expression and NO production (see below).

As discussed above, the inactivation of intramitochondrially generated free radicals may represent a protective mechanism in the chain of events that initiates destructive pathways by NF- κ B activation, leading to apoptosis of pancreatic β -cells. Our data provide a rationale for the protective effect of MnSOD overexpression in some insulin-producing cell lines (16) against the toxicity of IL-1 β and are in line with the results reported by Manna et al. (36), where the overexpression of MnSOD suppressed the TNF- α -induced activation of NF- κ B and AP-1 in human breast cancer MCF-7 cells. However, cytokine-mediated

activation of gene expression is dependent not only on NF- κ B activation but also on a complex interplay of different signal cascades including the MAP/JNK and the JAK/STAT pathways (1).

The rat iNOS promoter element is an example of a gene regulated by different cytokines through a complex cross-talk with transcription factors with particular emphasis on IL-1 β and IFN- γ (20). The rat iNOS promoter contains two binding motifs for NF- κ B (in contrast to the NF- κ B promoter construct with four binding motifs), an γ -IFN-activated site (GAS) and two adjacent IFN-stimulated response elements (IREs). The inversely regulated iNOS SEAP reporter activities in RINm5F MnSOD *sense* and *antisense* cells also could be the result of STAT activation in dependence on the mitochondrial antioxidative status in a complex cross-talk with NF- κ B (20). This interaction of different cytokine signaling pathways could explain why the iNOS reporter gene activation by IL-1 β alone was lower when compared with the NF- κ B enhancer element, where there is no further potentiation by IFN- γ responsive elements of the promoter (20).

The iNOS promoter activation was dependent on the mitochondrial MnSOD activity. In RINm5F cells with a reduced MnSOD expression (MnSOD *antisense*), the level of activation was higher, whereas it remained at basal levels after MnSOD overexpression (MnSOD *sense* cells). It will have to be verified by future gene expression profiling which genes from the NF- κ B-dependent repertoire in insulin-producing cells already characterized by microarray analyses (4,5) are affected by the mitochondrial MnSOD activity levels and whether factors secondary to NF- κ B may exert a regulatory function on cytokine-sensitive genes.

The mechanism by which MnSOD overexpression prevents the activation of NF- κ B and of NF- κ B-dependent genes remains to be clarified. This is unlikely to be due to unspecific radical inactivation, because the cytoplasmic isoform of SOD (Cu/ZnSOD) failed to reduce NF- κ B activation. MnSOD has been suggested to modulate cell proliferation, apoptosis, and differentiation through effects on the function of diverse transcription factors. Thus, in a fibrosarcoma cell line, the overexpression of MnSOD decreased the DNA-binding activity of the jun-associated proteins AP-1 and CREB without modulating the DNA-binding activity of NF- κ B and p53 (37). The transcriptional function of AP-1 was also inhibited, and the protein expression of its target gene *bcl-x_L* was reduced (37). However, stable overexpression of MnSOD diminished p53 gene expression, decreased p53-mediated apoptosis (38), inhibited the transcriptional and DNA binding ability of AP-1 and NF- κ B (by 50%), and downregulated their responsive genes in human breast cancer cells (39). Moreover, the overexpression of MnSOD in murine fibrosarcoma cells decreased the DNA-binding activity of CREB (37), which is a ligand of CBP, a coactivator required for transcriptional activation by NF- κ B (40). These results show that the activation of NF- κ B is differentially regulated by the redox state in dependence on the cell type (8).

In conclusion, our data clearly show the importance of the mitochondrial antioxidant defense status for protection against cytokine-mediated toxicity in insulin-producing cells. Increasing the ROS-inactivation capacity inside

the β -cell mitochondria could interrupt the cytokine-induced activation of NF- κ B and reduce the subsequent activation of the iNOS promoter. Therefore, overexpression of MnSOD may be an attractive approach for islet protection against autoimmune attack in insulin-dependent diabetes or after islet transplantation.

ACKNOWLEDGMENTS

We gratefully acknowledge the financial support of A.K.A.M. by a grant from the European Union within the ALFA (Amérique Latine-Formation Académique) Program and from the Juvenile Diabetes Research Foundation International to M.T. and D.L.E. This work has been conducted in collaboration with and supported by the JDRF Center for Prevention of β -cell Destruction in Europe.

We are grateful to Dr. Y.-S. Ho (Detroit, MI) for the cDNAs for rat Gpx and Cu/ZnSOD, to Dr. K. Asayama (Yamanashi, Japan) for the antibody against MnSOD, and to Dr. G.I. Bell (Chicago, IL) for the cDNA for human MnSOD.

REFERENCES

- Eizirik DL, Mandrup-Poulsen T: A choice of death—the signal-transduction of immune-mediated beta-cell apoptosis. *Diabetologia* 44:2115–2133, 2001
- Rabinovitch A, Suarez-Pinzon WL: Cytokines and their roles in pancreatic islet beta-cell destruction and insulin-dependent diabetes mellitus. *Biochem Pharmacol* 55:1139–1149, 1998
- Sekine N, Ishikawa T, Okazaki T, Hayashi M, Wollheim CB, Fujita T: Synergistic activation of NF-kappa b and inducible isoform of nitric oxide synthase induction by interferon-gamma and tumor necrosis factor-alpha in INS-1 cells. *J Cell Physiol* 184:46–57, 2000
- Kwon G, Corbett JA, Hauser S, Hill JR, Turk J, McDaniel ML: Evidence for involvement of the proteasome complex (26S) and NF κ B in IL-1 β -induced nitric oxide and prostaglandin production by rat islets and RINm5F cells. *Diabetes* 47:583–591, 1998
- Cardozo AK, Heimberg H, Heremans Y, Leeman R, Kutlu B, Kruhoffer M, Orntoft T, Eizirik DL: A comprehensive analysis of cytokine-induced and nuclear factor-kappa B-dependent genes in primary rat pancreatic beta-cells. *J Biol Chem* 276:48879–48886, 2001
- Heimberg H, Heremans Y, Jobin C, Leemans R, Cardozo AK, Darville M, Eizirik DL: Inhibition of cytokine-induced NF- κ B activation by adenovirus-mediated expression of a NF- κ B super-repressor prevents β -cell apoptosis. *Diabetes* 50:2219–2224, 2001
- Thanos D, Maniatis T: NF-kappa B: a lesson in family values. *Cell* 80:529–532, 1995
- Bonizzi G, Piette J, Merville MP, Bours V: Cell type-specific role for reactive oxygen species in nuclear factor- κ B activation by interleukin-1. *Biochem Pharmacol* 59:7–11, 2000
- Hutter D, Greene JJ: Influence of the cellular redox state on NF- κ B-regulated gene expression. *J Cell Physiol* 183:45–52, 2000
- Dalton TP, Shertzer HG, Puga A: Regulation of gene expression by reactive oxygen. *Annu Rev Pharmacol* 39:67–101, 1999
- Lenzen S, Drinkgern J, Tiedge M: Low antioxidant enzyme gene expression in pancreatic islets compared with various other mouse tissues. *Free Radic Biol Med* 20:463–466, 1996
- Tiedge M, Lortz S, Drinkgern J, Lenzen S: Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* 46:1733–1742, 1997
- Ho E, Bray TM: Antioxidants, NF κ B activation, and diabetogenesis. *Proc Soc Exp Biol Med* 222:205–213, 1999
- Tiedge M, Lortz S, Munday R, Lenzen S: Complementary action of antioxidant enzymes in the protection of bioengineered insulin-producing RINm5F cells against the toxicity of reactive oxygen species. *Diabetes* 47:1578–1585, 1998
- Tiedge M, Lortz S, Munday R, Lenzen S: Protection against the co-operative toxicity of nitric oxide and oxygen free radicals by overexpression of antioxidant enzymes in bioengineered insulin-producing RINm5F cells. *Diabetologia* 42:849–855, 1999
- Hohmeier HE, Thigpen A, Tran VV, Davis R, Newgard CB: Stable expression of manganese superoxide dismutase (MnSOD) in insulinoma cells

- prevents IL-1 β -induced cytotoxicity and reduces nitric oxide production. *J Clin Invest* 101:1811–1820, 1998
17. Lortz S, Tiedge M, Nachtwey T, Karlisen AE, Nerup J, Lenzen S: Protection of insulin-producing RINm5F cells against cytokine-mediated toxicity through overexpression of antioxidant enzymes. *Diabetes* 49:1123–1130, 2000
 18. Beck Y, Oren R, Amit B, Levanon A, Gorecki M, Hartman J: Human Mn superoxide dismutase cDNA sequence. *Nucleic Acids Res* 15:9076, 1987
 19. Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
 20. Darville MI, Eizirik DL: Regulation by cytokines of the inducible nitric oxide synthase promoter in insulin-producing cells. *Diabetologia* 41:1101–1108, 1998
 21. Schreiber E, Matthias P, Muller MM, Schaffner W: Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res* 17:6419, 1989
 22. Niemann A, Bjorklund A, Eizirik DL: Studies on the molecular regulation of the inducible form of nitric oxide synthase (iNOS) in insulin-producing cells. *Mol Cell Endocrinol* 106:151–155, 1994
 23. Rabinovitch A, Suarez-Pinzon WL, Strynadka K, Lakey JR, Rajotte RV: Human pancreatic islet beta-cell destruction by cytokines involves oxygen free radicals and aldehyde production. *J Clin Endocrinol Metab* 81:3197–3202, 1996
 24. Giannoukakis N, Rudert WA, Trucco M, Robbins PD: Protection of human islets from the effects of interleukin-1 β by adenoviral gene transfer of an IkB repressor. *J Biol Chem* 275:36509–36513, 2000
 25. Baker MS, Chen X, Cao XC, Kaufman DB: Expression of a dominant negative inhibitor of NF- κ B protects MIN6 beta-cells from cytokine-induced apoptosis. *J Surg Res* 97:117–122, 2001
 26. van den Berg R, Haenen GR, van den Berg H, Bast A: Transcription factor NF- κ B as a potential biomarker for oxidative stress. *Br J Nutr* 86:S121–S127, 2001
 27. Schmidt KN, Amstad P, Cerutti P, Baeuerle PA: The roles of hydrogen peroxide and superoxide as messengers in the activation of transcription factor NF-kappa B. *Chem Biol* 2:13–22, 1995
 28. Fridovich I: Superoxide radical and superoxide dismutases. *Annu Rev Biochem* 64:97–112, 1995
 29. Liu X, Kim CN, Yang J, Jemmerson R, Wang X: Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 86:147–157, 1996
 30. Raha S, Robinson BH: Mitochondria, oxygen free radicals, and apoptosis. *Am J Med Genet* 106:62–70, 2001
 31. Arteel GE, Sies H: Protection against peroxynitrite by cocoa polyphenol oligomers. *FEBS Lett* 462:167–170, 1999
 32. Suarez-Pinzon WL, Szabo C, Rabinovitch A: Development of autoimmune diabetes in NOD mice is associated with the formation of peroxynitrite in pancreatic islet beta-cells. *Diabetes* 46:907–911, 1997
 33. Suarez-Pinzon WL, Mabley JG, Strynadka K, Power RF, Szabo C, Rabinovitch A: An inhibitor of inducible nitric oxide synthase and scavenger of peroxynitrite prevents diabetes development in NOD mice. *J Autoimmun* 16:449–455, 2001
 34. Delaney C, Tyrberg B, Bouwens L, Vaghef H, Hellman B, Eizirik D: Sensitivity of human pancreatic islets to peroxynitrite-induced cell dysfunction and death. *FEBS Lett* 394:300–306, 1996
 35. Lakey JR, Suarez-Pinzon WL, Strynadka K, Korbutt GS, Rajotte RV, Mabley JG, Szabo C, Rabinovitch A: Peroxynitrite is a mediator of cytokine-induced destruction of human pancreatic islet beta cells. *Lab Invest* 81:1683–1692, 2001
 36. Manna SK, Zhang HJ, Yan T, Oberley LW, Aggarwal BB: Overexpression of manganese superoxide dismutase suppresses tumor necrosis factor-induced apoptosis and activation of nuclear transcription factor- κ B and activated protein-1. *J Biol Chem* 273:13245–13254, 1998
 37. Kiningham KK, St. Clair DK: Overexpression of manganese superoxide dismutase selectively modulates the activity of Jun-associated transcription factors in fibrosarcoma cells. *Cancer Res* 57:5265–5271, 1997
 38. Drane P, Bravard A, Bouvard V, May E: Reciprocal down-regulation of p53 and SOD2 gene expression-implication in p53 mediated apoptosis. *Oncogene* 20:430–439, 2001
 39. Li JJ, Oberley LW, Fan M, Colburn NH: Inhibition of AP-1 and NF- κ B by manganese-containing superoxide dismutase in human breast cancer cells. *FASEB J* 12:1713–1723, 1998
 40. Sheppard KA, Rose DW, Haque ZK, Kurokawa R, McInerney E, Westin S, Thanos D, Rosenfeld MG, Glass CK, Collins T: Transcriptional activation by NF- κ B requires multiple coactivators. *Mol Cell Biol* 19:6367–6378, 1999