MnSOD and Catalase Transgenes Demonstrate That Protection of Islets From Oxidative Stress Does Not Alter Cytokine Toxicity

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Reactive oxygen species (ROS) and nitric oxide (NO) are proposed mediators of cytokine-induced β-cell destruction in type 1 diabetes. We produced transgenic mice with increased β-cell expression of manganese superoxide dismutase (MnSOD) and catalase. Expression of these antioxidants increased β-cell ROS scavenging and improved β-cell survival after treatment with different sources of ROS. MnSOD or catalase conferred protection against streptozotocin (STZ)-induced β-cell injury. Coexpression of MnSOD and catalase provided synergistic protection against peroxynitrite and STZ. To determine the potential effect of these antioxidants on cytokine-induced toxicity, we exposed isolated islets to a cytokine mixture, including interleukin-1β and interferon-γ. Cytokine toxicity was measured as reduced metabolic activity after 6 days and reduced insulin secretion after 1 day. Cytokines increased ROS production, and both antioxidants were effective in reducing cytokine-induced ROS. However, MnSOD and/or catalase provided no protection against cytokine-induced injury. To understand this, the nuclear factor-κB (NF-κB) signaling cascade was investigated. Antioxidants reduced NF-κB activation by ROS, but none of the antioxidants altered activation by cytokines, as measured by inhibitor of κB phosphorylation, NF-κB translocation, inducible NO synthase activation, and NO production. Our data agree with previous reports that antioxidants benefit β-cell survival against ROS damage, but they are not consistent with reports that antioxidants reduce cytokine toxicity. ROS appear to have no role in cytokine toxicity in primary β-cells. *Diabetes* 54:1437–1446, 2005

Reactive oxygen species (ROS) have been proposed to play an important role in β-cell destruction. Several studies have shown that exposure of pancreatic islets to cytokines (1–3) greatly increases ROS production and leads to oxidative damage to β-cells. Since pancreatic β-cells contain very low levels of antioxidant enzymes (4), they may be more susceptible to the toxic actions of cytokines (5). In agreement with the proposed role of ROS, overexpression of antioxidant enzymes in insulin-producing tumor cell lines has achieved great success in improving resistance to both free radicals and cytokines. For instance, overexpression of antioxidant enzymes conferred protection to several insulinoma cell lines against the toxic effects of ROS (6), nitric oxide (NO) (7), interleukin-1β (IL-1β) (8), and a mixture of cytokines (9). However, other studies on primary islet cells, using various antioxidant compounds or enzymes, reported no protection against cytokine toxicity (10–13).

Inhibition of nuclear factor-κB (NF-κB) activity decreases cytokine-induced β-cell death (14–16). NF-κB activation is modulated by oxidative stress (17); compounds that generate ROS induce NF-κB activation (18,19), and some antioxidants can block NF-κB activation (20). Recently, it was reported that increased expression of mitochrondrial manganese superoxide dismutase (MnSOD), but not other antioxidant enzymes, prevented cytokine-induced NF-κB activation and inducible NO synthase (iNOS) promoter activity in rat insulinoma cells (21). If primary β-cells and insulinoma cells respond similarly to cytokines, then MnSOD should block activation of NF-κB in primary β-cells.

In the present study, we analyzed transgenic mice with β-cell overexpression of catalase and MnSOD. When used in combination, the two antioxidant transgenes were extremely effective against peroxyntitrite- and streptozotocin (STZ)-induced β-cell destruction. However, there was no indication that either antioxidant alone or in combination provided any protection from cytokine-induced toxicity or cytokine-induced activation of β-cell NF-κB. Our data clearly indicate that in primary mouse β-cells, unlike in insulinoma cells, ROS do not play a role in cytokine-induced injury, measured as either reduced viability determined by metabolic activity after 6 days exposure or reduced insulin secretion after 1 day exposure to cytokines.

RESEARCH DESIGN AND METHODS

Chemicals. 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA), picogreen, and ribogreen were from Molecular Probes

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Received for publication 14 December 2004 and accepted in revised form 9 February 2005.

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1400W, *N*-(3-(aminomethyl)enzyl)acetamidine; CM-H_2DCFDA, 5-(6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; IFN-γ, interferon-γ; IκB, inhibitor of κB; IL-1β, interleukin-1β; iNOS, inducible NO synthase; L-NAME, N^G-nitro-L-arginine methyl ester; MnSOD, manganese superoxide dismutase; NF-κB, nuclear factor κB; ROS, reactive oxygen species; SIN-1, 3-morpholinosydnonimine; SOD, superoxide dismutase; STZ, streptozotocin.

(Eugene, OR). Alarma Blue was from Biosource International (Camarillo, CA). H_2O_2, hypoxanthine, xanthine oxidase, 3-morpholinosydnonimine (SIN-1), STZ, pyrogallol, iNOS inhibitors (N^G-nitro-L-arginine methyl ester [L-NAME] and *N*-(3-(aminomethyl)enzyl)acetamidine [1400W]), mouse IL-1β, interferon-γ (IFN-γ), and anti-mouse actin antibody were from Sigma (St. Louis, MO). Nitrotyrosine antibody was from Upstate (Charlottesville, VA). Rabbit anti–NF-kB p50 was from Santa Cruz (Santa Cruz, CA). Donkey anti-rabbit fluorescein isothiocyanate and Cy3-conjugated IgG were from Jackson ImmunoResearch Laboratories (West Grove, PA).

Generation and treatment of transgenic mice. Catalase transgenic mice were as previously described (22). The MnSOD transgene was constructed from the catalase transgene by replacing the catalase sequences by digestion with the full-length human MnSOD cDNA. Transgenic mice were produced on the strain FVB as previously described (22). All mice were housed in ventilated cages at the University of Louisville Research Resources Center with free access to water and standard mouse diet. Some mice were treated with a single intraperitoneal injection of STZ in 0.1 mol/l sodium citrate (pH 4.5) at a dose of 220 mg/kg body wt. All animal procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care.

Measurement of islet superoxide dismutase activity, DNA, and insulin secretion. Islets were isolated and cultured as described (23). Islet total superoxide dismutase (SOD) activity was measured by inhibition of the auto-oxidation of pyrogallol (24) using extracts obtained from 200 islets. Glucose-stimulated insulin secretion was measured by static assay (23) performed in 96-well microplates. Insulin was determined by a radioimmunoassay kit (Diagnostic Products, Los Angeles, CA). DNA was quantified with picogreeen.

Immunohistochemistry. Immunostaining of MnSOD, catalase, insulin, and nitrotyrosine was carried out on paraffin sections (5 μm) based on previously described protocols (23,25). Briefly, sections were pretreated with target antigen retrieval solution (Dako), washed with PBS, blocked with serum, and incubated with primary antibody at 4°C overnight. At the end of the incubation, immune complexes were detected either by incubation with appropriate biotin-labeled IgG, followed by ABC reagent and diaminobenzidine chromogen (Vector Labs), or by incubation with appropriate fluorescein isothiocyanate– or Cy3-conjugated IgG.

For NF-κB localization, islets were treated with 50 μmol/l H_2O_2 or a cytokine mix (containing 10 units/ml IL-1β plus 100 units/ml IFN-γ) for 2 h. Islets were fixed with 4% formaldehyde (in PBS, pH 7.4) for 30 min and immobilized in 2% low-melting agarose in PBS by centrifugation. Cryostat sections were pretreated with 0.01% trypsin for 30 min at room temperature. After three washes with PBS and 1 h blocking with 5% donkey serum, the sections were incubated overnight with polyclonal antibodies to the rabbit NF-κB p50 unit. This was followed by detection with Cy3-conjugated anti-rabbit IgG.

Measurement of ROS production. ROS production in dispersed islet cells was measured by the ROS-sensitive fluorescent dye CM-H_2DCFDA as described previously (25). To determine ROS production in whole islets after cytokine treatment, islets were loaded with 5 μmol/l CM-H_2DCFDA for 1 h followed by three washes with fresh culture medium. Islets were incubated with cytokines for 5 h. After treatment, ROS production was visualized on a fluorescent microscope equipped with a digital camera. Images were analyzed with ImagePro software (Media Cybernetics, Silver Spring, MD).

Assessment of islet cell viability. The effects of ROS and cytokines on islet cell viability was assessed by Alamar Blue assay as previously described (25). Islets were exposed to ROS sources at concentrations and time periods as indicated. The islet cell viability was calculated as the ratio of Alamar Blue fluorescence after treatment to the fluorescence before treatment. Untreated islets were considered to be 100% viable.

Western blot analysis. Islets were lysed in 50 μl cold lysis buffer (20 mmol/l Tris, pH 7.5, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 1% Triton X-100, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, 1 mmol/l Na_3VO_4, 1 mmol/l dithiothreitol, 1 μg/ml leupeptin, and 1 mmol/l phenylmethylsulfonyl fluoride) by sonication and centrifuged at 11,000*g* for 30 min. Equal amounts of protein (2 or 10 μg) were separated by SDS-PAGE (Bio-Rad) and transferred to polyvinylidine fluoride membranes. After blocking with 5% nonfat dry milk in 0.1% Tween-20 Tris-buffered saline solution, blots were incubated with antibodies against human MnSOD (1:2,000), catalase (1:30,000), actin (1:4,000), or phospho-IkBα (1:2,000) overnight at 4°C. Blots were further incubated with appropriate peroxidase-labeled secondary antibodies (1:2,000 dilution). The immune complexes were identified using the enhanced chemiluminescence detection system (ECL; Amersham).

Islet iNOS expression and NO production. The messenger RNA level of iNOS in cultured islets was measured by real-time quantitative RT-PCR. Islet RNA was extracted with the RNA Microprep Kit from Stratagene according to the manufacturer's instructions. Fifty nanograms of RNA was reverse transcribed to cDNA with StrataScript reverse transcriptase (Stratagene) in the presence of oligo dT, based on the procedure provided by Stratagene. RT-PCR was carried out using the Brilliant plus Two-Step Quantitative RT-PCR Core Reagent kit (Stratagene). The probes were labeled with FAM at the 5′ end and a quencher TAMRA at the 3′ end. Sequences for the primer-probe sets for each gene were iNOS (forward 5′-CAACATCAGGTCGGCCATCAC-3′, reverse 5′-GTAGCCAGCGTACCGGATGA-3′, probe 5′-CCCCAGCGGACTGACGGCAAACAT-3′) and β-actin (forward 5′-CTGCCTGACGGCCAAG-3′, reverse 5′-GGAAAAGAGCCTCAGGGCAT-3′, probe 5′-CATCACTATTGGCAACGAGCGGTTCC-3′). Nitrite formation was measured in islet culture medium as described by Hohmeier et al. (8) using Griess reagent (Molecular Probes).

Data analysis. Data are presented as means ± SE. Statistical analysis was performed by ANOVA plus Dunnet's or Tukey's post hoc test for multiple comparisons.

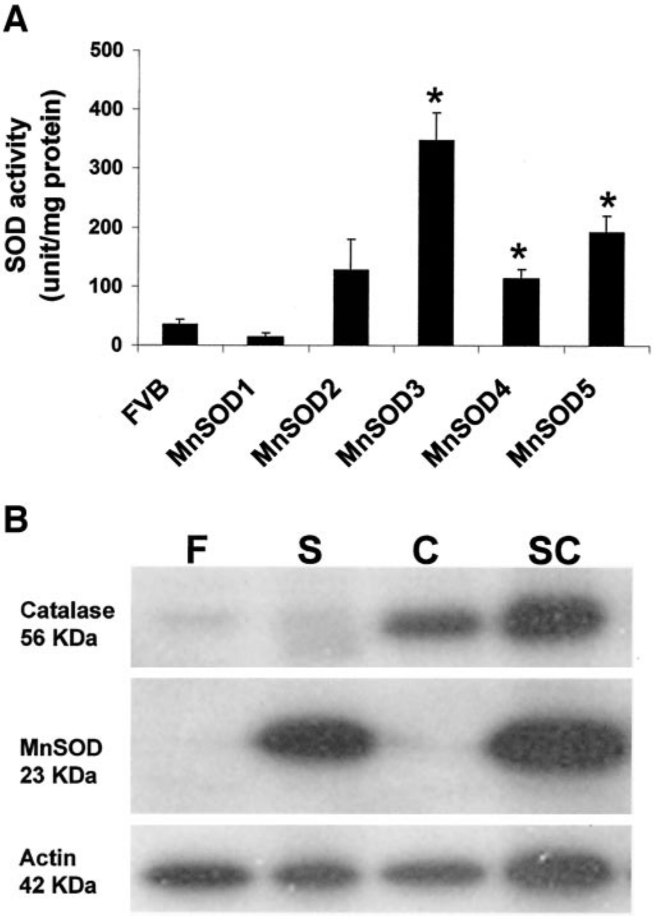

FIG. 1. SOD enzyme activity and protein expression of MnSOD and catalase in isolated islets from control and transgenic mice. *A*: Total islet SOD activity measured by inhibition of the auto-oxidation of pyrogallol, as described in RESEARCH DESIGN AND METHODS. Each bar represents the means ± SE of three to eight assays from three independent islet preparations. *$P < 0.01$ vs. FVB. *B*: Immunoblotting of MnSOD and catalase in isolated islets of FVB control (F), MnSOD3 (S), catalase (C), and MnSOD3 plus catalase (SC) mice. Whole-islet lysates (2 μg) were fractionated by SDS-PAGE and analyzed by Western blot using specific antibodies. One representative figure of three independent experiments with similar results is shown.

RESULTS

Transgenic mice with pancreatic β-cell–specific overexpression of MnSOD and/or catalase. Five independent lines of MnSOD transgenic mice were generated on the background FVB. Line MnSOD3 had the highest activity (~10-fold greater than control) (Fig. 1*A*). To analyze the complementary effects of MnSOD and catalase, MnSOD3 mice were bred to transgenic mice with β-cell–specific

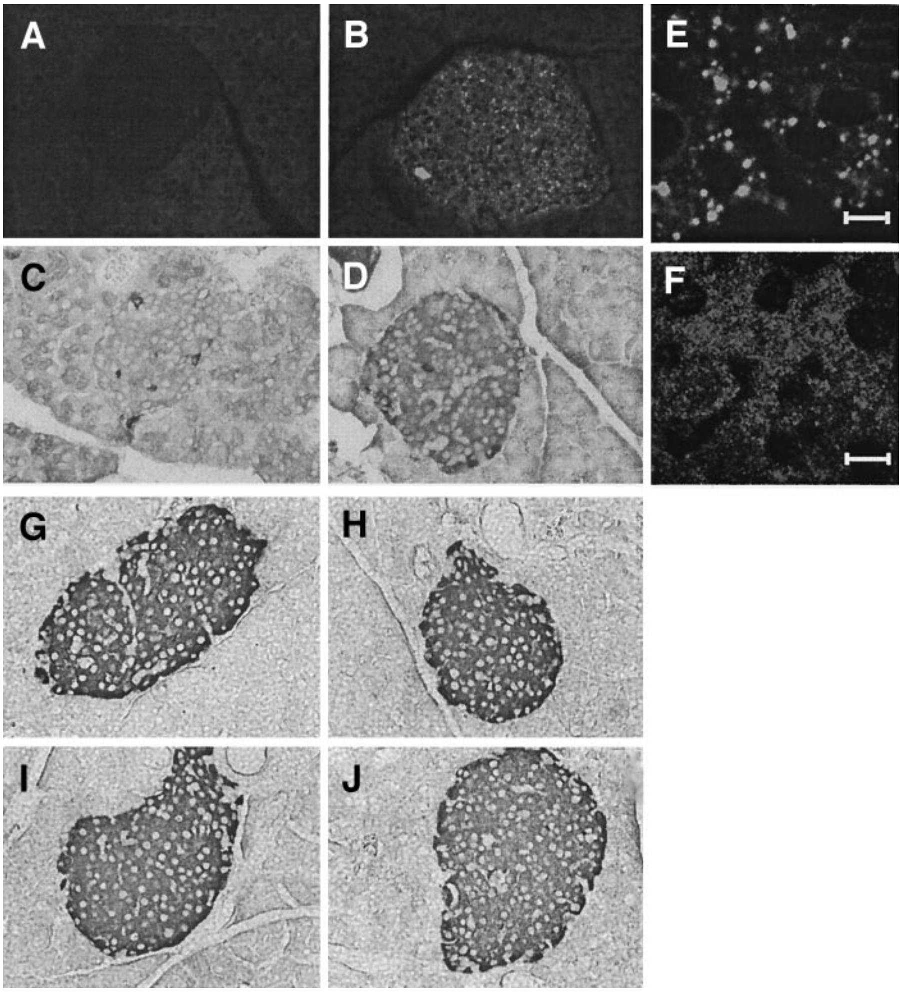

FIG. 2. MnSOD and catalase transgenes are specifically expressed in islets at high levels without influence on islet structure and insulin staining. *A–F*: Immunohistochemistry on pancreatic sections for MnSOD protein in FVB (*A*) and MnSOD (*B*) mice and for catalase expression in FVB (*C*) and catalase (*D*) mice. *E* and *F*: Confocal microscopy images for MnSOD (*E*) and catalase (*F*) immunostaining revealing the different subcellular distributions. MnSOD is excluded from nuclei with a punctate expression pattern consistent with a mitochondrial localization. Catalase staining is also excluded from nuclei but with a much more diffuse distribution. *G–J*: Insulin immunostaining in control FVB (*G*) and transgenic MnSOD (*H*), catalase (*I*), and MnSOD plus catalase (*J*) mice. Original magnification ×400. Scale bars, 5 μm.

overexpression of catalase (22). Expression of the transgenes was confirmed by Western blot analysis for catalase and MnSOD (Fig. 1*B*). As also demonstrated in Fig. 1, catalase and MnSOD did not impede one another's expression.

The immunohistochemistry results in Fig. 2*A–D* show overexpression of the antioxidant proteins in the core of the islet, presumably in the β-cells. Confocal microscopy indicated that the immunostaining of MnSOD was granular (Fig. 2*E*), which is a typical mitochondrial staining pattern (26). However, catalase staining was more diffusely distributed (Fig. 2*F*) throughout the cytoplasm.

Since some transgenes cause unintended β-cell dysfunction (27,28), morphology and function of β-cells in our transgenic mice were evaluated. Figure 2*G–J* shows that transgenic islets had normal morphology and insulin staining. Assays of islet DNA, protein, insulin content, and glucose-stimulated insulin secretion demonstrated that these characteristics were not altered by the antioxi-

TABLE 1
Characteristics of control and transgenic islets

	DNA (ng/islet)	Protein (μg/islet)	Insulin (ng/islet)	Ratio of insulin secreted at 20 and 3 mmol/l glucose
FVB	19.44 ± 1.33	0.45 ± 0.02	29.63 ± 1.49	6.34 ± 0.93
MnSOD	22.04 ± 1.63	0.46 ± 0.03	32.41 ± 1.66	6.03 ± 1.31
Catalase	21.50 ± 1.10	0.48 ± 0.01	32.25 ± 1.10	6.93 ± 1.65
MnSOD + catalase	20.64 ± 1.75	0.44 ± 0.01	30.86 ± 3.49	5.85 ± 1.44

Data are means ± SE for six or more assays per group for every type of assessment. No significant difference was found between groups by one-way ANOVA.

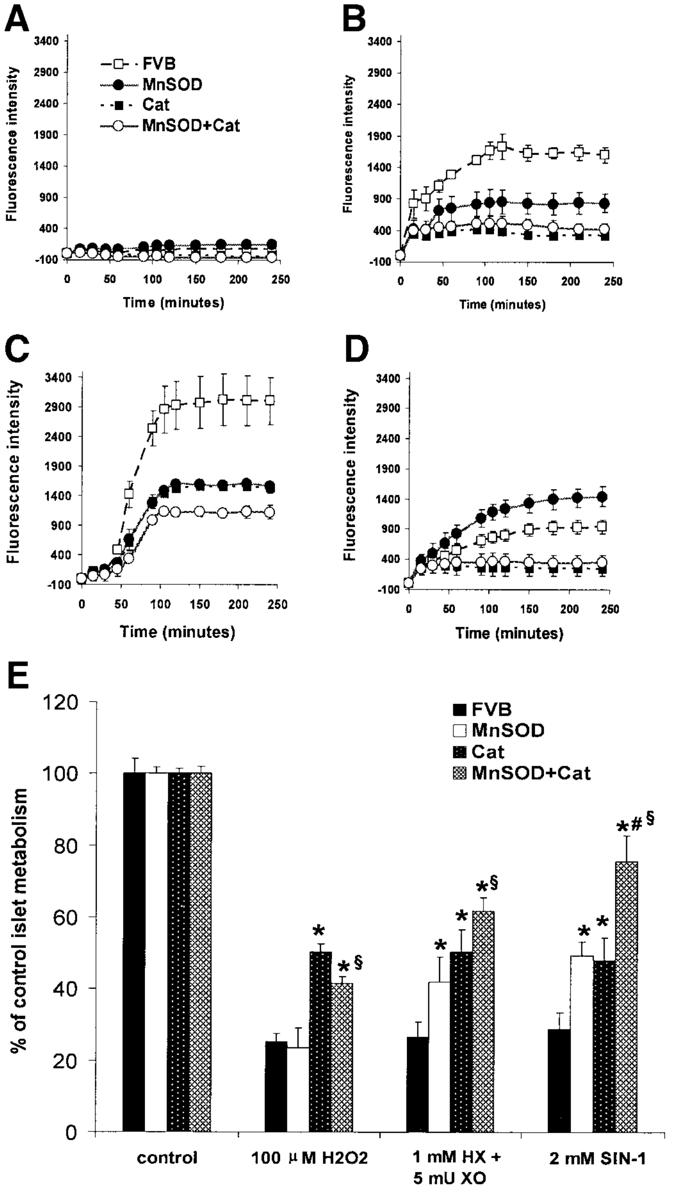

FIG. 3. ROS scavenging and islet cell viability in control and transgenic islets exposed to chemical ROS sources. Dispersed FVB control and transgenic islet cells were not treated (*A*), treated with 1 mmol/l hypoxanthine plus 2 mU xanthine oxidase (*B*), treated with 100 μmol/l SIN-1 (*C*), or treated with 20 μmol/l H_2O_2 (*D*). ROS production was measured by CM-H_2DCFDA fluorescence, as described in RESEARCH DESIGN AND METHODS. Data are mean values calculated from three or four independent experiments. *E*: Isolated islets were subjected to the indicated concentrations of H_2O_2 for 12 h, hypoxanthine (HX) and xanthine oxidase (XO) for 4 h, and SIN-1 for 24 h. Islet cell viability was measured by Alamar Blue assay. Data are the means from six to eight independent assays. Vertical bars indicate SE. **P* < 0.002 vs. FVB; #*P* < 0.01 vs. catalase (Cat); §*P* < 0.01 vs. MnSOD, by two-way ANOVA and Tukey's post hoc test.

dant transgenes (Table 1). Glucose tolerance tests (data not shown) revealed no distinctions among the transgenic and control animals. Therefore, overexpression of MnSOD, catalase, or both did not impair normal pancreatic β-cell function and structure.

ROS detoxification and protection from STZ-induced diabetes by MnSOD and/or catalase transgenes. To determine whether the antioxidant transgenes enhanced pancreatic β-cell ROS scavenging capacity, dispersed islet cells from control and transgenic mice were exposed to

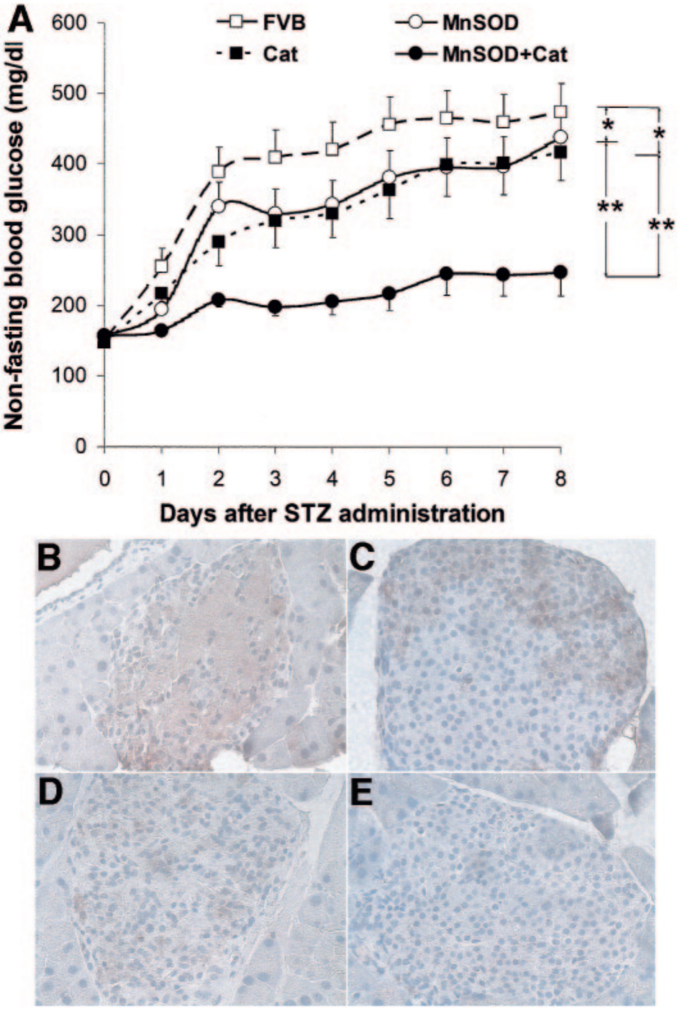

FIG. 4. Protection by MnSOD and catalase against STZ-induced diabetes and β-cell damage. *A*: Blood glucose levels in STZ-induced diabetic animals. Data are the means ± SE from 15 or 16 mice in each group. *B–E*: Representative micrographs of nitrotyrosine immunostaining in pancreatic islets of STZ-induced diabetic animals. Pancreata were obtained from FVB (*B*), MnSOD (*C*), catalase (*D*), and MnSOD plus catalase (*E*) mice that were injected with STZ 30 h earlier. Sections were stained with a polyclonal anti-rabbit nitrotyrosine antibody. Similar results were obtained from three or four animals in each group. Original magnification ×400. **P* < 0.05 vs. FVB mice; ***P* < 0.01 vs. MnSOD plus catalase mice, by two-way ANOVA and Tukey's post hoc test.

H_2O_2, superoxide generated by hypoxanthine and xanthine oxidase, and peroxynitrite produced from SIN-1. ROS production was measured by the ROS-sensitive fluorescent dye CM-H_2DCFDA. As shown in Fig. 3*A–D*, catalase conferred scavenging for every type of ROS tested ($P <$ 0.01 vs. FVB). MnSOD transgenic islet cells disposed of superoxide and peroxynitrite more efficiently than FVB control cells ($P < 0.01$ vs. FVB), but MnSOD increased the ROS signal in the presence of H_2O_2 ($P < 0.01$ vs. FVB). The reason for this increased signal is not certain, but may be due to the fact that CM-H_2DCFDA is more sensitive to oxidation by H_2O_2 than it is to superoxide (29). The combination of MnSOD plus catalase was more effective than MnSOD ($P < 0.01$) for all ROS and more effective than catalase against SIN-1 ($P < 0.01$ vs. MnSOD).

To confirm that increased scavenging capacity not only reduced ROS levels but also rendered transgenic islets more resistant to ROS-induced toxicity, we assessed cell

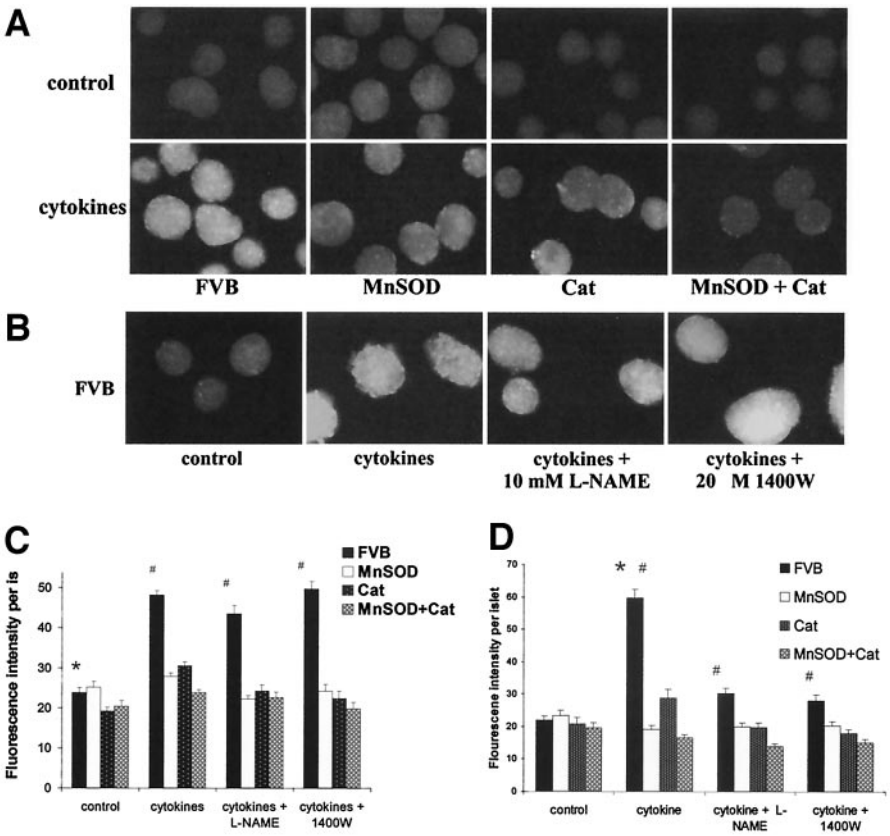

FIG. 5. Cytokine-induced ROS production measured by CM-H_2DCFDA fluorescence is reduced by antioxidant transgenes. *A*: Representative images from FVB and transgenic islets after 5 h culture in the absence (control) or presence (50 units/ml IL-1β plus 500 units/ml IFN-γ) of cytokines. *B*: Images of FVB islets showing that iNOS inhibitors, L-NAME (10 mmol/l), or 1400W (200 μmol/l) only slightly reduce cytokine-induced ROS production after 5 h cytokine treatment. *C*: Quantitation of fluorescence intensity in cultured islets after 5 h cytokine exposure. A 5-h exposure to cytokines results in about a twofold elevation of ROS production in FVB islets, which was not prevented by iNOS inhibitors ($*P < 0.001$, FVB control vs. all other FVB groups). The FVB group was different from all antioxidant transgene groups under that treatment condition ($\#P < 0.01$). *D*: Quantitation of fluorescence intensity in cultured islets after 24 h cytokine exposure. After 24 h, cytokine-induced ROS production becomes sensitive to iNOS inhibitors and remains sensitive to all antioxidant transgenes. FVB plus cytokines was different from all other FVB treatment groups ($*P < 0.001$). The FVB group was different from all antioxidant transgene groups under that treatment condition ($\#P < 0.01$). Statistic analysis was performed by two-way ANOVA and Tukey's post hoc test in a total of 40–110 islets from three or four separate islet isolations in each group. Original magnification ×100.

viability. The toxicity findings (Fig. 3*E*) closely paralleled the results obtained with CM-H_2DCFDA fluorescence. Catalase, MnSOD, and MnSOD plus catalase significantly improved resistance to ROS damage for all conditions, except that MnSOD alone did not protect islets from H_2O_2. Also, as seen with CM-H_2DCFDA fluorescence, catalase provided protection similar to MnSOD plus catalase, except in the case of SIN-1, where the combination of antioxidants was more effective than catalase alone. Similar results were obtained with other concentrations of ROS donors (data not shown).

To test whether β-cell overexpression of antioxidants was effective in vivo, mice were challenged with STZ. Either catalase or MnSOD significantly retarded diabetes by STZ (Fig. 4*A*). However, by 8 days after STZ, blood glucose in these groups had increased by at least 250 mg/dl to a peak >400 mg/dl. In contrast, the two transgenes were strikingly more effective in combination. Over 8 days, blood glucose climbed only 90 mg/dl to a plateau of <250 mg/dl in MnSOD plus catalase mice.

Nitrotyrosine staining was used as an indicator of STZ-induced damage (Fig. 4*B*). Within 30 h of STZ treatment, nitrotyrosine staining became prominent in FVB islets. Either the MnSOD or catalase transgene markedly reduced staining. However, the combination of these two antioxidant transgenes was especially effective, reducing nitrotyrosine staining to barely detectable levels.

MnSOD and catalase transgenes reduce cytokine-induced ROS production but not cytokine toxicity. We tested whether the transgenes could reduce cytokine-induced ROS production. Measurements were performed on CM-H_2DCFDA–preloaded islets following 5 h of cytokine treatment. This time period was selected because ROS production peaks 5 h after cytokine treatment (1), and iNOS induction is minimal at this time point. As shown in Fig. 5*A–C*, cytokines IL-1β plus IFN-γ led to a twofold elevation of ROS in FVB islets. MnSOD and catalase, either alone or in combination, reduced cytokine generation of ROS. At this 5-h time point, most ROS generation by cytokines does not appear to include NO production; two inhibitors of iNOS, L-NAME, and 1400W had only a slight effect on cytokine-induced CM-H_2DCFDA fluorescence at this time point, probably due to minimal induction of iNOS at 5 h. When the same experiment was carried out after a 24-h incubation, when iNOS protein expression is high, cytokine-induced fluorescence could now be blocked by the two iNOS inhibitors (Fig. 5*D*). At the same time point, the antioxidant transgenes remained effective inhibitors of

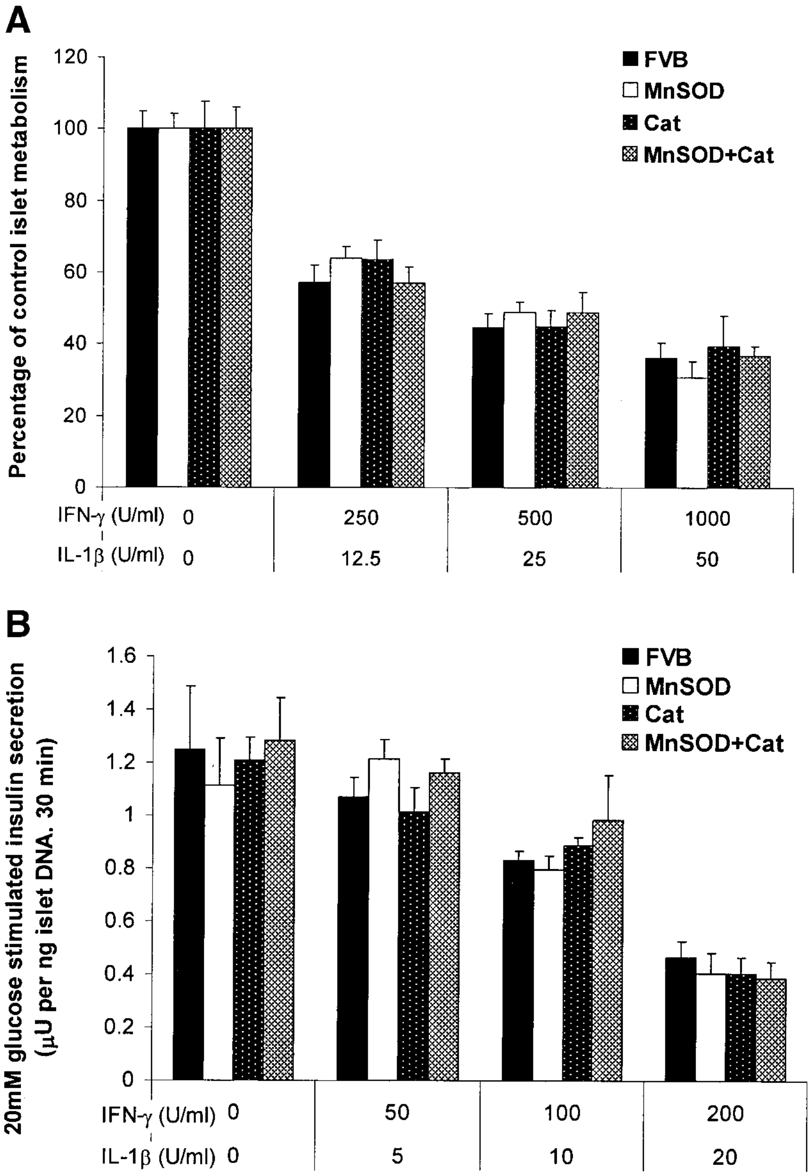

FIG. 6. MnSOD and/or catalase do not provide protection against cytokine toxicity. *A*: Cytokine-induced reduction in islet cell viability was measured by the Alamar Blue assay. Transgenic and control FVB islets were exposed to the indicated concentrations of IL-1β and IFN-γ for 6 days. *B*: Inhibition of glucose-stimulated insulin secretion was measured in islets exposed to the indicated concentrations of cytokines for 18 h. Data are the means ± SE calculated from 6 to 12 independent measurements obtained using three to five islet preparations per group. Cat, catalase.

cytokine-induced fluorescence. We suspect that much of the activation of CM-H2DCFDA fluorescence at 24 h is due to increased formation of peroxynitrite, which can be prevented by decreased levels of either NO using iNOS inhibitors or by decreased superoxide levels due to antioxidant transgenes.

Figure 6*A* shows islet cell viability after 6 days of cytokine treatment, assayed with the Alamar Blue metabolic assay. Alamar blue and other metabolic assays are widely used as a measure of β-cell viability, and they produce results that are similar to other tests of viability (30,31). The 6-day time period was used because cytokine-induced β-cell death requires several days to develop (32). All doses of cytokines significantly reduced viability, and none of our antioxidants had a significant beneficial effect. We tested if they might be protective for other parameters or after a shorter time period. Therefore, we evaluated insulin secretion after 24 h of cytokine treatment (Fig. 6*B*). However, neither MnSOD nor catalase transgene reversed the suppression of secretion.

In β-cell tumor lines, antioxidant transgenes are effective in preventing cytokine-induced iNOS activation and NO production (8,21). We tested this in our transgenic β-cells. The data in Fig. 7 demonstrated that MnSOD, catalase, and MnSOD plus catalase were all unable to decrease cytokine-induced iNOS expression and NO production at every cytokine concentration examined.

Antioxidants block NF-κB activation in β-cell tumor lines (21). We first assessed NF-κB activation in transgenic islets by measuring phosphorylation of serine 32 in the protein inhibitor of κB (IκB), whose phosphorylation initiates NF-κB activation. As shown in Fig. 8, IκB phosphorylation was induced by cytokines in a dose- and time-dependent manner. However, neither antioxidant trans-

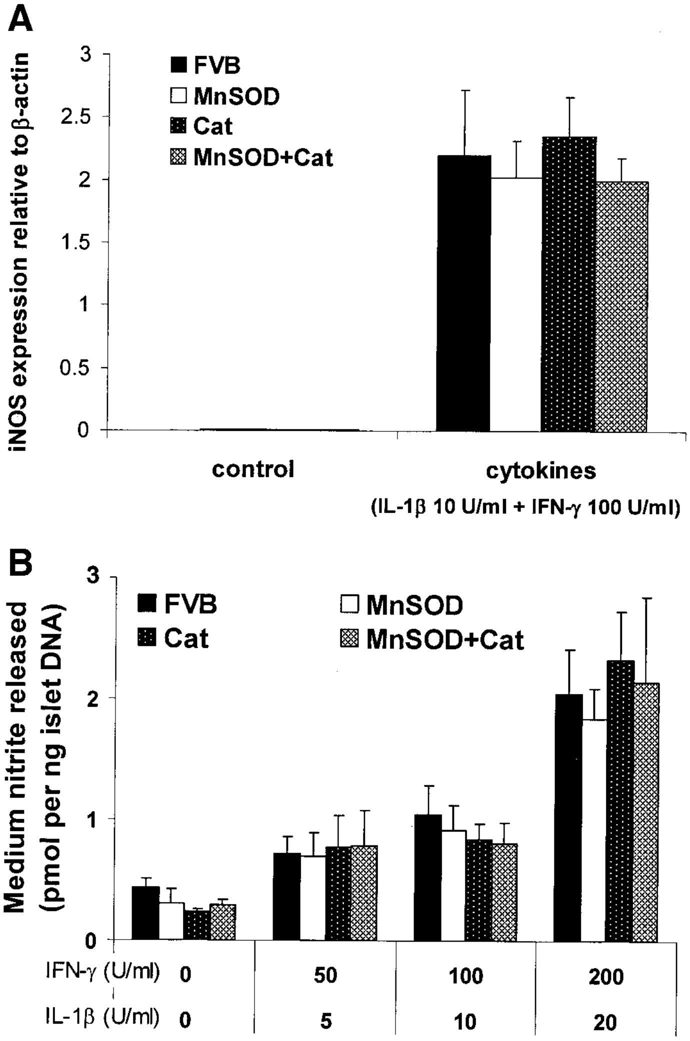

FIG. 7. Cytokine-induced iNOS expression and NO production in transgenic and control FVB islets. *A*: Real-time RT-PCR quantification of iNOS mRNA expression. Total RNA was isolated from cultured islets that were exposed to the indicated concentrations of cytokine mix for 6 h. The expression of iNOS was normalized against the expression of β-actin, and the results were calculated from a standard curve performed in each assay. Data are the means ± SE from three independent experiments in duplicate. (*B*) Cultured islets were treated with the indicated concentrations of cytokines for 24 h, and the amount of NO released in the culture medium was measured by Griess reagent. Data were calculated from four independent experiments. Neither single nor combined antioxidant transgenes were able to prevent cytokine-induced activation of iNOS or NO production ($P > 0.05$ by two-way ANOVA/Tukey's post hoc test). Cat, catalase.

gene, alone or in combination, inhibited IκB phosphorylation. When NF-κB is activated, it translocates into the nucleus. The immunohistochemistry results shown in Fig. 9 demonstrate that antioxidants were highly effective in preventing NF-κB activation by H_2O_2 but completely ineffective against cytokine-induced activation.

DISCUSSION

In the present study, transgenic mice with β-cell–specific overexpression of antioxidant proteins MnSOD and/or catalase were produced. Elevated expression of antioxidants enhanced β-cell ROS scavenging and rendered β-cells resistant to STZ- and ROS-mediated damage. Antioxidants were very effective in reducing cytokine-induced ROS production. However, alone or in combination, MnSOD and catalase could not alter cytokine-induced β-cell dysfunction or death. Moreover, none of these antioxidants could inhibit cytokine-induced activation of β-cell NF-κB. Our results confirm that primary β-cells are sensitive to ROS, but they indicate that ROS do not play a significant role in cytokine-mediated β-cell death.

Catalase and SOD have previously been shown (33) to be synergistic. In β-cells, we did not see synergistic protection of MnSOD plus catalase against hydrogen peroxide or superoxide. However, their coexpression did provide additive or synergistic protection against SIN-1–induced ROS production, SIN-1–induced cell death, STZ-induced diabetes, and STZ-induced nitrotyrosine formation. SIN-1 is thought to act by generation of peroxynitrite (7), and nitrotyrosine is a reaction product of peroxynitrite (34). This suggests that catalase plus MnSOD was especially effective against peroxynitrite. Synergistic protection against STZ may also be due to protection from peroxynitrite, since this reactive species is a mediator of STZ-induced injury to β-cells (35).

In several lines of insulinoma cells, antioxidants prevent toxic effects of cytokines, including induction of cell death (8,9). Therefore, we expected that our antioxidant transgenes would also protect primary β-cells from cytokine-induced dysfunction and death. However, cytokine injury to transgenic and nontransgenic β-cells was identical. This was especially surprising in view of the fact that the antioxidant transgenes dramatically reduced cytokine-induced ROS production. This indicated that the burst of ROS, which follows cytokine exposure in primary β-cells, does not contribute to cytokine-induced injury. In primary β-cells, antioxidants did not influence cytokine activation of the NF-κB pathway, including serine phosphorylation of IκB, nuclear recruitment of NF-κB, or iNOS induction. This is in complete contrast to results obtained in insulinoma cells, where antioxidants essentially block cytokine activation of NF-κB and iNOS (8,21). Clearly, continuously dividing insulinoma cells and nondividing primary β-cells differ significantly in their mode of regulation of NF-κB. A potential limitation of our conclusions is that we determined viability using the Alamar Blue metabolic assay. This is a widely used assay of viability, but under some conditions viability and metabolic activity can diverge. However, this would still not affect our findings on impaired insulin secretion, activation of NF-κB, or iNOS induction.

For at least 15 years, it has been proposed that ROS injury to the β-cell is an essential component in the onset of type 1 diabetes (36). One pillar of this hypothesis has been the protection antioxidants provide against cytokine-induced injury to insulinoma cells. Since our current findings cast doubt on the relevance of this evidence to primary β-cells, it is worthwhile to reassess the overall case for ROS as a cause of autoimmune destruction to the β-cell. Part of the frequently sited evidence is merely associative; β-cells have low levels of antioxidant defense enzymes, and markers of oxidative damage increase in islets exposed to cytokines (37). More critical to the hypothesis are results that imply a cause-and-effect relationship between ROS and β-cell injury. Those findings can be divided into two categories: experiments in which whole animal antioxidant status was increased and experiments

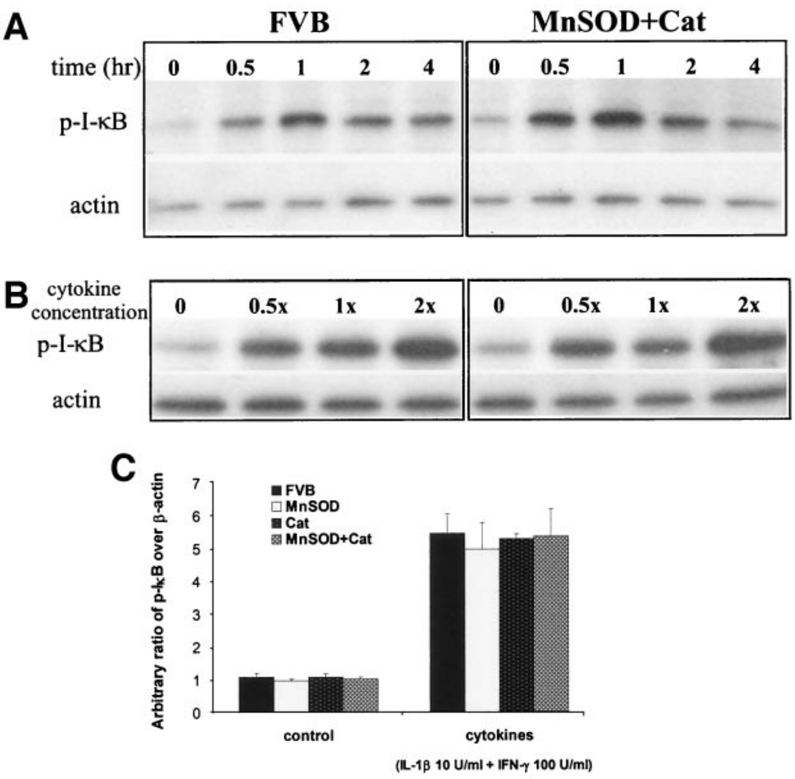

FIG. 8. Cytokine-mediated IκB serine phosphorylation. *A*: The time course of IκBα serine 32 phosphorylation in islets from FVB control mice and transgenic mice (MnSOD plus catalase [Cat]) after cytokine treatment. Isolated islets were treated with 1× cytokine mix (10 units/ml IL-1β plus 100 units/ml IFN-γ) for the indicated time periods. *B*: Dose dependency of IκBα serine phosphorylation following 1 h exposure to cytokines. Results in *A* and *B* are typical of three independent experiments. *C*: Quantitative densitometric analysis of phosphorylated IκBα in FVB control and transgenic islets after treatment with 1× cytokine mix for 1 h. Data are the means ± SE derived from three independent islet preparations in each group. No significant statistical differences were found between groups ($P > 0.05$ by two-way ANOVA/Tukey's post hoc test).

where only β-cell antioxidant status was increased. Multiorgan or whole-body protection from ROS has been successful in decreasing NOD (38–40) or multiple low-dose STZ-induced (41) diabetes. However, these experiments could not distinguish between changes in the immune system versus direct ROS protection of the islet. In fact, when these studies analyzed the effect of antioxidants on immune cell activation, it became clear that some and potentially all of the protection from diabetes may have been due to changes in the immune response (39,42). There is only one example where an antioxidant protein targeted to the β-cell has reduced type 1 diabetes: transgenic overexpression of the antioxidant protein thioredoxin in β-cells effectively reduced NOD diabetes (43). However, it is now recognized that thioredoxin has potent antiapoptotic actions (44) in addition to its antioxidant actions. β-Cell overexpression of another, more specific antioxidant enzyme, SOD3, provided no protection from NOD diabetes (45), and we have found (H.C., P.N.E., unpublished observations) that β-cell–specific antioxidant protection with catalase, MnSOD, or metallothionein provides no benefit whatsoever in NOD diabetes. Our results are consistent with other reports indicating that NO (46) and additional pathways (47) may mediate cytokine toxicity. One caveat to our conclusion about the limited role of ROS in cytokine toxicity is that humans and rodent β-cells differ significantly in their levels of antioxidants enzymes (48) and their response to cytokine exposure (49).

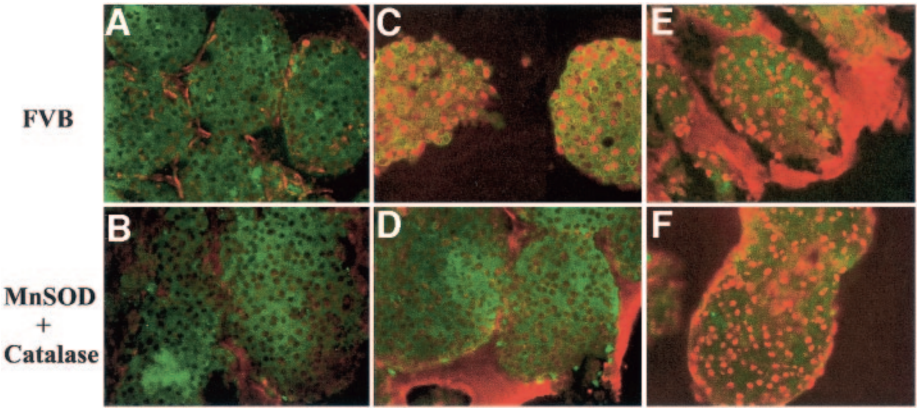

FIG. 9. Cytokine- and H_2O_2-induced NF-κB activation. FVB control (*top row*) and MnSOD plus catalase transgenic islets (*bottom row*) were left untreated (*A* and *B*), treated with 50 μmol/l H_2O_2 (*C* and *D*), or treated with cytokines (10 units/ml IL-1β plus 100 units/ml IFN-γ; *E* and *F*) for 2 h. Islet frozen sections were immunostained with rabbit anti–NF-κB (p50) antibody and visualized with Cy3-conjugated anti-rabbit IgG. When NF-κB is activated and translocates, nuclei become visible as bright red spots. *C* and *E*: H_2O_2 and cytokines activate NF-κB in FVB control islets. MnSOD and catalase block NF-κB activation by H_2O_2 (*D*), whereas the antioxidants do not block activation by cytokines (*F*). Cy3 staining outside of the islet is due to residual mounting material. Original magnification ×400. Results were replicated in three independent experiments for H_2O_2 and four independent experiments for cytokines.

In summary, we have demonstrated efficient antioxidant protection of pancreatic islets using two antioxidant transgenes. Both antioxidants were protective against chemical inducers of ROS, provided additive or synergistic protection from some β-cell toxins, and suppressed ROS generation by cytokines. However, our failure to see any protection from cytokine toxicity, when combined with a lack of convincing published evidence to the contrary, indicates that antioxidant protection of β-cells, at least in mice, is unlikely to benefit autoimmune diabetes.

ACKNOWLEDGMENTS

This work was supported by grants DK58100 and HL075080 and from the Commonwealth of Kentucky Research Challenge Trust Fund and the University of Louisville Center for Genetics and Molecular Medicine.

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