Prevention of Spontaneous and Experimentally Induced Diabetes in Mice With Zinc Sulfate-Enriched Drinking Water Is Associated with Activation and Reduction of NF-κB and AP-1 in Islets, Respectively

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Recently, we reported that zinc sulfate-enriched (25 m*M*) drinking water (Zn^{2+}) protected male C57BL/6 mice from diabetes induced by multiple low doses of streptozotocin (MLD-STZ) and that MLD-STZ activates the transcription factors nuclear factor (NF)-κB and activator protein (AP)-1 in islets of these mice. Therefore, we studied the effect of Zn^{2+} on spontaneous diabetes in female nonobese diabetic (NOD) mice and on the activity of NF-κB and AP-1 in islets of NOD and MLD-STZ–injected male C57BL/6 mice. We hypothesized that Zn^{2+} may affect NF-κB, which may play a key role in immune-mediated diabetogenesis. Here we continuously administered Zn^{2+} to NOD mice, to both parents and their F_1 offspring, and treated C57BL/6 male mice with MLD-STZ either alone or in addition to Zn^{2+}. We assessed effects of Zn^{2+} on insulitis and peri-insulitis in 8-week-old NOD mice and analyzed NF-κB and AP-1 activities in islets. Zn^{2+} significantly prevented diabetes in female F_1 offspring and significantly reduced insulitis and peri-insulitis. Zn^{2+} significantly stimulated NF-κB and AP-1 activation in NOD mice, in contrast, in C57BL/6 mice, Zn^{2+} significantly reduced their activation by MLD-STZ. These data demonstrate that NF-κB may play a critical role in immune-mediated diabetes. Depending on the mode of β-cell destruction, Zn^{2+} may prevent apoptosis through activation of NF-κB in NOD mice or prevent inflammatory immune destruction through inhibition of NF-κB in MLD-STZ–treated C57BL/6 mice. Exp Biol Med 229:1177–1185, 2004

Key words: activator protein (AP)-1; MLD-STZ diabetes; NOD mice; nuclear factor (NF)-κB; zinc sulfate

In type 1 diabetes, T-cell–dependent inflammatory immune reactions selectively destroy β-cells (1, 2). In nonobese diabetic (NOD) female mice, a bias toward pro-inflammatory T helper (Th) 1-type cytokines, which are locally produced in the pancreatic islets, promotes insulitis and diabetes, whereas anti-inflammatory Th2-type cytokines are protective (3, 4). In the putative type 1 diabetes model induced by multiple low doses of streptozotocin (MLD-STZ), this laboratory has reported that in addition to local upregulation of Th1-type cytokines in islets, it is the reduction of Th2-type cytokines that is tightly associated with disease susceptibility in male C57BL/6 recipients (5). Reactive oxygen species (ROS) have been implicated as mediators of β-cell destruction (2, 6) and are potential stimulators of proinflammatory cytokines (7), which, in turn, produce ROS. Finally, β-cells succumb to this vicious circle of ROS generation that is due to sustained local inflammatory cytokine responses and failure of antioxidative systems. Therefore, diabetes can be prevented by augmenting antioxidants (8–11) and/or by shifting proinflammatory toward anti-inflammatory reactions (12–15).

In chronic inflammatory diseases, activation of the ROS-sensitive nuclear factor (NF)-κB (16) and activator protein (AP)-1 (17) is essential in cytokine gene activation and disease progression. In MLD-STZ diabetes, activation of NF-κB has also been assigned a central role in the

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pathogenesis, because deficiency in the p50 subunit confers resistance (18). This finding is corroborated by recent data from our laboratory, which indicate that inhibition of NF-κB activation by the anti-inflammatory cytokine rhIL-11 prevents MLD-STZ diabetes (15). Furthermore, in primary islet cells of human (19) and rats (20) and in insulinoma cells of rats (20–22), NF-κB activation induces increased cell death. Contrary to these findings, a protective role of NF-κB activation against cell death has been observed in some tumor cell lines (23–25). Similarly, in pancreatic β-cells of NOD mice and SV40 T-transformed insulinoma cells derived from NOD mice, activation of NF-κB also protected against TNF-α-induced apoptosis (26). Apparently, depending on the cell type and mode of cell death, the interplay of cytokines and transcription factors may differ. The role of NF-κB in the pathogenesis of spontaneous diabetes in the NOD mouse, however, has yet not been investigated.

Previously, this laboratory demonstrated that zinc sulfate-enriched drinking (25 m*M*) water (Zn^{2+}) up-regulates the antioxidative protein metallothionein (MT) in islets and protects from MLD-STZ diabetes (27). The assumption that Zn^{2+}-induced MT may have exerted a β-cell protective effect by scavenging hydroxyl radicals (•OH), the most toxic species of ROS, has been supported by other investigators using different animal models of induced diabetes (28–30). Furthermore, overexpression of MT targeted to β-cells protected from diabetes induced with a single high dose of STZ (11). It is noteworthy to mention that a zinc ion–enriched diet also protected BioBreeding (BB) Wistar rats from spontaneous diabetes (31). Here, we analyzed the effect of Zn^{2+} on diabetes and insulitis in NOD mice and on NF-κB and AP-1 activity in islets that were isolated from both C57BL/6 mice injected with MLD-STZ and female NOD mice.

Materials and Methods

Animals. NOD mice were bred in our own colony, and male C57BL/6 mice were obtained from Harlan Winkelmann GmbH (Borchen, Germany). The mice were kept under specific pathogen-free conditions and received rodent diet (Ssniff M; Ssniff, Soest, Germany) and drinking water *ad libitum*. In our NOD colony, the diabetes prevalence reached a maximum of 80% in females at the age of 36 weeks. C57BL/6 mice were 7–8 weeks old at the beginning of the experiments. The experiments were conducted in accordance with the "Principles of Laboratory Animal Care" (NIH publication no. 85-23, revised 1995) as well as the current version of the German law on the protection of animals.

Reagents. All of the following reagents were purchased from companies residing in Germany. STZ, proteinase inhibitor, and T4 polynucleotide kinase were obtained from Roche Diagnostics GmbH (Mannheim); collagenase (0.42 U/mg) and trypsin (1.25 mg/ml) from Sigma (Deisenhofen); and PBS from Life Technologies GmbH (Karlsruhe). Reagents for the hematoxylin-eosin staining and zinc-sulfate were obtained from Merck (Darmstadt). NF-κB consensus oligonucleotides (5′-AGTTGAGGGGACTTTCCCAGGC-3′), AP-1 (5′-CGCTTGATGAGTCAGCCGGAA-3′), and Octamer (Oct)-1 (5′-TGTCGAATGCAAATCACTAGAA-3′) were commercially synthesized by Promega (Mannheim).

Treatment of Mice. To investigate the effect of Zn^{2+} on diabetes development in female NOD mice, both the breeding pairs and their offspring had free access to Zn^{2+} (27). Breeding pairs and their offspring that had received tap water served as controls. Of the offspring, 44 received Zn^{2+} and 30 remained as control; both groups were monitored for diabetes by determination of blood glucose levels every 2 weeks starting at the age of 4 weeks.

To investigate effects of Zn^{2+} on *ex vivo* activity of NF-κB and AP-1, islets were isolated from NOD and C57BL/6 mice. Groups of 10 female NOD mice were sacrificed at the age of 8 weeks. Groups of 12 C57BL/6 male mice each were injected ip with MLD-STZ (40 mg/kg body weight) on 5 consecutive days (32) or the solvent as control, either alone or in addition to treatment with Zn^{2+} that was started 1 week before the first intraperitoneal injection. Islets were isolated on Days 1 and/or 3 after the final injection. To investigate effects of Zn^{2+} on insulitis and peri-insulitis, groups of 5 female NOD mice each, which had received Zn^{2+} or not, were sacrificed at the age of 8 weeks.

Determination of Plasma Glucose. Blood samples were collected from the tail vein of nonfasted animals between 0900 and 1100 hrs using 20-μl capillary glass tubes. Glucose concentration was measured by the hexokinase method using an autoanalyzer (Eppendorf APC 5040; Eppendorf, Hamburg, Germany). Diabetes was

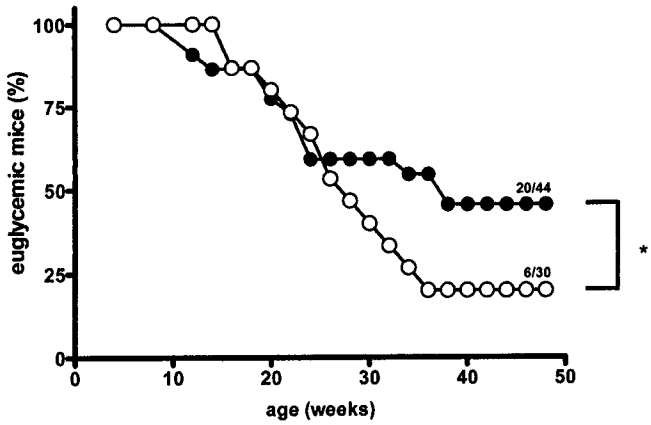

Figure 1. Percentage of euglycemic female nonobese diabetic (NOD) mice. Both the mice examined and their parents had (closed circle) or had not (open circle) received Zn^{2+}. Zn^{2+} protects NOD mice from diabetes. n/n, number of euglycemic mice/number tested. * $P < 0.05$.

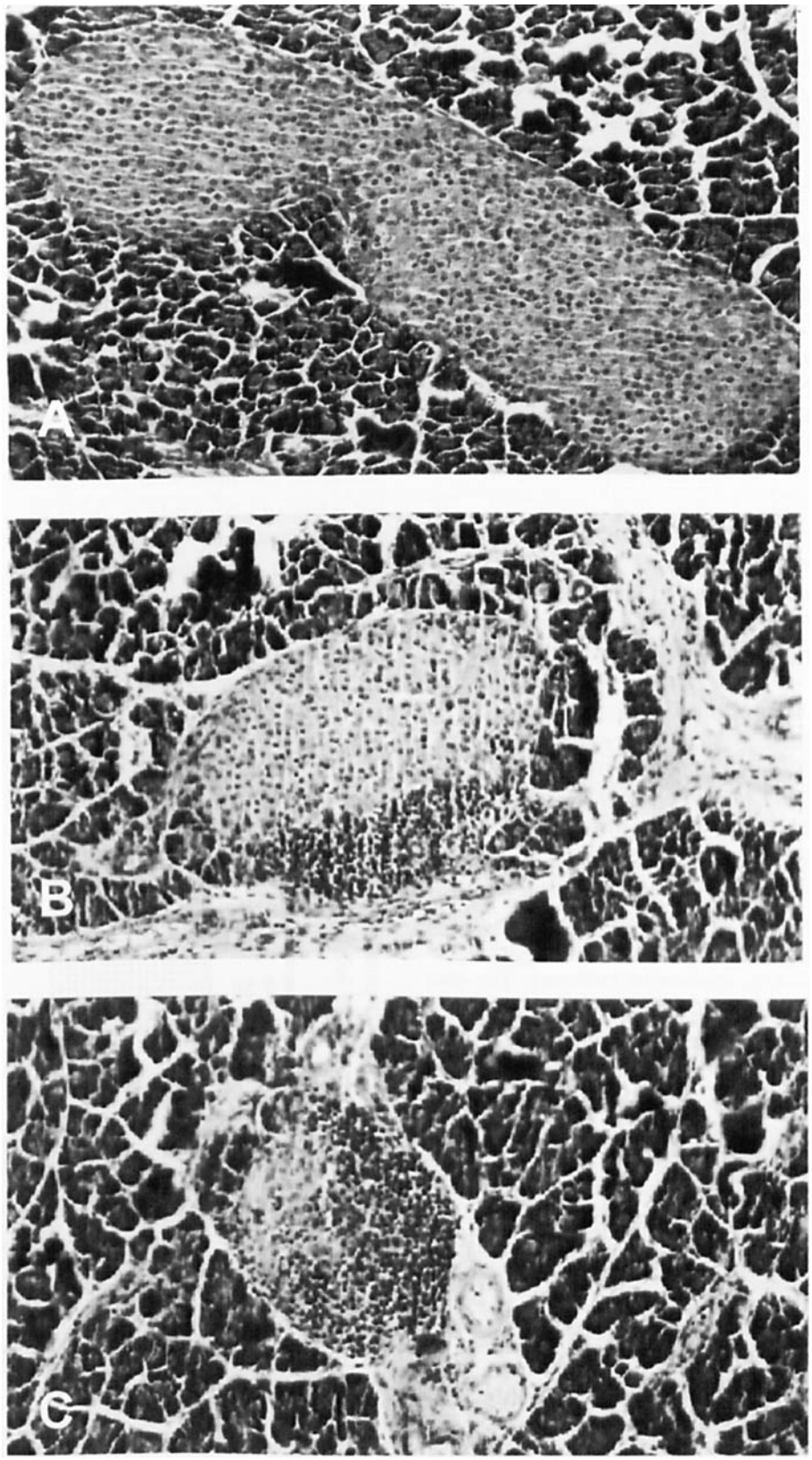

Figure 2. Representative histology of pancreatic sections prepared from 8-week-old nonobese diabetic (NOD) mice. Absence of insulitis (A); insulitis scored as 1+ (B) and 3+ (C). Hematoxylin and eosin stain. Magnification: ×200.

defined as a blood glucose concentration greater than 11.1 m*M* for at least 2 weeks.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA). The method applied has been recently described (15). Briefly, approximately 700–1000 islets isolated from each group of mice were separated into single cells by trypsin digestion. The cells were then separated into cytosol and nuclei. From the nuclei an extract was prepared for the determination of NF-κB, AP-1, and Oct-1 activities. Double-stranded synthetic oligonucleotide probes for the three targets were end-labeled using γ-[^{32}P]-dATP (Hartmann Analytic, Braunschweig, Germany) and T4 polynucleotide kinase. The specificity of the NF-κB, AP-1, and Oct-1 signals was approved by using an unlabeled (cold) consensus sequence in excess (10 pmol) for target competition. Binding reactions containing equal amounts of protein (4 μg) and labeled oligonucleotide probes were performed. The protein-DNA complexes were electrophoresed using a nondenatured 6% polyacrylamide gel. The gels were dried and exposed to autoradiographic films. The films were scanned and band intensity was quantified using TINA 2.09d quantification software (Raytest, Straubenhardt, Germany).

Histologic Examination. Serial sections of pancreata were prepared as described (27). After coding the slides, the sections were examined independently by two of the authors for presence of infiltrates with mononuclear cells at both islet-pole and intraislet sites. The degree of insulitis was scored as follows: 0 = no infiltrate; 1+ = mild infiltrate (≤30% of cells in islets); 2+ = moderate infiltrate (30%–75% of cells in islets); and 3+ = severe infiltrate (>75% of cells in islets). Perivascular or periductular sites were examined for presence or absence of infiltrates. The data were obtained from at least 300 islets per group.

Data Analysis. Data presenting NF-κB and AP-1 activities are means ± SE of four and three independent experiments with C57BL/6 and NOD mice, respectively. The chi-square test was applied for statistical analysis of diabetes prevalence and histology, the unpaired Student's *t* test for the data presenting activation of the transcription factors, and Fisher's exact test for comparing the total number of infiltrated islets in the Zn^{2+}-treated versus the untreated group. We considered a *P* value of <0.05 to be statistically significant.

Results

Zn^{2+} Protects NOD Mice from Diabetes and Reduces Insulitis. Continuous treatment of parents and their offspring with Zn^{2+} significantly reduced ($P < 0.05$) the prevalence of diabetes in female recipients compared with untreated litter mates (Fig. 1). The percentage of euglycemic mice increased from 20% (6 out of 30) up to 45.5% (20 out of 44). When Zn^{2+} was given only to either the breeding pairs or their offspring, the protective effect, however, was less pronounced and not significant (data not shown).

Applying a scoring system from 0 to 3+, the percentage of islets without infiltrates (Fig. 2A) or with 1+ (Fig. 2B), 2+, or 3+ (Fig. 2C) insulitis of Zn^{2+}-treated mice was compared with that of untreated donors. As shown in Figure 3, the percentage of islets with 1+, 2+, or 3+ insulitis decreased from 15.7% to 7.94%, from 7.36% to 2.71%, and from 4.66% to 1.01%, respectively. Peri-insulitis was absent after Zn^{2+} treatment. Zn^{2+} significantly reduced ($P = 0.008$) the degree of insulitis comparing the total number of infiltrated islets between the Zn^{2+}-treated versus the untreated group.

Contrary Effects of Zn^{2+} on NF-κB and AP-1 Activity in Islets. The *ex vivo* activation of NF-κB and AP-1 by MLD-STZ in male C57BL/6 mice was inhibited by additional treatment with Zn^{2+}. Zn^{2+} significantly reduced the activity of NF-κB ($P < 0.01$) and AP-1 ($P < 0.05$) on Day 3 after the last STZ injection, and the activities were comparable with those in untreated control groups. Zn^{2+} treatment alone did not alter the constitutive level of NF-κB and AP-1 activities (Fig. 5A and B, respectively). The activity of the ubiquitous transcription factor Oct-1 (Fig. 4) as internal control remained unchanged by the different treatments. In islets isolated from 8-week-old female NOD

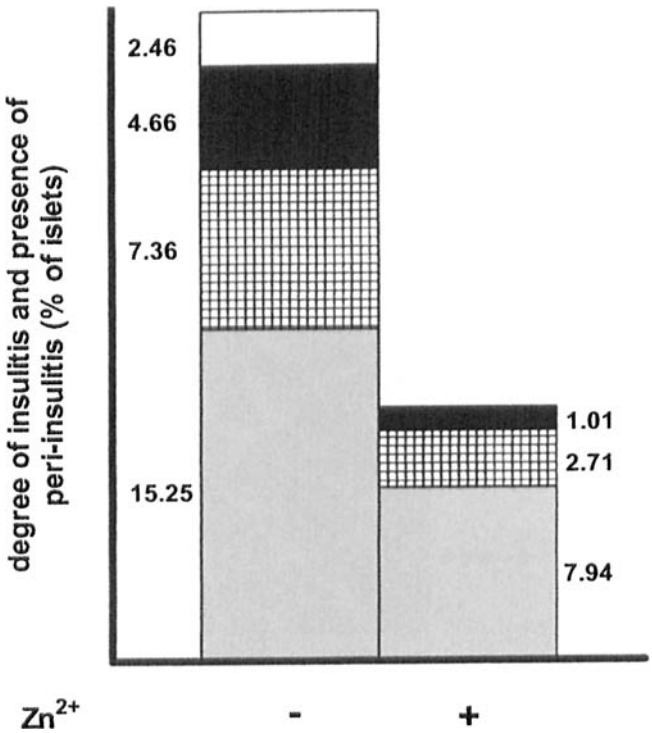

Figure 3. Histological degree of insulitis and peri-insulitis in 8-week-old female nonobese diabetic (NOD) mice. The mice and their parents received Zn^{2+} (+) or did not receive Zn^{2+} (–). Insulitis was scored as 1+ (dotted bar), 2+ (square-lined bar), and 3+ (black bar), or the presence of peri-insulitis (white bar). Zn^{2+} significantly ($P = 0.008$) reduces the degree of insulitis and prevents peri-insulitis. At least 300 islets of either group of 5 mice were examined.

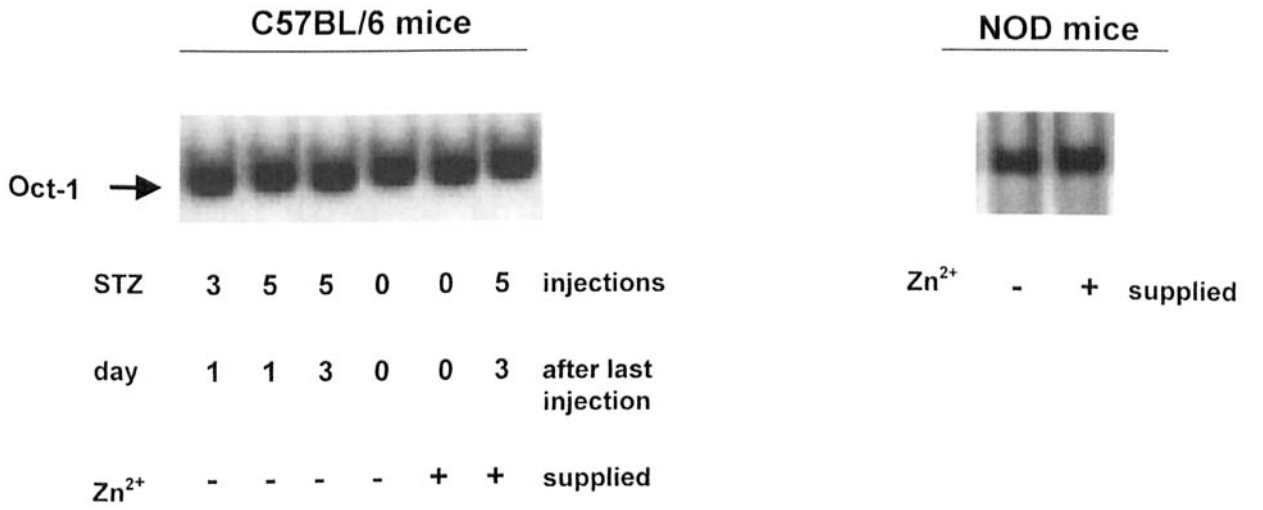

Figure 4. *Ex vivo* activity of Oct-1 in islets of male C57BL/6 mice and female nonobese diabetic (NOD) mice as internal control. C57BL/6 mice had received three or five injections of streptozotocin (STZ) or five injections of the solvent of STZ (0) as control, either alone or in addition to Zn^{2+}. Islets were isolated on Day 1 and/or Day 3 after the final injection. Of NOD mice, the mice examined and their parents received Zn^{2+} (+) or did not receive Zn^{2+} (–). The Oct-1 activity remains unchanged in all samples.

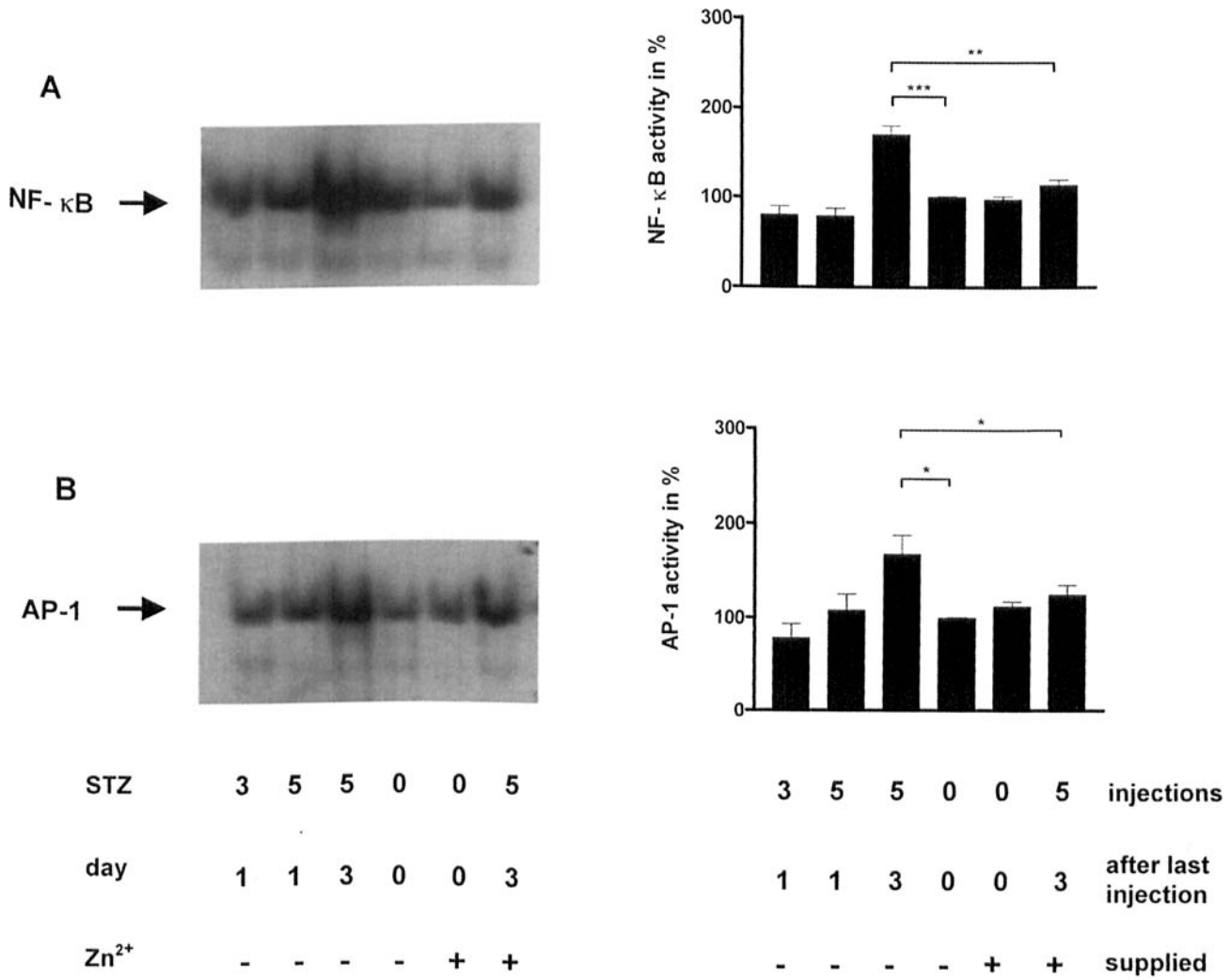

Figure 5. *Ex vivo* activity of NF-κB (A) and AP-1 (B) in islets of male C57BL/6 mice that had received three or five injections of STZ or five injections of the solvent of STZ (0) as control, either alone or in addition to Zn^{2+}. Islets were isolated on Day 1 and/or Day 3 after the final STZ injection. Multiple low doses of streptozotocin (MLD-STZ) induced a significant up-regulation of the activity of both NF-κB (*** $P < 0.001$) and AP-1 (* $P < 0.05$) that is significantly inhibited by Zn^{2+} (** $P < 0.01$ and * $P < 0.05$, respectively). Zn^{2+} alone does not affect NF-κB and AP-1 activity.

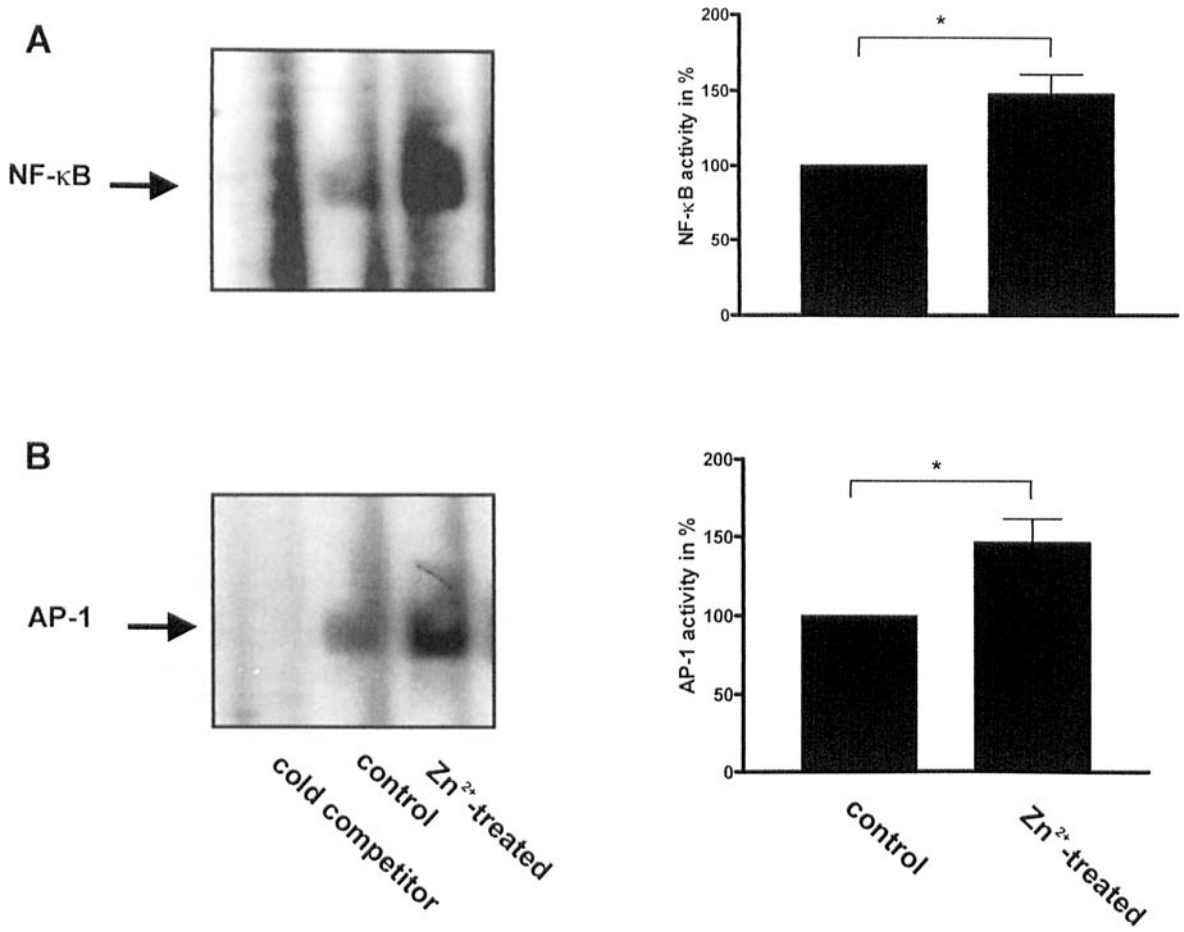

Figure 6. *Ex vivo* activity of NF-κB (A) and AP-1 (B) in islets of female nonobese diabetic (NOD) mice. The mice examined and their parents received Zn^{2+} or did not. Zn^{2+} increases both the NF-κB and AP-1 activity compared with untreated controls. The unlabeled consensus sequences completely prevented registration of signals. * $P < 0.05$ versus control.

mice, in contrast, Zn^{2+} significantly increased ($P < 0.05$) the activity of NF-κB and AP-1 (Fig. 6A and B, respectively), and the activity of Oct-1 (Fig. 4) as internal control again remained unchanged by Zn^{2+}. The specificity of NF-κB and AP-1 activity determinations was verified by cold target competition analyses in islets of C57BL/6 mice (15) and of NOD mice (Fig. 6). The unlabeled consensus sequences completely prevented registration of signals.

Discussion

The present results are first to demonstrate that Zn^{2+} prevents diabetes in female NOD mice. Zn^{2+} is most effective when given continuously to both the breeding pairs and their F_1 offsprings. Above that, Zn^{2+} reduces insulitis and induces activation of the transcription factors NF-κB and AP-1 in islets. As reported previously, in the MLD-STZ–induced model, Zn^{2+} also protects male C57BL/6 mice from diabetes (27). In contrast to NOD mice, however, Zn^{2+}, as shown here, inhibits activation of NF-κB and AP-1 by MLD-STZ in islets. The beneficial effect of Zn^{2+} on spontaneous diabetes in NOD mice corresponds to our previous findings in MLD-STZ–injected C57BL/6 and (C57BL/6 × SJL)F_1-hybrid mice (27) and in alloxan-induced diabetes (30). In concordance with our data are those of other investigators (28, 29, 31) who have reported on Zn^{2+} supplementation as an effective approach to prevent diabetes in different animal models. Presumably, Zn^{2+} protects from diabetes through upregulation of the antioxidative protein MT, which can scavenge $^{\bullet}OH$, the most toxic species of the group of ROS (27, 33, 34). In support of MT as a β-cell protective antioxidant are the results indicating resistance to STZ diabetes in male mice overexpressing MT targeted to β-cells (11). The assumption that MT may scavenge noxious $^{\bullet}OH$ is substantiated by data of our laboratory indicating that $^{\bullet}OH$ generation is stimulated by STZ in islets *in vitro* (35). On that point, we have also found an increased generation of hydrogen peroxide (H_2O_2), a precursor of $^{\bullet}OH$, in both islets incubated with STZ *in vitro* and islets of MLD-STZ-injected C57BL/6 male mice (36). Because Zn^{2+} has induced MT in islets of several mouse strains tested (27), it is likely that a comparable effect occurs in islets of NOD mice.

The finding that the protective effect of Zn^{2+} was less pronounced when given only to either the breeding pairs or

their offspring is of particular importance because it indicates that diabetes development and its prevention is already programmed *in utero*.

To analyze the complex mechanism underlying the beneficial effects of Zn^{2+}, we studied the activation of the ROS-sensitive transcription factors NF-κB and AP-1. These factors regulate genes of proinflammatory cytokines such as TNF-α and IFN-γ, which are involved in the pathogenesis of diabetes in NOD mice (2) and MLD-STZ–injected C57BL/6 mice (5, 37). In islets of male C57BL/6 mice, MLD-STZ stimulates NF-κB and AP-1 activity, which is inhibited by treatment with the anti-inflammatory cytokine rhIL-11 that protects against diabetes (15). Similarly, Zn^{2+} protects against MLD-STZ diabetes and abrogates NF-κB and AP-1 activation, as presented here. In line with these data are those of increased cell death in pancreatic islet cells and insulinoma cells due to NF-κB activation (19–22). In contrast, in NOD mice, as reported here, the diabetes-protective Zn^{2+} effect is associated with activation of NF-κB and AP-1.

Which molecular mechanisms underlie the controversial effects of Zn^{2+} on NF-κB activity in islets? We assume that in NOD mice the MT induction by Zn^{2+} is primarily specified to the nucleus and results in direct interaction with and activation of NF-κB. In concordance with this notion are data indicating (i) interaction between MT and the p50 subunit of NF-κB and (ii) activation of NF-κB in human breast cancer MCF-7 cells by MT (38). Because MT overexpression (39) and NF-κB activation (40) suppressed TNF-α–signalling death in various cell cultures, comparable effects could be exerted by Zn^{2+} in our experiments in NOD mice. It is likely that TNF-α–mediated β-cell apoptosis is abrogated by NF-κB activation, as recently demonstrated in primary pancreatic β-cells of NOD mice *in vitro* (26). Conceivably, the inverse findings of Zn^{2+}-stimulated effects on the NF-κB activity in MLD-STZ–induced and spontaneous diabetes depend on a different mode of β-cell destruction. In NOD mice, Zn^{2+} may prevent cytokine-signaled apoptosis through activation of NF-κB, whereas in C57BL/6 mice, cytokine-mediated toxicity is prevented through inhibition of MLD-STZ–induced NF-κB activation.

The supposed interruption of β-cell apoptosis in NOD mice by NF-κB activation is supported by results indicating that murine fibroblasts and macrophages deficient in the RelA subunit of NF-κB are more susceptible to TNF-α–induced apoptotic cell death than are cells from wild-type donors (41). For the Zn^{2+}-induced NF-κB activation in NOD islets, nuclear localization of MT may be essential, because this pathway is contingent with MT-mediated antiapoptotic and cytoprotective effects (42, 43).

A controversial mechanism may be initiated in C57BL/6 mice (27). Here, Zn^{2+} primarily results in upregulation of cytoplasmic MT, which may scavenge $^{\bullet}OH$ and thus prevent activation of NF-κB by MLD-STZ and, hence, diabetes development. Above that, MT may protect the inhibitor (IκB) of NF-κB from degradation and subsequent NF-κB–dependent gene activation, a pathway described *in vitro* using MT-transfected cells (44).

It is unlikely that Zn^{2+} *per se* is a β-cell–protecting trace element. Although required for insulin production and activity (45), Zn^{2+} did not affect the mRNA expression of proinsulin and insulin in mice (27) and rats (46), nor did it change blood glucose concentrations due to increased insulin release (27). Undoubtedly, Zn^{2+} controversially affects NF-κB activity: Zn^{2+} can inhibit NF-κB activation as demonstrated by us and others (47, 48), yet certain levels of Zn^{2+} are required to sustain NF-κB activity, since Zn^{2+} deficiency correlates with reduced NF-κB activity (49). Thus, in NOD mice, Zn^{2+} supplementation may increase primarily low levels in islets and NF-κB activation, with beneficial effects. Furthermore, Zn^{2+} can exert effects also on other transcription factors such as the signal transducer and activator of transcription (STAT) involved in immune cell activation (50). Besides affecting β-cells, Zn^{2+} may interact with other cell types such as islets infiltrating immune cells. Such knowledge will contribute to design schedules for monitoring treatment with Zn^{2+} in clinical settings.

In conclusion, our data suggest that NF-κB plays a critical role in immune-mediated diabetes. Zn^{2+} supplementation either activates NF-κB and may prevent apoptosis in NOD mice or inhibits NF-κB activation and prevents ROS-mediated inflammatory immune reactions in MLD-STZ–treated mice. Thus, the mode of β-cell destruction seems to be crucial for the mode of action of Zn^{2+}. Because Zn^{2+} prevented diabetes in our mouse models as well as in those of other investigators, further research may be considered to study its effect in individuals at risk for type 1 diabetes.

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