A Salen-Manganese Catalytic Free Radical Scavenger Inhibits Type 1 Diabetes and Islet Allograft Rejection

Angelica P. Olcott,[1] Georges Tocco,[2] Jide Tian,[1] Dan Zekzer,[1] Jon Fukuto,[1] Louis Ignarro,[1] and Daniel L. Kaufman[1]

Reactive oxygen species, such as superoxide, and nitrogen oxides, such as peroxynitrite, are thought to contribute to β-cell destruction during the disease process that leads to type 1 diabetes. EUK-8 is a member of a new class of synthetic salen-manganese compounds with low toxicity that possess catalytic superoxide dismutase, peroxidase, and catalase activity that can inactivate superoxide and nitrogen oxides (e.g., peroxynitrite and nitrogen dioxide). We observed that EUK-8 administration inhibited the adoptive transfer of type 1 diabetes to NOD mice. In addition, administration of EUK-8 to NOD mice with established autoimmunity completely prevented the development of type 1 diabetes for up to 1 year in age, even though the treatment was discontinued after 35 weeks of age. EUK-8 treatment also prolonged the survival of islet allografts in newly diabetic NOD mice. Thus, reactive oxygen and nitrogen species contribute to the pathoetiology of both spontaneous type 1 diabetes and allograft rejection. In cultures of NIT-1 cells, EUK-8 inhibited cytotoxicity caused by superoxide as well as nitric oxide. Collectively, our findings implicate a greater role for nitrogen oxides (other than peroxynitrite) in β-cell damage. Antioxidants designed to prevent the formation of both cytotoxic reactive oxygen and nitrogen species may effectively protect β-cells from spontaneous autoimmunity and alloresponses. *Diabetes* 53:2574–2580, 2004

The pathology of type 1 diabetes is characterized by the infiltration of the pancreatic islets by T- and B-cells and macrophages. These cells mediate β-cell destruction by the production of proinflammatory cytokines as well as reactive oxygen and nitrogen species (1–6). The latter include products derived from nitric oxide (NO), namely the reactive nitrogen species (RNS) peroxynitrite, nitrogen dioxide, and nitrite, as well as the reactive oxygen species (ROS) superoxide ion, hydrogen peroxide, and hydroxyl radical, the latter

formed by the dismutation of superoxide (4,5,7–11). In addition, islets contain high levels of the iron-containing protein ferritin (12,13), which in the context of an inflammatory response, may release free iron and lead to the generation of hydroxyl radical via a Fenton-like reaction (14,15) (Fig. 1). Islets also express low levels of antioxidant genes, which may make the β-cells particularly vulnerable to free radical–mediated cytotoxicity (7,16,17). Consistent with this notion, the expression of antioxidant proteins in β-cells enhances resistance to the cytotoxic effects of ROS and RNS (7,10,18–21), and ROS inhibition protects against cytokine-mediated islet degeneration in vitro (4).

In the NOD mouse model, previous studies have shown that the adenovirus-mediated expression of manganese superoxide dismutase (SOD) in NOD mouse islets can protect them from diabetogenic T-cells (22). Additionally, administration of a metalloporphyrin-based SOD mimic could inhibit the adoptive transfer of autoimmune diabetes by a diabetogenic T-cell clone (BDC2.5) (23). Moreover, the administration of nonspecific ROS and NOS inhibitors such as aminoguanidine, lazaroid, and probucol can prevent disease onset (2,8,24). However, the production of certain free radicals such as NO can also be beneficial. For example, NO protects against ROS-mediated toxicity (25), and the inhibition of NO synthase exacerbates experimental autoimmune encephalomyelitis (EAE) (26) Therefore, rather than inhibiting NO formation, intervention strategies may be best directed at the prevention of highly reactive NO-derived species.

EUK-8 is one of a new class of synthetic salen-manganese compounds with SOD, peroxidase, and catalase activities (27) (Fig. 1). These enzymatic activities give these compounds the ability to inactivate superoxide and its dismutation product hydrogen peroxide, thereby preventing hydroxyl radical formation. They also catalyze the breakdown of numerous NO products to more benign species, limiting the formation of the nitrogen oxides, peroxynitrite, nitrogen dioxide, and nitrite (28). These salen-manganese compounds have been shown to inhibit EAE (29) and skin allograft rejection (30). Moreover, EUK-8 has very low toxicity in vivo (27,31,32).

Here, we show that a synthetic salen-manganese compound (EUK-8) can completely inhibit disease progression in NOD mice with established autoimmunity and can prolong islet allograft survival in diabetic NOD mice. To obtain insight into the protective mechanism(s) of EUK-8 treatment, we performed in vitro studies in which NIT cells were exposed to NO donor compounds that generate
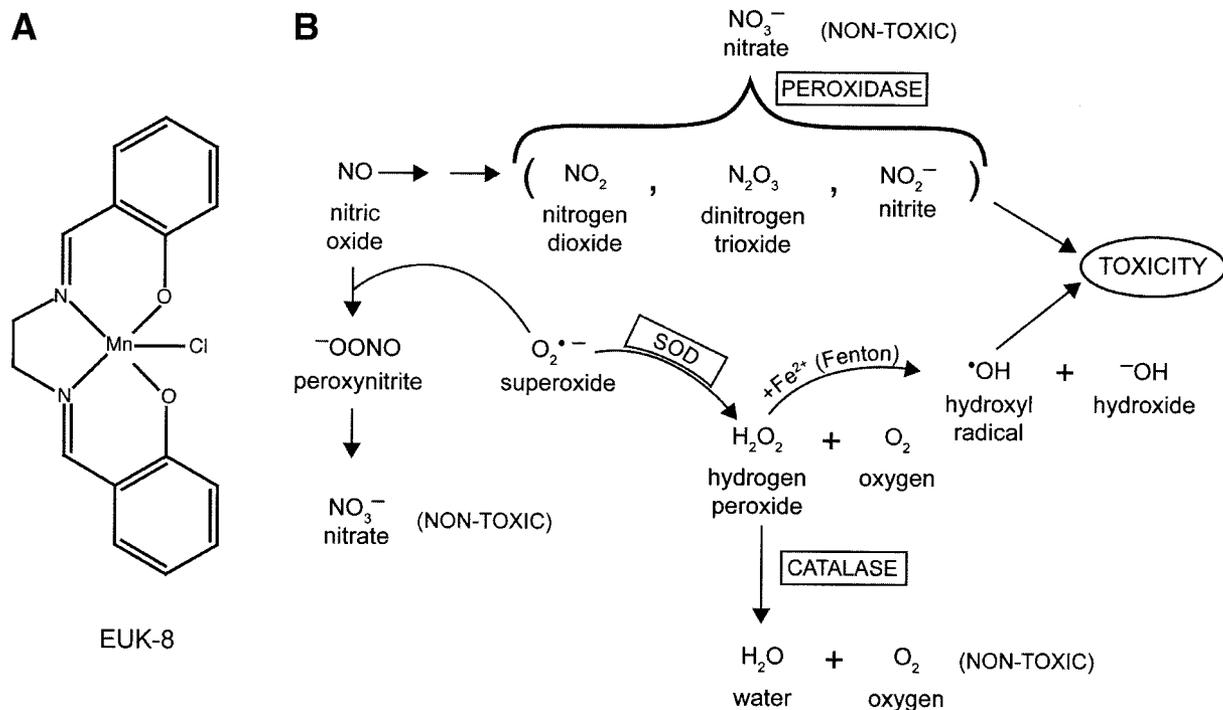
FIG. 1. *A*: Structure of EUK-8. *B*: Potential pathways of superoxide and NO-mediated cytotoxicity. The SOD, peroxidase, and catalase activities of EUK-8 (boxed) act to prevent or inactivate potential cytotoxic species.

different combinations of the free-radical species thought to contribute to β-cell damage. Our results suggest that RNS (other than peroxynitrite) play a more significant role in β-cell destruction than previously appreciated. Accordingly, antioxidants that inhibit the formation of both cytotoxic reactive oxygen and nitrogen species may more effectively protect β-cells from spontaneous autoimmunity and alloresponses.

RESEARCH DESIGN AND METHODS

Reagents. The salen-manganese compound EUK-8 was a gift from Eukarion (Bedford, MA). EUK-8 was dissolved in autoclaved water at 30 mg/ml, filtered, and stored at room temperature for up to 2 months. For in vivo studies, EUK-8 was mixed with sterile PBS to the indicated concentration (5–100 mg/kg). For in vitro studies, EUK-8 was initially dissolved as a $20\times$ stock in PBS and then diluted to a final concentration of 80 μmol/l in media for the NIT cell viability assays.

3-Morpholinosydnonimine-*N*-ethylcarbamide (SIN-1) was purchased from Cayman Chemical (Ann Arbor, MI). SIN-1 is an NO donor that rapidly releases superoxide anion and hydrogen peroxide concomitantly with NO when dissolved in aqueous solutions. This primarily leads to peroxynitrite formation but can also produce some nitrate and nitrite as well (33–35). DETA-NONOate [(Z)-1-[2-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate (Alexis, San Diego, CA)] is an NO donor with a half-life of NO release that is comparable with that of SIN-1 (36,37), but it does not produce peroxynitrite. SIN-1 or DETA-NONOate was dissolved directly in culture medium (Click's media containing 10% fetal bovine serum, 2 mmol/l L-glutamine, and 2 mmol/l penicillin-streptomycin) immediately before treatment of cells for the in vitro assays.

Mice. Female NOD mice (Taconic Farms) and BALB/c mice (The Jackson Laboratories) were bred and housed under specific pathogen-free conditions. Food and water were provided ad libitum, and the animals were housed under 12-h light and dark cycles. The UCLA Animal Research Committee approved all animal care and experimental procedures.

Diabetes assessment. NOD mice were screened for the onset of hyperglycemia by monitoring urine glucose levels by Tes-tape (Lilly). NOD mice were tested every other day after reaching 12 weeks in age or after receiving an infusion of diabetogenic T-cells. After detection of abnormal glucose in the urine, blood glucose concentration was monitored daily by measurement of glucose with a Glucometer Elite (Bayer, Pittsburgh, PA). Two consecutive

blood glucose levels of >13 mmol/l were considered as type 1 diabetes onset. NOD mice that received islet allografts were monitored daily for blood glucose levels. Diabetes incidence or recurrence data were analyzed by SAS statistical software (SAS, Cary, NC) using the Kaplan-Meier method to generate survival curves, followed by log-rank test comparisons to generate the P value.

Adoptive transfer of type 1 diabetes. Female NOD mice (6–8 weeks old) were irradiated (500 rad) and 24 h later received 10 million splenic mononuclear cells intravenously from newly diabetic female NOD mice. The recipients were randomly separated into experimental and control groups ($n = 10$ per group). One group was treated daily with 5 mg/kg EUK-8 in 0.1 ml PBS i.p., whereas the other received PBS alone. The EUK-8 dose was based on previous results from EAE studies (29). Diabetes incidence was monitored as described above.

Effect of EUK-8 treatment on spontaneous type 1 diabetes in NOD mice. To test the ability of EUK-8 to prevent spontaneous TID in NOD mice, female NOD mice were treated from 6 to 35 weeks of age with 100 mg/kg of EUK-8 given in 0.5 ml PBS i.p. every other day. Age-matched control female NOD mice received 0.5 ml PBS every other day. Their blood glucose levels were monitored for up to 52 weeks in age as described above.

Islet allograft studies. Islets were isolated from pancreata of 6- to 8-week-old female BALB/c mice by digestion with collagenase P (Sigma) at 37°C in Dulbecco's modified Eagle's media (10% FCS, 2 mmol/l L-glutamine, and 2 mmol/l penicillin-streptomycin) as previously described (38). After digestion, islets were rinsed three times in chilled media (Click's media with 10% FCS, 2 mmol/l L-glutamine, and 2 mmol/l penicillin-streptomycin) and maintained in chilled media during isolation by hand picking using a dissecting scope. Approximately 500 islets were implanted under the kidney capsule of newly diabetic female NOD mice. After islet transplantation, recipient mice were treated daily with PBS or 100 mg/kg EUK-8 in intraperitoneal PBS, and their blood glucose was monitored daily.

In vitro toxicity assays. NIT-1 cells (ATCC) were plated at $2–5 \times 10^5$ cells/well in 4 ml incubation media (Click's media with 10% FCS, 2 mmol/l L-glutamine, and 2 mmol/l penicillin-streptomycin). Cultures were exposed to dose ranges of SIN-1 or DETA-NONOate. Some of the cultures were pretreated with EUK-8 (80 μmol/l) for 2 h before addition of SIN-1 or DETA-NONOate. Control cultures were incubated in media alone. Twelve hours after SIN-1 or DETA-NONOate addition, the NIT cells were harvested and stained with trypan blue, and their viability was determined ($n = 4–6$ wells/group). The experiments were repeated twice. The results are expressed as the average percentage of viable cells in each treatment group. Statistical analyses were performed using pairwise t tests.
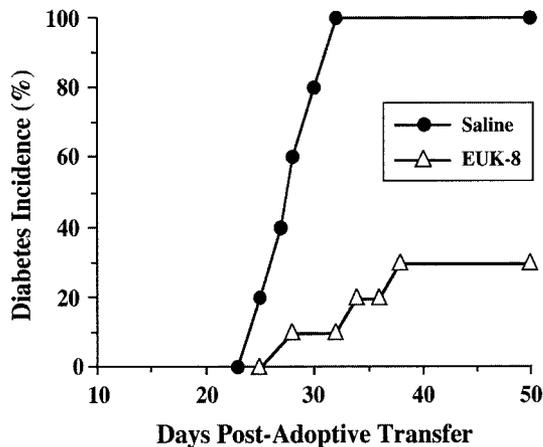
FIG. 2. EUK-8 treatment inhibits the adoptive transfer of type 1 diabetes. After the adoptive transfer of splenic T-cells from newly diabetic NOD mice, the recipients were treated with EUK-8 or saline daily (as described in RESEARCH DESIGN AND METHODS). EUK-8 treatment inhibited adopted transfer of type 1 diabetes ($P < 0.0001$). Data are from two separate experiments ($n = 10$ for each group).

RESULTS

Salen-manganese treatment can prevent the adoptive transfer of type 1 diabetes. A number of new salen-manganese compounds have been generated, of which EUK-8 is a promising therapeutic antioxidant based on its strong SOD, peroxidase, and catalase activities (27,31,39) (Fig. 1). To begin to evaluate whether EUK-8 may have therapeutic potential to protect β-cells from autoreactivity, we first tested its ability to inhibit the adoptive transfer of type 1 diabetes. The 6- to 8-week-old irradiated female NOD mice were transfused with 10 million splenic mono-nuclear cells from newly diabetic NOD mice. Recipient mice were treated daily with EUK-8 or PBS alone. All of the mice that were treated with PBS alone became diabetic within 32 days (Fig. 2). In contrast, only 10% of the EUK-8–treated mice were diabetic at this time. Over the 50-day observation period, 30% of the EUK-8–treated mice became diabetic, a significant protective effect compared with the PBS-treated group (survival analysis, $P < 0.0001$). Thus, EUK-8 treatment can significantly inhibit a highly diabetogenic immune cell population. Accordingly, inactivation of ROS, such as superoxide and hydroxyl radical, as well as prevention of nitrogen oxide products, are important components of therapeutic strategies aimed at protecting β-cells from immune responses.

Salen-manganese treatment completely prevents the spontaneous development of type 1 diabetes in NOD mice with established β-cell autoimmunity. We next examined the ability of EUK-8 treatment to inhibit the progression of spontaneous disease in NOD mice. We began treating female NOD mice at 6 weeks in age, when T-cell autoreactivity and insulitis are already well established. The mice received EUK-8 or PBS alone, every other day, from 6 to 35 weeks in age, and their blood glucose was monitored for up to 1 year in age. All of the mice that received EUK-8 thrived throughout the lengthy treatment period and displayed no loss in body weight. In the PBS treatment group, 66% of the mice became diabetic by 25 weeks in age, and 80% were diabetic within the 1-year observation period (Fig. 3). In contrast, none of the EUK-8–treated mice became hyperglycemic, even up to 1
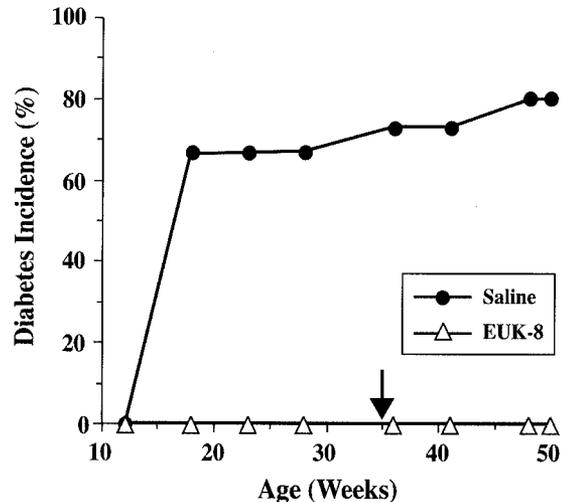

FIG. 3. Administration of EUK-8 completely inhibits spontaneous autoimmunity. Female NOD mice treated with either EUK-8 or saline starting at 6 weeks in age, every other day, until 35 weeks in age (arrow indicates when treatment ceased). The mice were monitored for the spontaneous development of type 1 diabetes. All EUK-8–treated mice remained normoglycemic for up to 1 year in age ($P < 0.0001$) ($n = 15$ mice per group.

year in age ($P < 0.0001$). Thus, even though EUK-8 treatment was discontinued at 35 weeks in age, the entire group of mice remained euglycemic until 52 weeks in age, at which point the study was terminated.

Salen-manganese treatment delays recurrent diabetes in islet allograft recipients. We next examined whether EUK-8 administration could promote the survival of islet allografts in newly diabetic NOD mice. A total of 500 islets isolated from BALB/c mice were implanted under the kidney capsule of newly diabetic female NOD mice. The allograft recipients were treated daily with EUK-8 or PBS. Islet implantation restored euglycemia in all recipients. However, hyperglycemia recurred in all of the control PBS treated mice within 7–10 days after alloislet transplantation, as is expected in immunocompetent mice (Fig. 4). In contrast, all of the EUK-8–treated allograft recipients remained euglycemic until 10 days after transplantation. Thereafter, hyperglycemia recurred, but at a slower rate, with three of six mice remaining euglycemic at 20 days after transplantation and one of six of the islet allograft recipients remaining disease free at 50 days after transplantation ($P < 0.0011$ by survival analysis). These data suggest that ROS, such as superoxide and hydroxyl radical, and RNS, such as peroxynitrite and nitrite, contribute to the islet graft rejection process.

EUK-8 inhibits superoxide and NO-mediated cytotoxicity during formation of nitrogen oxides. The ability of EUK-8 to prevent spontaneous type 1 diabetes and inhibit islet allograft rejection suggests that the production of ROS and RNS contributes to β-cell death in these models. Conceivably, the reactivity of NO, superoxide, hydroxyl radical, and/or NO/superoxide-derived products may contribute to β-cell cytotoxicity. To further explore the mechanisms of β-cell injury and the protective mechanism(s) of EUK-8, we used an in vitro system in which we could modulate the putative free radicals that were present.

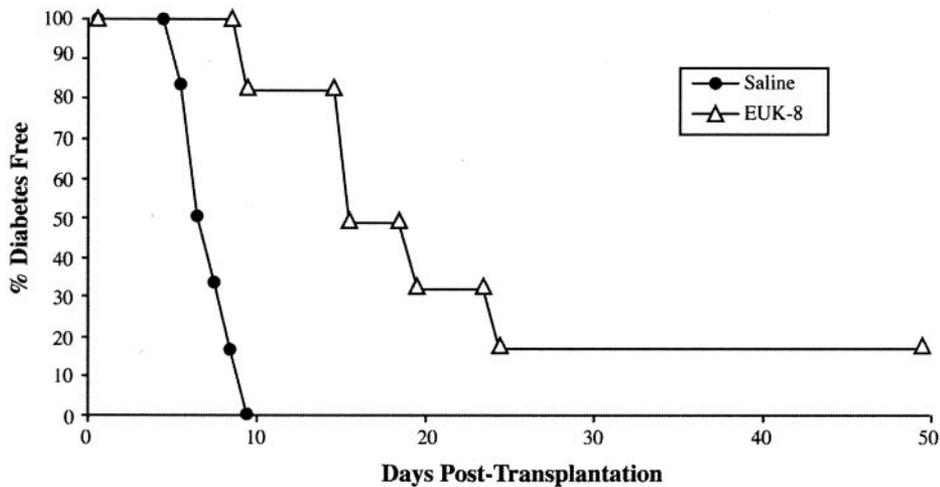The insulinoma cell line NIT-1 was exposed to SIN-1, an

FIG. 4. EUK-8 treatment delays diabetes recurrence in islet allograft recipients. Newly diabetic NOD mice received 500 islets from BALB/c mice under their kidney capsule and were treated with either EUK-8 daily or an equal volume of saline. Mice that did not have restored normoglycemia after implantation were not included in the study. All saline-treated recipients redeveloped hyperglycemia within 8–10 days after transplantation. Treatment with EUK-8 significantly prolonged islet allograft survival ($P < 0.0011$). $n = 6$ mice per group.

NO donor that rapidly releases superoxide anion concomitantly with NO in aqueous solutions, producing peroxynitrite (predominantly) as well as nitrite and nitrate (33–35). We also added EUK-8 (80 μmol/l) to some of the cultures to inactivate superoxide and hydrogen peroxide and thereby prevent, or catalyze the breakdown of, cytotoxic NO products. Twelve hours after adding SIN-1 (0, 1, or 3 mmol/l), we determined cell viability by trypan blue exclusion. Cells grown in media alone or that were treated with EUK-8 had similar viability (82 and 76% viability, respectively; Fig. 5). Addition of SIN-1 to 1 mmol/l reduced cell viability to 63%. However, inclusion of EUK-8 with SIN-1 protected NIT cells, such that NIT viability was similar to that of NIT cells cultured in media alone (78% viability). When SIN-1 was added to 3 mmol/l, cell viability in cultures without EUK-8 was 47%, whereas in cultures with EUK-8, 64% of the cells remained viable ($P < 0.0001$). These results indicate that NIT cells are sensitive to ROS

and/or RNS generated by SIN-1 and support the hypothesis that production of NO, superoxide, and their derived products may lead to β-cell death.

To further evaluate the contribution of NO versus superoxide to cytotoxicity in our model, we cultured NIT cells with DETA-NONOate, an agent that only generates NO (at a rate similar to that of SIN-1). We observed that increasing dosages of DETA-NONOate (0.01–0.1 mmol/l) induced progressively greater NIT cell death, but that addition of EUK-8 (80 μmol/l) effectively inhibited the cytotoxicity of DETA-NONOate (Fig. 6). For example, in cultures exposed to 1 mmol/l DETA-NONOate, only 12% of cultured NIT cells were viable. However, inclusion of EUK-8 with 1 mmol/l DETA-NONOate led to 67% cell viability. Therefore, in the absence of superoxide, NO production alone can lead to cytotoxicity, which can be prevented by a salen-manganese compound.
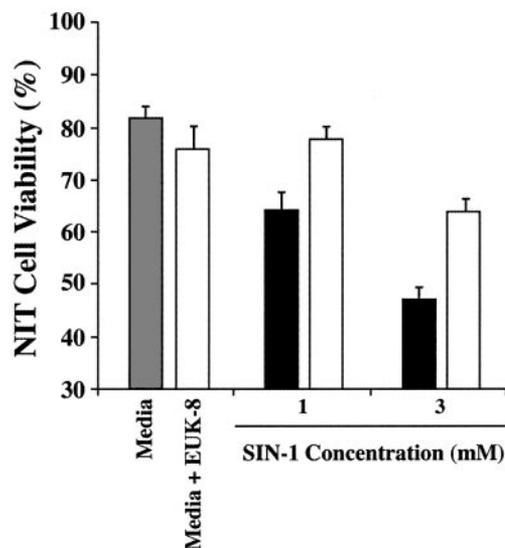
DISCUSSION

The formation of ROS and RNS has been implicated in the pathoetiology of type 1 diabetes. Accordingly, limitation of deleterious ROS and RNS products may provide new therapeutic strategies to mitigate β-cell destruction. Previous studies showed that adenovirus-mediated expression of manganese SOD in islets could protect them from diabetogenic T-cells after implantation in syngeneic mice (22). Recently, a metalloporphyrin-based SOD mimic was shown to inhibit the adoptive transfer of autoimmune diabetes by a diabetogenic T-cell clone (BDC2.5) (23). However, these studies did not examine the compound's ability to prevent spontaneous type 1 diabetes or prolong islet graft survival. Here, we used a low–molecular weight synthetic salen-manganese compound that has SOD, peroxidase, and catalase activity and tested its ability to inhibit both spontaneous type 1 diabetes and islet allograft rejection in diabetic NOD mice. The enzymatic activities of this compound may make it well suited to protect islets from immune-mediated damage, because it inactivates superoxide and hydrogen peroxide and prevents the formation of a highly cytotoxic hydroxyl radical. This compound also catalyzes the breakdown of numerous NO-derived products, including cytotoxic nitrogen oxides.

Our initial studies demonstrated that EUK-8 treatment could inhibit the adoptive transfer of disease by diabeto-

FIG. 5. EUK-8 treatment can inhibit superoxide and/or NO-mediated cytotoxicity in NIT cells. NIT cells were cultured in media alone (▦) or media plus different concentrations of SIN-1 in the presence (□) or absence (■) of EUK-8 (80 μmol/l) (as described in RESEARCH DESIGN AND METHODS). Twelve hours after SIN-1 addition, cell viability was measured by cellular exclusion of trypan blue dye. EUK-8 pretreatment inhibited SIN-1–associated cytotoxicity at both 1 and 3 mmol/l ($P < 0.0001$). Data show the means ± SD of four samples. The data were analyzed by ANOVA pairwise comparisons.
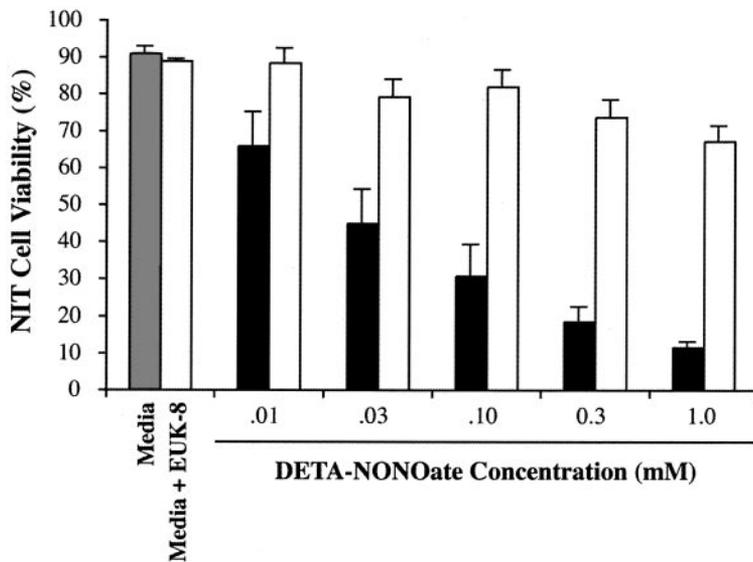
FIG. 6. EUK-8 treatment can inhibit NO-mediated cytotoxicity in NIT cells. NIT cells were cultured in media alone (▨) or media plus different concentrations of DETA-NONOate in the presence (□) or absence (■) of EUK-8 (80 μmol/l). Twelve hours after DETA-NONOate addition, cell viability was measured by cellular exclusion of trypan blue dye. EUK-8 pretreatment inhibited DETA-NONOate–associated cytotoxicity. Data show the means ± SD of six samples. $P = 0.0003$ for 0.01 mmol/l dose; $P < 0.0001$ for 0.1 and 1.0 mmol/l doses of DETA-NONOate compared with cultures without EUK-8 at each dose.

genic spleen cells from newly diabetic mice. This finding supports the hypothesis that at late stages of the disease process, effector cells producing ROS and/or RNS play a major role in β-cell destruction. We went on to show that when given to pre-diabetic NOD mice with established autoimmunity, EUK-8 completely inhibited the spontaneous development of type 1 diabetes for up to 52 weeks in age, even though treatment was discontinued after the mice reached 35 weeks in age. Histological analysis of pancreata taken from 52-week-old EUK-8–treated NOD mice showed most islets to be free of infiltrates, although some had a slight insulitis (data not shown). Because EUK-8 treatment was initiated at 6 weeks in age, some insulitis is expected. It is unclear whether the slight insulitis at 52 weeks of age (17 weeks after ceasing EUK-8 treatment) represents a long-term benign insulitis or the beginning of a potentially pathogenic response.

We also found that salen-manganese treatment significantly delayed the recurrence of diabetes in newly diabetic NOD mice that received an islet allograft. The observation that salen-manganese treatment completely inhibited spontaneous disease progression in pre-diabetic NOD mice with established β-cell autoimmunity and delayed recurrent diabetes in islet allograft recipients suggests that ROS and RNS contribute to the pathogenesis of spontaneous type 1 diabetes and allograft rejection. Because EUK-8 has very low toxicity in vivo (27,31,32), it will be of interest to further investigate the potential of such salen-manganese treatments to inhibit type 1 diabetes and prolong islet allograft survival.

Nitrogen oxide formation is considered to contribute to cytotoxicity during type 1 diabetes pathogenesis because of NO and/or superoxide release. To model this process in vitro, we exposed insulinoma cells to two different NO donors (SIN-1 and DETA-NONOate), which have equivalent rates of NO release but vary in the proportions of their NO products. SIN-1 releases superoxide anion concomitantly with NO in aqueous solutions (40) and provides two potential pathways of cytotoxicity. The first is a coordinated release of superoxide with NO, leading to the formation of peroxynitrite predominantly, as well as some other nitrogen oxides. The second involves superoxide

releasing iron from ferritin (which is known to be present at high levels in islets [12,13]), and concomitant dismutation of superoxide to generate hydrogen peroxide. The combined presence of hydrogen peroxide and iron in the ferrous state can then yield cytotoxic hydroxyl radicals by the Fenton reaction (14,15,41) (Fig. 1). Using cultured NIT cells, we found that SIN-1 mediated NIT cell death in a dose-dependent manner and that a micromolar concentration of EUK-8 could inhibit this cytotoxicity. DETA-NONOate, which only releases NO with no superoxide, had a higher cytotoxicity profile than that of SIN-1, whereas the median lethal dose for SIN-1 was ~3 mmol/l, and the median lethal dose for DETA-NONOate was only ~0.03 mmol/l. The cytotoxicity of DETA-NONOate was also inhibited by EUK-8. Collectively, these data indicate that NO production with or without the presence of superoxide is cytotoxic, either through nitrogen oxide formation or via the generation of hydroxyl radical.

The ability of EUK-8 to inhibit SIN-1– and DETA-NONOate–mediated cytotoxicity helps clarify previous conflicting evidence regarding the contribution of the joint products of superoxide and NO, namely peroxynitrite, to islet destruction. Previous studies have pointed to peroxynitrite as a primary mediator of β-cell cytotoxicity based on its ability to form hydroxyl radical, the formation of nitrotyrosine in NOD mouse islets, and the ability of pharmacological agents that block peroxynitrite formation to partially inhibit disease in NOD mice (5,8,42–45). Peroxynitrite can also give rise to the cytotoxic nitrogen dioxide. However, other lines of evidence suggest that peroxynitrite may not be particularly cytotoxic to β-cells. Peroxynitrite can readily degrade spontaneously to nitrate (which is innocuous), and, accordingly, its formation may not yield substantial cytotoxicity (46). Moreover, studies of peroxynitrite cytotoxicity have used supraphysiological concentrations in vitro, far exceeding the levels of peroxynitrite that are generated locally by inflammatory responses in vivo (6,47). Finally, nitrotyrosine may be formed by other nitrogen oxides besides peroxynitrite, such as nitrogen dioxide (48,49). In our studies, both DETA-NONOate and SIN-1 produce NO at similar rates (36,37), but SIN-1 also generates superoxide, leading to

peroxynitrite formation. Our finding that DETA-NONOate was more cytotoxic than SIN-1 suggests that NO and its derived products (such as nitrogen dioxide, dinitrogen trioxide, and nitrite) are more cytotoxic than the release of superoxide and NO, leading to their peroxynitrite derivative (as well as its NO-derived products). Indeed, the formation of peroxynitrite may provide a detoxification mechanism, because its rapid spontaneous degradation to the innocuous nitrate limits the production of potentially more cytotoxic nitrogen oxides from NO (Fig. 1). This latter category of nitrogen oxides (e.g., nitrogen dioxide, dinitrogen trioxide, and nitrite) cannot spontaneously degrade to nitrate and thus are available to nitrate tyrosine residues in proteins (50).

Salen-manganese compounds (such as EUK-8) are able to convert nitrogen oxides (such as nitrogen dioxide, dinitrogen trioxide, and nitrite) to nitrate during NO "detoxification," presumably by its peroxidase activity (28). Our finding that nitrogen oxides can be a major contributor to NIT cell toxicity may explain the ability of EUK-8 to inhibit the cytotoxicity of NO donors in vitro, as well as its therapeutic efficacy in preventing spontaneous type 1 diabetes and prolonging allograft survival in vivo.

In summary, we have shown that treatment with a salen-manganese antioxidant, even after the onset of autoimmunity, completely prevented spontaneous type 1 diabetes and delayed islet allograft rejection in newly diabetic NOD mice. Our study also broadens our understanding of the cytotoxicity of reaction products resulting from NO and/or superoxide formation in β-cell destruction. Specifically, the superoxide product hydroxyl radical, as well as its specific reaction products with NO, appears to contribute to cytotoxicity in our models. The SOD, peroxidase, and catalase activity of EUK-8 to prevent both hydroxyl radical formation and inactivate nitrogen oxides may account for its therapeutic efficacy. Salen-manganese compounds have been shown to inhibit EAE, skin allograft rejection, and neuronal damage in neuropathological conditions (27,29,30). This new class of antioxidants may provide a potent and safe pharmacological approach to inhibit spontaneous type 1 diabetes and aid in prolonging survival of islet allografts.

ACKNOWLEDGMENTS

REFERENCES

1. Rabinovitch A: Free radicals as mediators of pancreatic islet beta-cell injury in autoimmune diabetes. *J Lab Clin Med* 119:455–456, 1992
2. Rabinovitch A, Suarez WL, Power RF: Lazaroid antioxidant reduces incidence of diabetes and insulitis in nonobese diabetic mice. *J Lab Clin Med* 121:603–607, 1993
3. Corbett JA, Mikhael A, Shimizu J, Frederick K, Misko TP, McDaniel ML, Kanagawa O, Unanue ER: Nitric oxide production in islets from nonobese diabetic mice: aminoguanidine-sensitive and -resistant stages in the immunological diabetic process. *Proc Natl Acad Sci U S A* 90:8992–8995, 1993
4. Corbett JA, Wang JL, Misko TP, Zhao W, Hickey WF, McDaniel ML: Nitric oxide mediates IL-1 beta-induced islet dysfunction and destruction: prevention by dexamethasone. *Autoimmunity* 15:145–153, 1993
5. Suarez-Pinzon WL, Szabo C, Rabinovitch A: Development of autoimmune diabetes in NOD mice is associated with the formation of peroxynitrite in pancreatic islet β-cells. *Diabetes* 46:907–911, 1997
6. 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
7. 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
8. 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
9. Grankvist K, Marklund S, Taljedal IB: Superoxide dismutase is a prophylactic against alloxan diabetes. *Nature* 294:158–160, 1981
10. 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
11. Thomas PD, Mao GD, Rabinovitch A, Poznansky MJ: Inhibition of superoxide-generating NADPH oxidase of human neutrophils by lazaroids (21-aminosteroids and 2-methylaminochromans). *Biochem Pharmacol* 45:241–251, 1993
12. MacDonald MJ, Cook JD, Epstein ML, Flowers CH: Large amount of (apo)ferritin in the pancreatic insulin cell and its stimulation by glucose. *FASEB J* 8:777–781, 1994
13. Iancu TC, Ward RJ, Peters TJ: Ultrastructural changes in the pancreas of carbonyl iron-fed rats. *J Pediatr Gastroenterol Nutr* 10:95–101, 1990
14. Wink DA, Nims RW, Saavedra JE, Utermahlen WE Jr, Ford PC: The Fenton oxidation mechanism: reactivities of biologically relevant substrates with two oxidizing intermediates differ from those predicted for the hydroxyl radical. *Proc Natl Acad Sci U S A* 91:6604–6608, 1994
15. Ignarro LJ, Fukuto JM, Griscavage JM, Rogers NE, Byrns RE: Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proc Natl Acad Sci U S A* 90:8103–8107, 1993
16. Grankvist K, Marklund SL, Taljedal IB: CuZn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase in pancreatic islets and other tissues in the mouse. *Biochem J* 199:393–398, 1981
17. 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
18. Kubisch HM, Wang J, Luche R, Carlson E, Bray TM, Epstein CJ, Phillips JP: Transgenic copper/zinc superoxide dismutase modulates susceptibility to type I diabetes. *Proc Natl Acad Sci U S A* 91:9956–9959, 1994
19. Kubisch HM, Wang J, Bray TM, Phillips JP: Targeted overexpression of Cu/Zn superoxide dismutase protects pancreatic β-cells against oxidative stress. *Diabetes* 46:1563–1566, 1997
20. Benhamou PY, Moriscot C, Richard MJ, Beatrix O, Badet L, Pattou F, Kerr-Conte J, Chrobocek J, Lemarchand P, Halimi S: Adenovirus-mediated catalase gene transfer reduces oxidant stress in human, porcine and rat pancreatic islets. *Diabetologia* 41:1093–1100, 1998
21. Hohmeier HE, Thigpen A, Tran VV, Davis R, Newgard CB: Stable expression of manganese superoxide dismutase (MnSOD) in insulinoma cells prevents IL-1beta-induced cytotoxicity and reduces nitric oxide production. *J Clin Invest* 101:1811–1820, 1998
22. Bertera S, Crawford ML, Alexander AM, Papworth GD, Watkins SC, Robbins PD, Trucco M: Gene transfer of manganese superoxide dismutase extends islet graft function in a mouse model of autoimmune diabetes. *Diabetes* 52:387–393, 2003
23. Piganelli JD, Flores SC, Cruz C, Koepp J, Batinic-Haberle I, Crapo J, Day B, Kachadourian R, Young R, Bradley B, Haskins K: A metalloporphyrin-based superoxide dismutase mimic inhibits adoptive transfer of autoimmune diabetes by a diabetogenic T-cell clone. *Diabetes* 51:347–355, 2002
24. Rabinovitch A, Suarez WL, Power RF: Combination therapy with an antioxidant and a corticosteroid prevents autoimmune diabetes in NOD mice. *Life Sci* 51:1937–1943, 1992
25. Wink DA, Hanbauer I, Krishna MC, DeGraff W, Gamson J, Mitchell JB: Nitric oxide protects against cellular damage and cytotoxicity from reactive oxygen species. *Proc Natl Acad Sci U S A* 90:9813–9817, 1993
26. O'Brien NC, Charlton B, Cowden WB, Willenborg DO: Inhibition of nitric oxide synthase initiates relapsing remitting experimental autoimmune

encephalomyelitis in rats, yet nitric oxide appears to be essential for clinical expression of disease. *J Immunol* 167:5904–5912, 2001

27. Doctrow SR, Huffman K, Marcus CB, Tocco G, Malfroy E, Adinolfi CA, Kruk H, Baker K, Lazarowych N, Mascarenhas J, Malfroy B: Salen-manganese complexes as catalytic scavengers of hydrogen peroxide and cytoprotective agents: structure-activity relationship studies. *J Med Chem* 45:4549–4558, 2002

28. Sharpe MA, Ollosson R, Stewart VC, Clark JB: Oxidation of nitric oxide by oxomanganese-salen complexes: a new mechanism for cellular protection by superoxide dismutase/catalase mimetics. *Biochem J* 366:97–107, 2002

29. Malfroy B, Doctrow SR, Orr PL, Tocco G, Fedoseyeva EV, Benichou G: Prevention and suppression of autoimmune encephalomyelitis by EUK-8, a synthetic catalytic scavenger of oxygen-reactive metabolites. *Cell Immunol* 177:62–68, 1997

30. Tocco G, Illigens B, Iwamoto Y, Akiyama Y, Benichou GA: Prolongation of skin and heart allograft survival by synthetic catalytic scavengers of reactive oxygen species. *Hum Immunol* 64:S51, 2003

31. Gonzalez PK, Zhuang J, Doctrow SR, Malfroy B, Benson PF, Menconi MJ, Fink MP: EUK-8, a synthetic superoxide dismutase and catalase mimetic, ameliorates acute lung injury in endotoxemic swine. *J Pharmacol Exp Ther* 275:798–806, 1995

32. Doctrow SR, Huffman K, Marcus CB, Musleh W, Bruce A, Baudry M, Malfroy B: Salen-manganese complexes: combined superoxide dismutase/catalase mimics with broad pharmacological efficacy. *Adv Pharmacol* 38:247–269, 1997

33. Feelisch M, Ostrowski J, Noack E: On the mechanism of NO release from sydnonimines. *J Cardiovasc Pharmacol* 14:S13–S22, 1989

34. Hogg N, Darley-Usmar VM, Wilson MT, Moncada S: Production of hydroxyl radicals from the simultaneous generation of superoxide and nitric oxide. *Biochem J* 281:419–424, 1992

35. Ioannidis I, de Groot H: Cytotoxicity of nitric oxide in Fu5 rat hepatoma cells: evidence for co-operative action with hydrogen peroxide. *Biochem J* 296:341–345, 1993

36. Mooradian DL, Hutsell TC, Keefer LK: Nitric oxide (NO) donor molecules: effect of NO release rate on vascular smooth muscle cell proliferation in vitro. *J Cardiovasc Pharmacol* 25:674–678, 1995

37. Kurozumi R, Tokuzumi S, Kojima S: Does peroxynitrite involve in the elevation of cellular glutathione induced by sodium nitroprusside (SNP) in RAW 264.7 cells? *Biol Pharm Bull* 26:1070–1075, 2003

38. Tian J, Clare-Salzler M, Herschenfeld A, Middleton B, Newman D, Mueller R, Arita S, Evans C, Atkinson MA, Mullen Y, Sarvetnick N, Tobin AJ, Lehmann PV, Kaufman DL: Modulating autoimmune responses to GAD inhibits disease progression and prolongs islet graft survival in diabetes-prone mice. *Nat Med* 2:1348–1353, 1996

39. Baudry M, Etienne S, Bruce A, Palucki M, Jacobsen E, Malfroy B: Salen-manganese complexes are superoxide dismutase-mimics. *Biochem Biophys Res Commun* 192:964–968, 1993

40. Cunningham JM, Green IC: Cytokines, nitric oxide and insulin secreting cells. *Growth Regul* 4:173–180, 1994

41. Wink DA, Mitchell JB: Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. *Free Radic Biol Med* 25:434–456, 1998

42. van der Veen RC, Hinton DR, Incardonna F, Hofman FM: Extensive peroxynitrite activity during progressive stages of central nervous system inflammation. *J Neuroimmunol* 77:1–7, 1997

43. Beckman JS, Beckman TW, Chen J, Marshall PA, Freeman BA: Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* 87:1620–1624, 1990

44. Beckman JS, Chen J, Ischiropoulos H, Crow JP: Oxidative chemistry of peroxynitrite. *Methods Enzymol* 233:229–240, 1994

45. Delaney CA, Tyrberg B, Bouwens L, Vaghef H, Hellman B, Eizirik DL: Sensitivity of human pancreatic islets to peroxynitrite-induced cell dysfunction and death. *FEBS Lett* 394:300–306, 1996

46. Fukuto JM, Ignarro L: In vivo aspects of nitric oxide (NO) chemistry: does peroxynitrite ($-$OONO) play a major role in cytotoxicity? *Accounts of Chemical Research* 30:149–152, 1997

47. Virag L, Szabo E, Gergely P, Szabo C: Peroxynitrite-induced cytotoxicity: mechanism and opportunities for intervention. *Toxicol Lett* 140–141:113–124, 2003

48. Wink DA, Miranda KM, Espey MG: Cytotoxicity related to oxidative and nitrosative stress by nitric oxide. *Exp Biol Med (Maywood)* 226:621–623, 2001

49. Kroncke KD: Cysteine-Zn2+ complexes: unique molecular switches for inducible nitric oxide synthase-derived NO. *FASEB J* 15:2503–2507, 2001

50. Halliwell B: What nitrates tyrosine? Is nitrotyrosine specific as a biomarker of peroxynitrite formation in vivo? *FEBS Lett* 411:157–160, 1997