

# The Combined Inducible Nitric Oxide Synthase Inhibitor and Free Radical Scavenger Guanidinoethylsulfide Prevents Multiple Low-Dose Streptozotocin-Induced Diabetes *In Vivo* and Interleukin-1 $\beta$ -Induced Suppression of Islet Insulin Secretion *In Vitro*

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**Abstract:** Inhibition of inducible nitric oxide synthase has been shown to be antiinflammatory in a variety of disease states. Type I diabetes is an autoimmune disease resulting from the specific destruction of the insulin-producing pancreatic  $\beta$  cells. Here we demonstrate that guanidinoethylsulfide (GED), a combined inducible nitric oxide synthase inhibitor and peroxynitrite/reactive oxygen species scavenger reduces the hyperglycemia and incidence of type I diabetes induced in mice by multiple low-dose streptozotocin treatment. GED treatment (10 and 30 mg/kg/d) protected against the decrease in pancreatic insulin content as well as completely attenuating the increased pancreatic oxidative stress as determined by tissue levels of malondialdehyde. GED treatment also decreased neutrophil infiltration into the pancreas and reduced pancreatic levels of the chemokine MIP-1 $\alpha$  and the proinflammatory cytokines IL-1 and IL-12. We hypothesize that GED exerts these latter effects by protecting  $\beta$  cells from destruction reducing autoantigen release and decreasing the autoimmune response. *In vitro* GED treatment of isolated rat islets of Langerhans protected glucose-stimulated insulin secretion from inhibition by IL-1 $\beta$ . In conclusion, inhibiting formation and/or scavenging reactive nitrogen or oxygen species with GED protects against development of diabetes *in vivo* and isolated pancreatic islets of Langerhans from cytokine inhibitory effects *in vitro*.

**Key Words:** diabetes, nitric oxide, peroxynitrite, streptozotocin, cytokines

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Type I diabetes is a disease characterized by the specific destruction of the insulin-producing  $\beta$  cells of the pancreatic islets of Langerhans by the immune system.<sup>1</sup>  $\beta$  cells are

destroyed by an autoimmune process, the trigger for which has yet to be fully elucidated.<sup>1</sup> The islet is invaded by immune cells, particularly T cells that are CD4<sup>+</sup> and CD8<sup>+</sup>,<sup>2</sup> forming a pancreatic inflammation termed insulinitis. Islet  $\beta$ -cell destruction may result from direct contact with cytotoxic T lymphocytes as well as the resulting production of immune cell mediators such as cytokines and free radicals including nitric oxide and related oxidant species such as peroxynitrite inhibiting  $\beta$ -cell function and ultimately inducing  $\beta$ -cell death.<sup>3</sup> A variety of cytokines have been found expressed in the insulinitis lesion of both animal models of diabetes as well as in the pancreata of humans with type I diabetes. It has been proposed that the insulinitis lesion is  $\beta$ -cell destructive when T<sub>H</sub>1 cytokines (IL-12, IFN- $\gamma$ , IL-1, TNF- $\alpha$ ) produced by islet infiltrating T cells dominate over T<sub>H</sub>2 cytokines (IL-4, IL-10).<sup>4</sup> The proinflammatory cytokines IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  are cytotoxic to islet  $\beta$  cells via mechanisms that may involve  $\beta$ -cell production of nitric oxide and/or oxygen radicals.<sup>3,5–7</sup>

Peroxyntirite (ONOO<sup>-</sup>) is a highly reactive oxidant species formed following the reaction of superoxide with nitric oxide. Peroxyntirite has been implicated in the pathogenesis of diabetes<sup>7–9</sup> and is considered more cytotoxic than nitric oxide or superoxide. Therefore, the aim of this study was to determine the efficacy of the inducible nitric oxide synthase inhibitor and free radical scavenger guanidinoethylsulfide (GED) in preventing type I diabetes *in vivo* induced in mice by multiple low-dose streptozotocin (MLDS) and the *in vitro* suppression of glucose-stimulated islet insulin secretion by IL-1 $\beta$ .

## METHODS

### *In Vivo* Studies

#### Induction of Diabetes

Male Balb/c mice were treated with streptozotocin (40 mg/kg dissolved in citrate buffer, pH) or vehicle (citrate buffer) intraperitoneally for 5 consecutive days. Mice were

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treated every day starting on day 1 with GED (10 or 30 mg/kg/d) or vehicle (water) orally by gavage. Blood glucose was monitored over the following 21 days using a 1-touch blood glucose meter (Lifescan). Blood glucose was measured on days 1, 7, 14, and 21 from blood obtained from the tail vein. Hyperglycemia was defined as nonfasting blood glucose level higher than 200 mg/dL. The cumulative incidence of diabetes was calculated as a percentage of hyperglycemic mice per treatment group at each time point. Biopsy specimens of the pancreas were removed on day 21 for further biochemical analysis.

#### Determination of Pancreatic Insulin, Myeloperoxidase, Malondialdehyde, and Cytokines

The insulin content of the pancreas of BALB/c mice was determined from a pancreas biopsy specimen that was homogenized in acidified ethanol (75% ethanol, 1.5% 12 mol/L HCl, and 23.5% H<sub>2</sub>O), then incubated for 72 hours at 4°C, and centrifuged.<sup>10</sup> The insulin content of the supernatant was determined using an ELISA kit (Alpco). The pancreatic insulin content was expressed as ng insulin/mg protein, which was determined by the Bradford assay.<sup>11</sup>

To assay myeloperoxidase (MPO) activity in pancreas, the pancreas biopsy was homogenized in 0.5% hexadecyltrimethylammonium bromide dissolved in 10 mmol/L 3-*N*-morpholinopropanesulfonic acid and centrifuged at 15,000g for 40 minutes. The suspension was then sonicated 3 times for 30 seconds. An aliquot of supernatant was mixed with a solution of 1.6 mmol/L tetramethylbenzidine and 1 mmol/L hydrogen peroxide. MPO activity was measured spectrophotometrically as the change in absorbance at 650 nm, using a Spectramax microplate reader (Molecular Devices, Sunnyvale, CA).<sup>12,13</sup> Results are expressed as milliunits of MPO activity per milligram of protein.

Malondialdehyde (MDA) content in the pancreas was determined from a pancreatic biopsy specimen homogenized in 1.15% KCl buffer. Two hundred microliters of the homogenate was added to a reaction mixture consisting of 1.5 mL 0.8% thiobarbituric acid, 200  $\mu$ L 8.1% sodium dodecyl sulfate, 1.5 mL 20% acetic acid (pH 3.5), and 600  $\mu$ L distilled H<sub>2</sub>O. The mixture was incubated at 90°C for 45 minutes. After cooling to room temperature, the sample was cleared by centrifugation (10,000g, 10 minutes) and the absorbance measured at 532 nm, using 1,1,3,3-tetramethoxypropane as an external standard.<sup>12,14</sup> Results are expressed as pmol MDA/mg protein.

Cytokines expressed in the pancreas were determined from a pancreas biopsy specimen that was snap-frozen in liquid nitrogen. The biopsies were homogenized in 700  $\mu$ L of a Tris-HCl buffer containing protease inhibitors<sup>15</sup>; centrifuged for 30 minutes, and then the supernatant was removed and frozen at -80°C until assay. The cytokine content in the pancreas

was determined using specific ELISA kits (R&D Systems) and expressed as pg cytokine protein/mg protein.

## In Vitro Studies

### Isolation and Culture of Islets of Langerhans

Rat islets of Langerhans were isolated under aseptic conditions from collagenase-digested pancreata of adult female rats (175–200 g). Batches of 500 islets were cultured in RPMI 1640 media containing 5.5 mmol/L glucose, penicillin (50 U/mL<sup>-1</sup>), streptomycin (50  $\mu$ g/mL<sup>-1</sup>), and 5% fetal calf serum for 48 hours prior to being divided into experimental groups.

### Islet Insulin Secretion Response

Batches of 100 isolated rat islets were treated for 24 hours with IL-1 $\beta$  100 pmol/L, determined from previous studies<sup>16,17</sup> in the absence or presence of GED (10–100  $\mu$ mol/L). The islets were preincubated in Gey and Gey physiological buffer<sup>18</sup> containing 2 mmol/L glucose for 1 hour at 37°C. The islets were then washed in fresh 2 mmol/L glucose containing buffer before groups of 6 islets were handpicked into 1 mL of Gey and Gey buffer containing 20 mmol/L glucose and again incubated for 1 hour at 37°C.<sup>19,20</sup> At the end of the incubation, 0.4 mL was removed and assayed for insulin using a commercially available ELISA kit from Alpco. Results were expressed as ng insulin/islet/h.

### PARS Assay

NIT-1 cells were treated with various doses of GED 15 minutes prior to the cells being treated with peroxynitrite or hydrogen peroxide at 750  $\mu$ mol/L for 15 minutes. For the measurement of PARS activity,<sup>21</sup> the media was removed and replaced with 0.5 mL HEPES (pH 7.5) containing 0.01% digitonin and <sup>3</sup>H-NAD (0.5  $\mu$ Ci/mL<sup>-1</sup>) for 20 minutes. The cells were then scraped from the wells and placed in an Eppendorf tubes containing 200  $\mu$ L of ice-cold 50% TCA (wt/vol); the tubes were then placed at 4°C. After 4 hours, the tubes were centrifuged at 1800g for 10 minutes and the supernatant removed, the pellet was washed twice with 500  $\mu$ L ice-cold 5% TCA. The pellet was solubilized in 250  $\mu$ L NaOH (0.1 mol/L) containing 2% SDS overnight at 37°C, the PARS activity was then determined by measuring the radioactivity incorporated using a Wallac scintillation counter (Perkin-Elmer). The solubilized protein (250  $\mu$ L) was mixed with 5 mL of scintillant (ScintiSafe Plus; Fisher) before being counted for 10 minutes. Results are expressed as the percentage of inhibition of stimulated PARS activity by either peroxynitrite or hydrogen peroxide.

### Statistical Analysis

The data are presented as mean  $\pm$  SEM; statistical analysis was performed using either the  $\chi^2$  test or Student *t* test as appropriate, with *P* < 0.05 considered significant.

## RESULTS

## In Vivo Effects of GED on MLDS-Induced Diabetes

MLDS treatment induced a progressive hyperglycemia and an increased incidence of diabetes (Fig. 1). Daily treatment with GED (100 or 200 mg/kg) dose dependently reduced the MLDS-induced hyperglycemia and incidence of diabetes (Fig. 1). MLDS treatment markedly decreased pancreas insulin content (Fig. 2A) and increased the pancreas levels of the chemokine MIP-1 $\alpha$  and the proinflammatory cytokines IL-1 and IL-12 (Fig. 2B), as measured on day 21. MLDS treatment also increased pancreatic levels of MDA (Fig. 2C), indicative of increased oxidative stress, and MPO (Fig. 2D), indicative of increased pancreatic infiltration by mononuclear cells. GED dose dependently reversed the decrease in pancreatic insulin content (Fig. 2A) and the increases in MIP-1 $\alpha$ , IL-1, and IL-12 (Fig. 2B). The increased pancreatic oxidative stress and neu-

trophil infiltration were also significantly inhibited by GED treatment (Fig. 2C and D).

In Vitro Effects of GED on IL-1 $\beta$ -Induced Suppression of Glucose-Stimulated Insulin Secretion and Free Radical-Mediated Activation of Poly (ADP-ribose) Polymerase (PARP) Activity

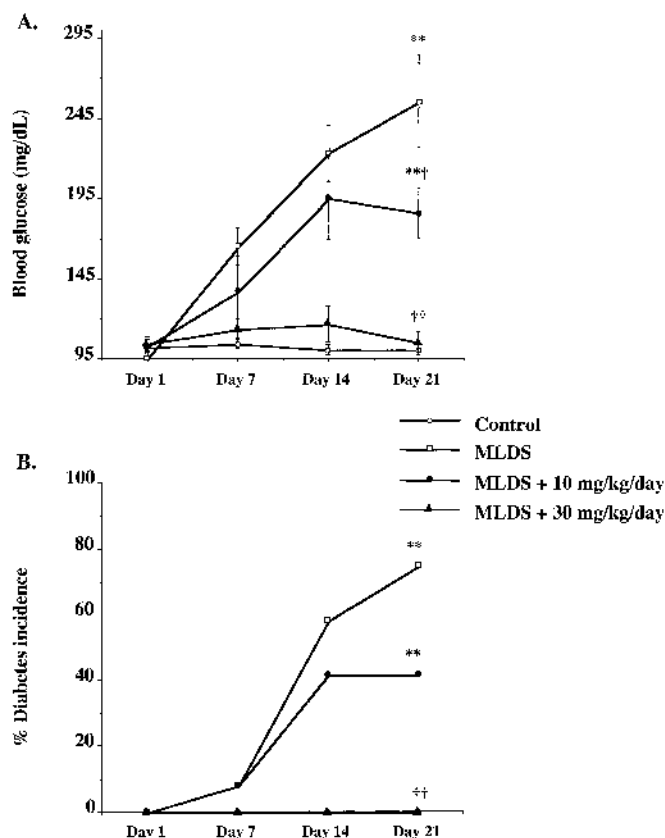
Treatment of rat islets of Langerhans for 24 hours with IL-1 $\beta$  (100 pmol/L) inhibited glucose-stimulated insulin secretion by >50% (Fig. 3). This inhibition was completely reversed by both 30 and 100  $\mu$ mol/L GED (Fig. 3). GED alone at 100  $\mu$ mol/L had no effect on glucose-stimulated insulin secretion (Fig. 3).

Treatment of the insulin-secreting cell line NIT-1 for 15 minutes with either peroxynitrite or hydrogen peroxide at 750  $\mu$ mol/L increases PARP activity [untreated cells 3181  $\pm$  157 cpm to 25,524  $\pm$  1521 ( $P < 0.01$ ) and 24,813  $\pm$  596 ( $P < 0.01$ ) with peroxynitrite and hydrogen peroxide, respectively], treatment of the cells with GED at various concentrations dose dependently inhibited PARP activation (Fig. 4). GED alone at 1000  $\mu$ mol/L has no effect on basal PARP activity (3193  $\pm$  211 cpm,  $P > 0.05$  vs. untreated cells).

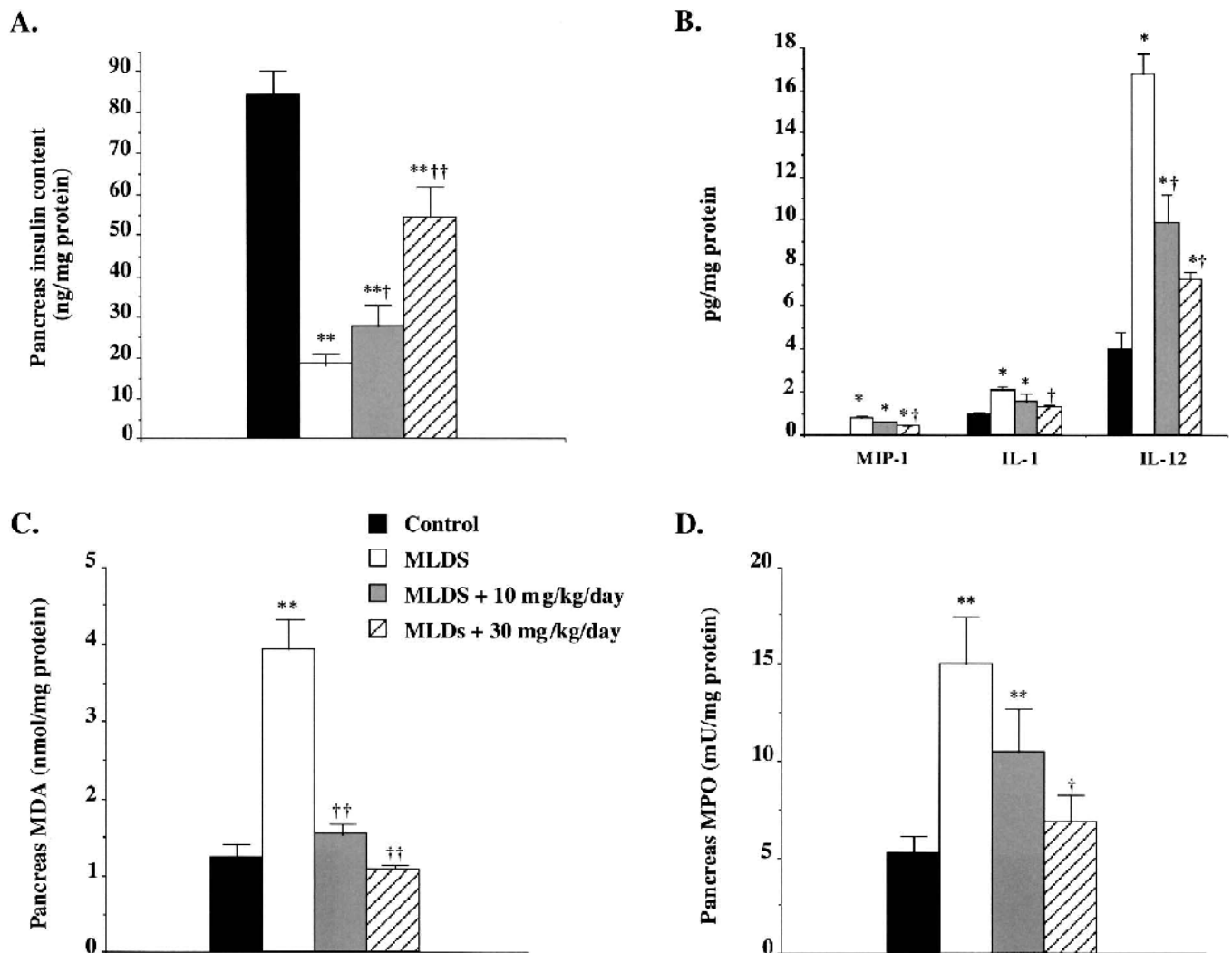
## DISCUSSION

The results from this study demonstrate for the first time that the combination of inhibiting inducible nitric oxide synthase and scavenging cytotoxic oxidant species such as peroxynitrite and hydrogen peroxide can protect against MLDS-induced diabetes. The protective effect of GED on pancreas insulin content and oxidative damage suggests that GED can directly protect the  $\beta$  cells. The attenuating effect of GED on pancreas infiltration by neutrophils and on pancreatic levels of the chemokine MIP-1 $\alpha$  and the proinflammatory cytokines IL-1 and IL-12 may be related to the reduction in  $\beta$ -cell damage and release of autoantigens resulting in a decreased autoimmune response. In vitro GED proved very effective in preventing IL-1 $\beta$ -mediated inhibition of insulin secretion and also inhibited the activation of poly (ADP-ribose) synthetase in a  $\beta$ -cell line treated with either peroxynitrite or hydrogen peroxide.

IL-1 $\beta$  induces  $\beta$ -cell expression of iNOS<sup>5,22-24</sup> and the subsequent nitric oxide formation that inhibits insulin secretion<sup>24,25</sup> and also causes  $\beta$ -cell death.<sup>24,26</sup> Interestingly, most nitric oxide synthase inhibitors only partially protect insulin secretion from inhibition by IL-1 $\beta$ ,<sup>5,22,23</sup> indicating there is a nitric oxide-independent component of this inhibition of insulin secretion, an observation recently demonstrated from islets isolated from iNOS-deficient mice whose glucose-stimulated insulin secretion remains susceptible to inhibition by cytokine treatment.<sup>27</sup> Interestingly, GED completely reverses IL-1 $\beta$ -mediated inhibition of insulin secretion, apparently also protecting against the nitric oxide-independent component, sug-



**FIGURE 1.** Daily treatment with GED (10 or 30 mg/kg) for 21 days decreases hyperglycemia (A) and the incidence of diabetes (B) following MLDS treatment of BALB/c mice on days 1–5. Diabetes incidence is expressed as a cumulative percentage of mice with a blood glucose  $\geq 200$  mg/dL. Results are mean  $\pm$  SEM or n = 20 mice in 2 separate experiments with 10 mice per experimental group. \*\* $P < 0.01$  versus vehicle-treated mice; † $P < 0.05$ ; †† $P < 0.01$  versus MLDS-treated mice.

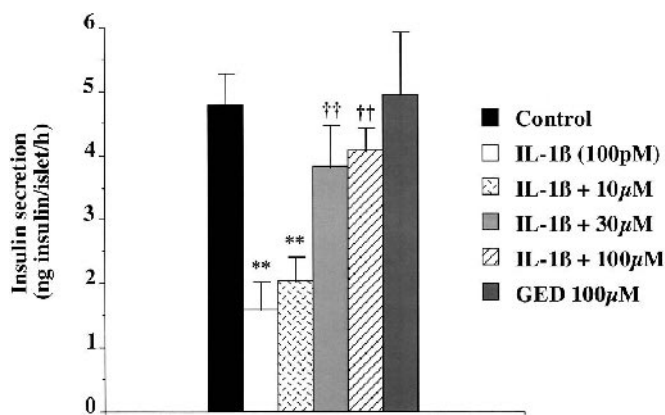


**FIGURE 2.** GED treatment dose dependently attenuates the effects of MLDS on pancreatic insulin content (A), chemokine and cytokine levels (B), MDA content (C), and MPO activity (D) in BALB/c mice. Results are mean  $\pm$  SEM or  $n = 10$ – $20$  mice in 2 separate experiments with 10 mice per experimental group. \* $P < 0.05$  versus vehicle-treated mice; † $P < 0.05$  versus MLDS-treated mice.

gesting that this component may be mediated by oxygen radical species, which GED is able to scavenge.<sup>7</sup>

Nonisoform-specific inhibitors of nitric oxide synthase have been demonstrated to protect in both genetic animal models of diabetes, the NOD mouse<sup>28</sup> and the BB rat.<sup>29,30</sup> GED has itself proved effective in protecting NOD mice from diabetes,<sup>9</sup> which was associated with a decreased formation of peroxynitrite. However, there have been no reports of protection by pharmacological inhibition of nitric oxide synthase in the MLDS model of diabetes, only the iNOS gene-deficient mouse has been shown to be less susceptible to MLDS.<sup>31</sup> In fact, in several studies, various inhibitors of nitric oxide synthase failed to protect against MLDS-induced diabetes.<sup>32–34</sup> In a recent report, the isoform-selective iNOS inhibitor 1400W

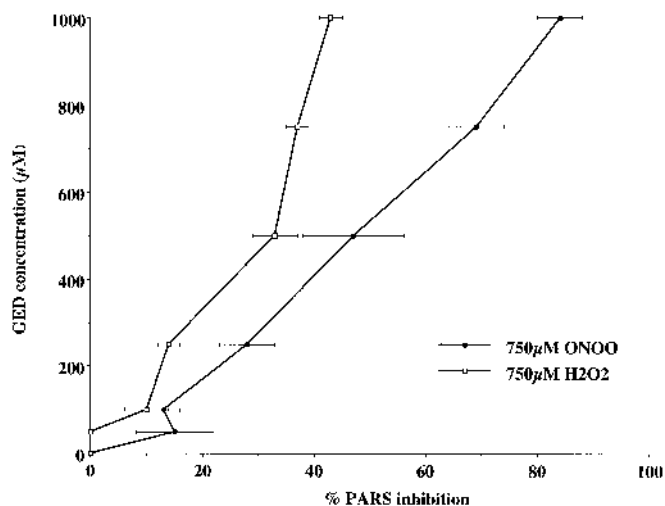
offered no protection in the MLDS model of diabetes<sup>35</sup> but was able to protect islets in vitro from the inhibitory effects of IL-1 $\beta$ .<sup>35</sup> Therefore, an interesting question arises to whether inhibition of iNOS is sufficient to protect against MLDS-induced diabetes. Alternatively, it is conceivable that the reason that GED was effective in attenuating MLDS-induced diabetes was related to its additional activities as a scavenger of peroxynitrite and hydrogen peroxide. The apparent need for this extra free radical scavenging action in addition to inhibition of nitric oxide synthase to protect against MLDS-induced diabetes may stem from the speed and severity of this model in which development of diabetes occurs over weeks rather than months as with the genetic models of type I diabetes, the NOD mouse, and BB rat. There is a clear role of both peroxynitrite<sup>8</sup>



**FIGURE 3.** Effect of GED on IL-1 $\beta$ -mediated inhibition of glucose-stimulated insulin secretion. Isolated rat islets of Langerhans were treated for 24 hours with IL-1 $\beta$   $\pm$  GED (10–100  $\mu$ mol/L) before islet responsiveness to glucose was determined; insulin secretion was expressed as nanograms of insulin released per islet per hour in response to 20-mmol/L glucose stimulation. Results are mean  $\pm$  SEM from 3 separate experiments. \*\* $P$  < 0.01 versus untreated islets and †† $P$  < 0.01 versus IL-1 $\beta$ -treated islets.

and oxidative stress<sup>3,36</sup> in the pathogenesis of diabetes, and we recently demonstrated that a specific peroxynitrite decomposition catalyst was remarkably effective in protecting against type I diabetes in both the MLDS model and the NOD mouse.<sup>37</sup>

The multiple molecular targets of GED represent a distinct advantage in protecting against type I diabetes. We propose that not only does GED inhibit nitric oxide formation and hence reduces  $\beta$ -cell damage by cutting off the source of reactive nitrogen species, but through its scavenger activity, it also interferes with the other pathway of  $\beta$ -cell death, that induced by reactive oxygen species.<sup>38</sup> Previous work demonstrating protection from MLDS-induced diabetes following treatment with desferrioxamine,<sup>39</sup> an iron chelator that inhibits the formation of hydroxyl radicals from hydrogen peroxide via the Fenton reaction, or dimethyl urea,<sup>40</sup> a scavenger of hydroxyl radicals, has demonstrated the potential role of oxygen radicals in MLDS-induced diabetes. The pancreas has been shown to have low levels of the antioxidant enzymes superoxide dismutase and catalase.<sup>41</sup> The already low level of antioxidant defense in the pancreas is compromised further as peroxynitrite, formed during diabetes development,<sup>8,37</sup> can inhibit the activity of both superoxide dismutase<sup>42,43</sup> and catalase.<sup>44</sup> It is possible, therefore, that GED not only directly assists these enzymes in their protective role of reducing oxygen free radical-mediated  $\beta$ -cell damage but also indirectly prevents the oxidant-mediated loss of superoxide dismutase and catalase activity. Increasing  $\beta$ -cell levels of these antioxidant enzymes not only protects them from cytokine and free radical damage<sup>45–47</sup> but also extends islet graft function when transplanted into diabetic NOD mice,<sup>48</sup> demonstrating a clear link between



**FIGURE 4.** Peroxynitrite and hydrogen peroxide activate PARS in the NIT-1 cell line: inhibition by GED. Pretreatment of NIT-1 cells with GED dose dependently reduces the percentage of PARS activation observed with both peroxynitrite and hydrogen peroxide. Results are expressed as the percentage of PARS activity inhibition.

oxygen free radicals and in vivo destruction of  $\beta$  cells in diabetes. Therefore, the maintenance of antioxidant enzyme activity may be a relevant factor for protection of  $\beta$  cells from destruction.

In conclusion, due to the severity of disease development and progression following MLDS-induced diabetes, inhibition of nitric oxide synthase alone is not sufficient for protection. Only when combined with scavenging of oxygen and nitrogen free radicals is there protection against type I diabetes. GED, a combined iNOS inhibitor and reactive oxidant species scavenger, prevented both the hyperglycemia and incidence of diabetes associated with MLDS treatment. The current study emphasizes the importance of nitric oxide synthase and reactive oxidant species in the pathogenesis of islet cell dysfunction and destruction associated with the MLDS model of type I diabetes. Further studies using clinically applicable nitric oxide synthase inhibitors alone and in combination with antioxidant therapy must address the relevance of those pathways in the pathogenesis of the human disease.

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