



# An Inhibitor of Inducible Nitric Oxide Synthase and Scavenger of Peroxynitrite Prevents Diabetes Development in NOD Mice

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Peroxynitrite (ONOO<sup>-</sup>) is a highly reactive oxidant produced by the interaction of the free radicals superoxide (O<sub>2</sub><sup>-</sup>) and nitric oxide (NO<sup>•</sup>). In a previous study, we found that peroxynitrite is formed in islet β-cells of nonobese diabetic (NOD) mice. Here, we report that guanidinoethylidithiolate (GED), a selective inhibitor of inducible nitric oxide synthase (iNOS) and scavenger of peroxynitrite prevents diabetes in NOD mice. GED treatment of female NOD mice, starting at age 5 weeks, delayed diabetes onset (from age 12 to 22 weeks) and significantly decreased diabetes incidence at 30 weeks (from 80% to 17%). GED did not prevent pancreatic islet infiltration by leukocytes; however, β-cells that stained positive for nitrotyrosine (a marker of peroxynitrite) were significantly decreased in islets of GED-treated mice (1±1%) compared with vehicle-treated mice (30±9%). In addition, GED significantly inhibited nitric oxide and nitrotyrosine formation and decreased destruction of β-cells in NOD mouse islets incubated *in vitro* with the combination of proinflammatory cytokines interleukin 1-beta (IL-1β), tumour necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ). These findings indicate that both superoxide and nitric oxide radicals contribute to islet β-cell destruction in autoimmune diabetes via peroxynitrite formation in the β-cells.

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## Introduction

Insulin dependent diabetes mellitus results from destruction of the insulin-producing pancreatic islet β-cells by a response considered to be autoimmune [1]. Pancreatic islets are infiltrated by mononuclear cells of the immune system, mostly macrophages and T lymphocytes and this is followed by destruction of the β-cells. Islet β-cell destruction may result from direct contact with β-cell specific cytotoxic T-lymphocytes, as well as from exposure to inflammatory products of activated macrophages and T-lymphocytes, such as cytokines, oxygen radicals, and nitric oxide [2–5]. In addition, the proinflammatory cytokines, interleukin 1-beta (IL-1β), tumour necrosis factor-alpha (TNF-α), and interferon gamma (IFN-γ) are cytotoxic to islet β-cells via mechanisms that may involve production of oxygen radicals and/or nitric oxide in the β-cells [4–11].

Peroxynitrite (ONOO<sup>-</sup>) is a highly reactive oxidant species produced by the combination of the free

radicals, superoxide (O<sub>2</sub><sup>-</sup>) and nitric oxide (NO<sup>•</sup>) [12, 13]. Peroxynitrite production has been observed in many inflammatory conditions [14, 15] and current evidence suggests that peroxynitrite is a more potent and cytotoxic mediator than superoxide or nitric oxide alone [13, 15, 16]. Also, both rodent and human islets are highly sensitive to peroxynitrite-induced damage [17]. In a previous study, we found that peroxynitrite is formed in islet β-cells of acutely-diabetic NOD mice [18]. The aim of the present study was to determine if prevention of peroxynitrite formation in β-cells of autoimmune diabetes-prone NOD mice would prevent β-cell destruction and diabetes development.

## Materials and Methods

### Animals

Female and male NOD mice, 4 weeks of age, were purchased from Taconic (Germantown, NY, USA). The mice were housed and fed under specific pathogen-free conditions and were cared for according to the guidelines of the Canadian Council on Animal Care. Female NOD mice of this colony develop pancreatic islet infiltration by immune system cells (insulinitis)

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beginning at ~6 weeks of age, followed by  $\beta$ -cell destruction and diabetes beginning at ~10–12 weeks of age, and increasing to ~80% by 30 weeks of age.

### Studies in vivo

Guanidinoethylidysulphide-2HCl (GED), a selective inhibitor of inducible nitric oxide synthase (iNOS) and scavenger of peroxynitrite [19, 20] was provided by Dr G. J. Southan (Inotek Corp., Beverly, MA, USA). The effects of GED were examined on diabetes development in NOD mice. Female NOD mice, 5 weeks of age, were randomly allocated to treatment with GED, 10 mg/kg ( $n=18$  mice) dissolved in phosphate-buffered saline (PBS), pH 7.2 (vehicle), or vehicle alone ( $n=18$ ), given twice a day by gavage. In another study, GED ( $n=19$  mice) and vehicle ( $n=18$  mice) treatments were started at 10 weeks of age. The mice were monitored daily by urine glucose testing, using Testape (Eli Lilly, Toronto, Ontario, Canada). Diabetes onset was diagnosed by the presence of glucosuria and a tail vein blood glucose  $\geq 12$  mmol/l measured on a glucose meter (Glucometer Elite, Bayer, Etobicoke, Ontario, Canada). The mice were killed by sodium pentobarbital overdose after diabetes onset or at age 30 weeks if still normoglycemic. In another study, GED ( $n=15$  mice) or vehicle ( $n=16$  mice) treatments were started at age 5 weeks, then the mice were killed and pancreases were removed at age 12 weeks for study of islet leukocytic infiltration and  $\beta$ -cell survival. Pancreases from some GED-treated mice ( $n=6$ ) and vehicle-treated mice ( $n=6$ ) were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 4.5  $\mu$ m, and stained with haematoxylin and eosin. Coded slides were read by light microscopy. Pancreases from other GED-treated mice ( $n=9$ ) and vehicle-treated mice ( $n=10$ ) were processed for islet isolation.

### Islet cell preparations

Pancreatic islets were isolated by collagenase digestion of the pancreas and Ficoll density-gradient purification, followed by handpicking of the isolated islets [21]. The islets were dispersed into single cells by incubation at 37°C for 10 min in  $\text{Ca}^{2+}$ -/ $\text{Mg}^{2+}$ -free PBS containing 0.2 mg/ml EDTA, followed by syringe injection through progressively narrower gauge needles from size 16 to 22. The islet cells were washed twice in PBS, then fixed with 4% paraformaldehyde in PBS for 10 min and washed twice in PBS. The fixed cells ( $50 \times 10^3$  in 10  $\mu$ l) were placed on glass slides coated with 3-aminopropyltriethoxysilane (Sigma, St Louis, MO, USA) and the slides were stored at -70°C until cell staining was done.

### Immunohistochemical studies

The slides with fixed cells attached were thawed and the cells were incubated with 1% paraformaldehyde

in PBS at 4°C for 10 min. Cell staining was performed as previously described [18]. Briefly, the cells were incubated in 10% normal goat serum and 1.5% bovine serum albumin to block non-specific binding of antibodies. The cells were incubated first with a rat monoclonal antibody (mAb) to a cell-surface leukocyte common antigen, CD45/T200 (mAb Ly-5, IgG2, Cedarlane Laboratories, Hornby, Ontario, Canada), 10  $\mu$ g/ml in 10% rabbit serum, a mouse mAb to an islet  $\beta$ -cell surface ganglioside antigen (mAb R2D6, IgM, provided by Dr R. Alejandro, Miami, FL, USA), 20  $\mu$ g/ml in PBS [22], or isotype-matched control mAbs. Next, the cells were incubated with secondary antibodies, either biotinylated goat anti-rat mouse-absorbed IgG or biotinylated goat anti-mouse IgM, as appropriate. The cells were incubated with streptavidin-alkaline phosphatase conjugate, followed by a substrate chromogen, alkaline phosphatase Fast-blue which stained leukocytes ( $\text{CD45}^+$  cells) and  $\beta$ -cells ( $\text{R2D6}^+$  cells) blue on the cell surface. To identify nitrotyrosine-staining cells, unstained cells and R2D6-stained cells ( $\beta$ -cells) were first permeabilised by incubation in 1.5% saponin in PBS (PBS-saponin). Endogenous peroxidase in the cells was blocked by incubation in 1%  $\text{H}_2\text{O}_2$  in PBS saponin, then in 10% normal rabbit serum and 1.5% bovine serum albumin; then the cells were incubated with an affinity-purified rabbit anti-nitrotyrosine antibody (Cedarlane), 10  $\mu$ g/ml in PBS-saponin, or a rabbit isotype-matched IgG control antibody. Next, the cells were incubated with a secondary antibody, biotinylated goat anti-rabbit IgG, then with streptavidin-peroxidase conjugate, followed by a substrate chromogen, 3-amino 9-ethylcarbazole which stained nitrotyrosine-containing cells red.  $\beta$ -cells containing nitrotyrosine (NT) were stained blue on the surface ( $\text{R2D6}^+$ ) and red intracellularly ( $\text{NT}^+$ ). Stained cells were mounted on the slides using Crystal Mount and a total of 3,000 cells were scored blindly by two independent observers who each scanned 60 different microscopic fields (oil immersion, 100 $\times$ ).

### Studies in vitro

Islets were isolated from pancreases of male NOD mice, 4–5 weeks of age, by collagenase digestion and Ficoll density-gradient purification, followed by handpicking of the isolated islets [21]. For some experiments, the islets were dispersed into single cells by incubation at 37°C for 10 min in  $\text{Ca}^{2+}$ -/ $\text{Mg}^{2+}$ -free PBS containing 0.2 mg/ml EDTA, followed by syringe injection through progressively narrower gauge needles from size 16 to 22. Islets (500) were incubated in 1.6 ml medium in 35 $\times$ 10 mm Falcon tissue culture dishes (Becton Dickinson, Lincoln Park, NJ, USA). Islet cells ( $10^5$ ) were incubated in 170  $\mu$ l medium in 96-well tissue culture plates (A/2, Sarstedt, Montreal, Quebec, Canada). For immunohistochemical studies, islet cells ( $10^4$ ) were seeded in 10  $\mu$ l medium in eight-well tissue culture chamber slides (Lab-Tek II, Nalge Nunc International, Napier, IL, USA) and incubated at 37°C for 30 min to allow the cells to attach to the slide

before adding 200  $\mu$ l medium. Islets and islet cells were incubated for 4–6 days at 37°C in RPMI-1640 medium containing 11 mmol/l D-glucose and supplemented with 2 mmol/l L-glutamine, 0.1 mmol/l sodium pyruvate, 10% heat-inactivated fetal calf serum (FCS), 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.25  $\mu$ g/ml amphotericin B, and 12 mmol/l HEPES, and the medium was changed every 2 days. The islets and islet cells were then washed in phenol red-free RPMI-1640 medium containing 0.3 mmol/l L-arginine, 11 mmol/l D-glucose, 10% heat-inactivated FCS, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.25  $\mu$ g/ml amphotericin B, and 12 mmol/l HEPES (test medium). In the first study, islet cells were incubated at 37°C for 3 days in test medium alone, medium with the cytokine combination of IL-1 $\beta$  (30 U/ml), TNF- $\alpha$  ( $10^3$  U/ml), and IFN- $\gamma$  ( $10^3$  U/ml), medium with GED (100  $\mu$ mol/l), and medium with cytokines plus GED. Recombinant human IL-1 $\beta$  ( $2\text{--}4\times 10^7$  U/mg) was provided by the Upjohn Co. (Kalamazoo, MI, USA) and recombinant murine TNF- $\alpha$  ( $1.2\times 10^7$  U/mg) and murine IFN- $\gamma$  ( $8\times 10^6$  U/mg) were provided by Genentech (South San Francisco, CA, USA). The islet cells were washed three times in PBS. Islet cells seeded on tissue culture chamber slides were processed for immunohistochemical studies to quantitate nitrotyrosine-positive  $\beta$ -cells. Islet cells seeded in wells in tissue culture plates were examined for cell viability by MTT assay, and for insulin content. In the second study, islets were incubated at 37°C for 3 days in test medium alone, medium with IL-1 $\beta$  (30 U/ml), TNF- $\alpha$  ( $10^3$  U/ml), and IFN- $\gamma$  ( $10^3$  U/ml), medium with GED (100  $\mu$ mol/l), and medium with cytokines plus GED. Media were collected and assayed for nitrite content and the islets were washed in PBS and assayed for nitrotyrosine content. In the third study, islets were incubated at 37°C for 14 h in test medium alone, medium with 500  $\mu$ mol/l peroxynitrite (Alexis Biochemicals, San Diego, CA, USA), medium with 100  $\mu$ mol/l GED, and medium with peroxynitrite plus GED. The islets were washed in PBS and assayed for nitrotyrosine content.

### MTT assay

Islet cell viability was determined by a colorimetric assay that detects the reduction of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, Sigma) into soluble blue colored formazan crystals, as described by Mosmann [23] and modified by Sladowski *et al.* [24].

### Insulin assay

Insulin was extracted from islets by incubation in acidified ethanol (75% ethanol, 1.5% 12 mmol/l HCl, and 23.5% H<sub>2</sub>O) for 18 h at 4°C. The ethanol extracts of islets were diluted in insulin assay buffer and insulin was measured using an RIA kit (Pharmacia, Uppsala, Sweden) and rat insulin as standard.

### Nitrite assay

Nitrite, the stable end product of nitric oxide in aqueous solution, was determined in islet incubation media by a modification of the method of Green *et al.* [25], an on-line semiautomated procedure using high performance liquid chromatography (HPLC), as previously reported [9].

### Nitrotyrosine assay

Sample preparation was as described by Hensley *et al.* [26]. Islets were briefly sonicated in 400  $\mu$ l sodium acetate (10 mmol/l, pH 6.5), then rapidly vortexed for 1 h and centrifuged for 10 min at 12,000 $\times$ g. A 50  $\mu$ l aliquot of the supernatant was removed for protein assay by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA). To 150  $\mu$ l of the supernatant, 25  $\mu$ l sodium acetate buffer and 50  $\mu$ l pronase (1 mg/ml in acetate buffer) was added. The solution was then heated at 50°C for 18 h and dried in a Speed Vac system. The dried extract was dissolved in 100  $\mu$ l ethanol:H<sub>2</sub>O (70:30) by rapid vortexing and then centrifuged at 12,000 $\times$ g for 10 min. The supernatant was frozen at -20°C until derivatization and quantification was done by HPLC, as described by Kamisaki *et al.* [27]. Derivatization of nitrotyrosine was done by adding 10  $\mu$ l sodium borate, 0.1 mol/l, pH 8.7 and 10  $\mu$ l 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-F, 10 mg/ml in ethanol) to 50  $\mu$ l of the ethanol:H<sub>2</sub>O solution containing islet extract and incubating at 60°C for 2 min. The reaction was terminated by addition of 15  $\mu$ l 0.1 mol/l HCl and an aliquot (50–80  $\mu$ l) was injected into the HPLC column. The chromatography procedure was as described by Kamisaki *et al.* [27]. The detection limit for nitrotyrosine was approximately 1 pmole at a signal to noise ratio of 5.

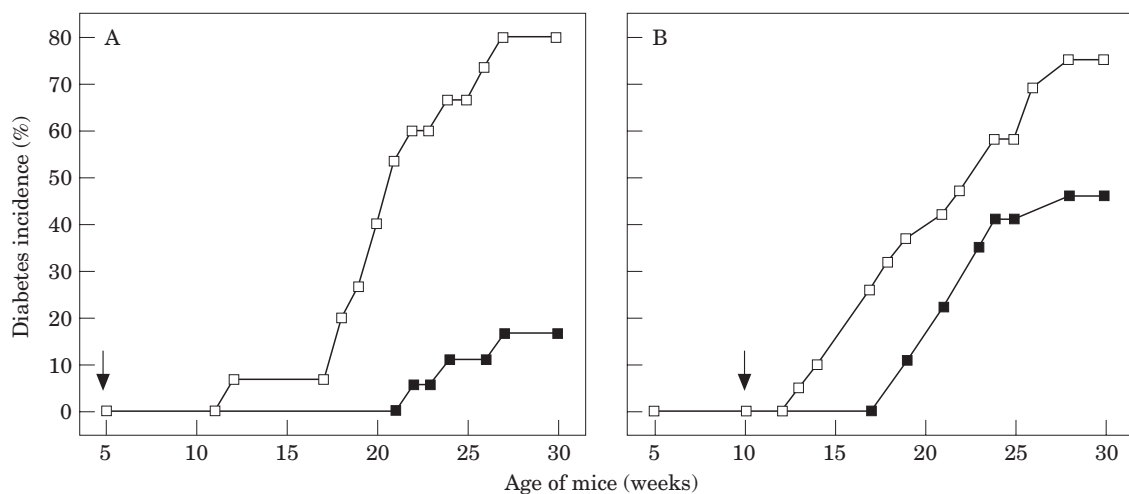
### Statistical analyses

Diabetes incidence data were compared for significant differences by the chi-squared test, 2 $\times$ 2 tables, df=1. All other data are expressed as mean $\pm$ SEM values. Differences between groups were analysed by Student's unpaired *t*-test where appropriate or by ANOVA with Tukey-Kramer's multiple comparisons test; *P*<0.05 was considered to be significant.

## Results

### Effects of GED on diabetes development

Treatment of diabetes-prone female NOD mice with GED, 10 mg/kg, given twice a day by gavage from age 5 weeks, delayed diabetes onset from age 12 weeks (vehicle-treated mice) to age 22 weeks, and diabetes incidence at age 30 weeks was significantly decreased from 81% to 17% (Figure 1A). GED

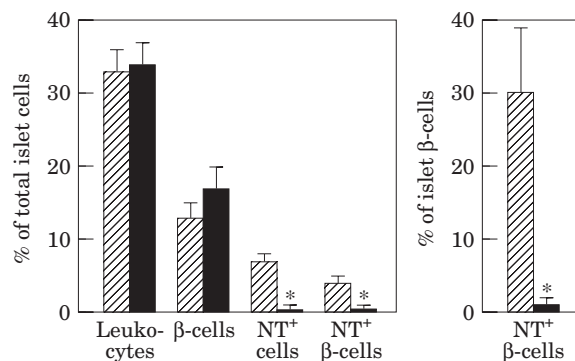


**Figure 1.** Diabetes incidence in female NOD mice treated from age 5 weeks (A) and 10 weeks (B) with GED, 10 mg/kg (■) or vehicle (□) twice daily by gavage. GED treatment begun at age 5 weeks significantly decreased diabetes incidence at age 30 weeks (17%, 3/18 mice) compared with vehicle treatment (81%, 13/16 mice,  $P < 0.001$ ). GED treatment begun at age 10 weeks significantly decreased diabetes incidence at age 30 weeks (45%, 8/18 mice) compared with vehicle treatment (74%, 14/19 mice,  $P < 0.05$ ).

treatment started later (at age 10 weeks) was less effective, delaying diabetes onset from age 13 weeks (vehicle-treated mice) to age 19 weeks, and diabetes incidence at age 30 weeks was partially decreased from 74% to 45% (Figure 1B). No adverse effects of GED on food intake or body weight were observed in these studies.

### Effects of GED on insulinitis and $\beta$ -cells

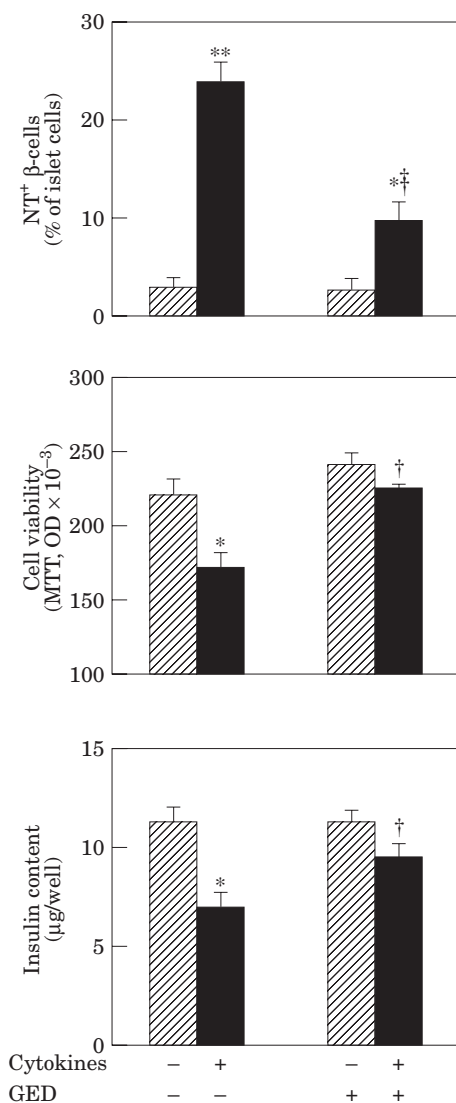
Although diabetes incidence was significantly decreased by GED treatment, insulinitis was not prevented. Histological examination of pancreatic sections of NOD mice treated with GED or vehicle from age 5 to 12 weeks revealed similar insulinitis scores in GED-treated mice ( $1.83 \pm 0.41$ ,  $n = 6$  mice) and vehicle-treated mice ( $1.88 \pm 0.27$ ,  $n = 6$  mice), where the insulinitis score represents the percentage of the islet area infiltrated by leukocytes (0 = none; 1 < 10%; 2 = 10–50%; 3 > 50%). The cellular composition of islets of NOD mice treated with GED or vehicle from age 5 to 12 weeks was examined further by isolating islets from these mice. This study confirmed that islet-associated leukocytes were present in similar numbers in islets of GED- and vehicle-treated mice, and  $\beta$ -cells were slightly reduced in vehicle-treated mice (Figure 2). Importantly, nitrotyrosine was detected in ~30% of  $\beta$ -cells in vehicle-treated mice compared with 1% of  $\beta$ -cells in GED-treated mice. Thus, insulinitis in vehicle-treated NOD mice was associated with peroxynitrite and nitrotyrosine formation in islet  $\beta$ -cells (Figure 2) and subsequent diabetes development (Figure 1), whereas insulinitis in GED-treated NOD mice did not lead to peroxynitrite and nitrotyrosine formation in islet  $\beta$ -cells (Figure 2) and diabetes was delayed and diabetes incidence was significantly decreased (Figure 1).



**Figure 2.** Immunohistochemical analysis of cells in islets isolated at age 12 weeks from NOD mice treated from age 5 weeks with GED, 10 mg/kg (■,  $n = 9$ ) or vehicle (▨,  $n = 10$ ), twice daily by gavage. Leukocytes were stained with an antibody to the CD45 cell surface leukocyte common antigen,  $\beta$ -cells were stained with a specific  $\beta$ -cell surface antibody (R2D6), and nitrotyrosine (NT) was stained with a specific antibody to NT. NT<sup>+</sup>  $\beta$ -cells were stained on the surface with the  $\beta$ -cell antibody and intracellularly with the NT antibody (see Methods). Data are expressed as mean  $\pm$  SEM values. \* $P < 0.01$  vs. vehicle group.

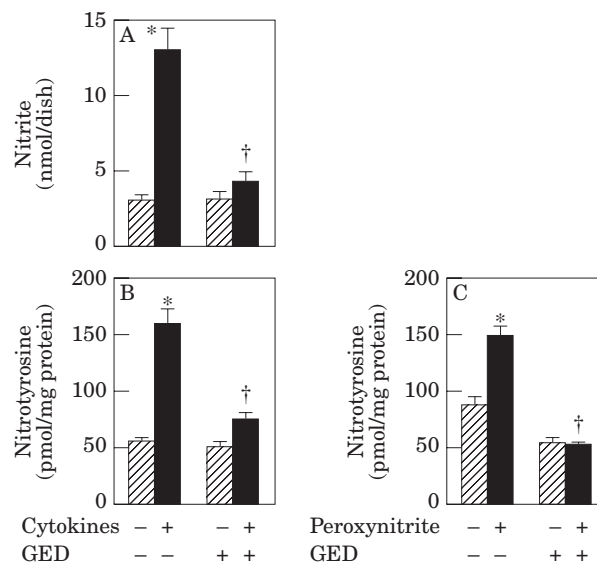
### Studies in vitro

To determine whether GED prevention of peroxynitrite formation in islet  $\beta$ -cells was the result of a direct effect of GED on  $\beta$ -cells, we examined the effects of the proinflammatory cytokines, IL-1 $\beta$ , TNF- $\alpha$ , and IFN- $\gamma$  on NOD mouse islets *in vitro*. We found that this cytokine combination significantly increased NT<sup>+</sup>  $\beta$ -cells, and decreased cell viability and insulin content of islet cell cultures (Figure 3). GED significantly reduced cytokine-induced increases in NT<sup>+</sup>  $\beta$ -cells and this was accompanied by improved cell viability and insulin content of the islet cell cultures. Next, we examined the mechanism of the protective effects of GED against cytokine-induced nitrotyrosine



**Figure 3.** GED protects NOD mouse islet cells from cytokine-induced nitrotyrosine formation in β-cells, decreased cell viability, and decreased insulin content in islet cell cultures. NOD mouse islet cells were incubated for 3 days without (-) and with (+) the cytokine combination of IL-1β (30 U/ml), TNF-β (10<sup>3</sup> U/ml), and IFN-γ (10<sup>3</sup> U/ml), alone and together with 100 μmol/l GED. Nitrotyrosine-stained β-cells (NT<sup>+</sup> β-cells) were identified by immunohistochemistry. Islet cell viability was determined by MTT assay. Insulin content of the islet cells was determined by RIA. Data are expressed as mean±SEM values for 5 experiments. \**P*<0.05, \*\**P*<0.01 vs. cytokines-GED-, †*P*<0.05, ‡*P*<0.01 vs. cytokines+GED-.

formation in islet β-cells. We found that cytokines significantly increased production of nitric oxide (measured as nitrite) and peroxynitrite (measured as nitrotyrosine) in islets, and GED significantly decreased cytokine-induced increases in both nitric oxide and peroxynitrite (Figure 4). In addition, GED prevented exogenous peroxynitrite from inducing nitrotyrosine formation in islets. We concluded that GED acted directly on islet cells to prevent cytokine-induced nitrotyrosine formation by preventing



**Figure 4.** GED prevents cytokine-induced production of nitrite (A) and nitrotyrosine (B) in NOD mouse islets. NOD mouse islets were incubated for 3 days without (-) and with (+) the cytokine combination of IL-1β (30 U/ml), TNF-α (10<sup>3</sup> U/ml), and IFN-γ (10<sup>3</sup> U/ml), alone and together with 100 μmol/l GED. Nitrite in islet incubation media and nitrotyrosine in islets were assayed by HPLC. \**P*<0.01 vs. cytokines-GED-, †*P*<0.05 vs. cytokines+GED-. Also, GED prevents peroxynitrite-induced nitrotyrosine formation (C) in NOD mouse islets. NOD mouse islets were incubated for 14 h without (-) and with (+) 500 μmol/l peroxynitrite, alone and together with 100 μmol/l GED. \**P*<0.01 vs. peroxynitrite-GED-, †*P*<0.01 vs. peroxynitrite+GED-. All data are expressed as mean±SEM values for 5 experiments.

production of nitric oxide, a precursor of peroxynitrite, and by scavenging peroxynitrite.

## Discussion

Autoimmune diabetes mellitus in NOD mice, as in humans, is characterised by infiltration of the pancreatic islets by mononuclear leukocytes (insulinitis), followed by destruction of the insulin-producing islet β-cells. Oxygen free radicals and nitric oxide produced by cytokine-activated macrophages, as well as by β-cells exposed to proinflammatory cytokines, have been implicated as mediators of islet β-cell destruction in autoimmune diabetes [2–11]. The nitric oxide synthesising enzyme, iNOS, is expressed in islet-infiltrating macrophages as well as in β-cells of prediabetic NOD mice [28]. In addition, peroxynitrite (the reaction product of nitric oxide and the oxygen free radical, superoxide) is expressed in islet β-cells of NOD mice in association with β-cell destruction and diabetes development [18]. In the present study, we found that prevention of peroxynitrite formation in islet β-cells of NOD mice prevented diabetes development, thereby indicating a pathogenic role for peroxynitrite in autoimmune β-cell destruction.

The agent that we used to prevent peroxynitrite formation, GED, is a mercaptoalkylguanidine

compound that inhibits NOS with selectivity towards the inducible isoform, iNOS [19]. Importantly, GED is able to reverse endotoxin-induced hypotension and vascular hyporeactivity—typical iNOS-mediated phenomena [29]—at doses used in the current study, without inducing a pressor response (a physiological response to inhibition of the constitutive endothelial isoform of NOS, eNOS). *In vivo*, guanidinoethyl-disulphide (GED) may convert to its monomer, mercaptoethylguanidine (MEG). While GED is a relatively weak scavenger of peroxynitrite, MEG (a free thiol-containing compound) is a potent scavenger of peroxynitrite [30]. Therefore, GED may have prevented peroxynitrite formation in islet  $\beta$ -cells of NOD mice in the current study by inhibiting iNOS and, in addition, by scavenging peroxynitrite. GED and MEG are very weak inhibitors of the cyclooxygenase enzyme [31], an effect which is unlikely to contribute to the findings described in the current study. Finally, MEG has been identified as a compound that decreases translation and stability of iNOS [32, 33]. These additional effects may also contribute to its inhibitory action on iNOS-related cytotoxic pathways in different disease states [33].

We confirmed that GED prevented cytokine-induced production of nitric oxide and peroxynitrite in NOD mouse islet cells *in vitro*. Peroxynitrite was measured as nitrotyrosine, which is produced by peroxynitrite-induced nitration of tyrosine residues on cellular proteins. Also, we found that GED prevented exogenously added peroxynitrite from inducing nitrotyrosine formation in NOD mouse islet cells, confirming its peroxynitrite scavenging action. These findings in NOD islets *in vitro* suggest that GED prevented nitrotyrosine formation in islet  $\beta$ -cells in NOD mice *in vivo* by inhibiting nitric oxide and peroxynitrite production and by scavenging any peroxynitrite produced.

The ultimate mechanism by which peroxynitrite leads to  $\beta$ -cell death remains to be clarified. Peroxynitrite has been reported to cause both mitochondrial and DNA damage, and DNA damage may indirectly amplify the direct toxic effects of peroxynitrite on mitochondria. Thus, peroxynitrite triggered the development of DNA single-strand breakage in murine thymocytes, leading to activation of the DNA repair enzyme, poly (ADP-ribose) synthetase, and consequent depletion of  $\text{NAD}^+$  which potentiated the mitochondrial dysfunction and free radical generation induced by peroxynitrite, resulting in cell necrosis [34]. This sequence of events may also occur when peroxynitrite is generated in islet  $\beta$ -cells. Thus, peroxynitrite addition to rat and human islets was found to cause DNA strand breaks in islet cells, and this was accompanied by mitochondrial dysfunction, detected as impaired glucose oxidation, followed by cell death by necrosis [17]. Our finding that peroxynitrite is a mediator of cytokine-induced  $\beta$ -cell destruction in NOD mouse islets is consistent with the observation that cytokine-induced necrosis of mouse islet cells is nitric oxide-dependent [35]. Because GED protection against  $\beta$ -cell destruction was only partial both *in vivo* and *in vitro*, however, nitric oxide-independent

mechanisms of  $\beta$ -cell destruction may still occur. In fact, cytokines can induce death by apoptosis in  $\beta$ -cells isolated from mice lacking iNOS expression (iNOS gene knockout mice) [35]. Also, islets deficient in iNOS could still be destroyed by adoptive transfer of  $\text{CD4}^+$  T cells from diabetic BDC 2.5 T cell receptor transgenic NOD mice [36]. It is not known, however, whether iNOS gene knockout might prevent normal course autoimmune diabetes development in NOD mice.

In summary, this study demonstrates that prevention of peroxynitrite production and action in islet  $\beta$ -cells is associated with prevention of  $\beta$ -cell destruction and autoimmune diabetes in NOD mice. These findings suggest that both superoxide and nitric oxide free radicals, and their reaction product, peroxynitrite, participate in pancreatic islet  $\beta$ -cell destruction in autoimmune diabetes.

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