

# Peroxynitrite Is a Mediator of Cytokine-Induced Destruction of Human Pancreatic Islet $\beta$ Cells

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**SUMMARY:** The proinflammatory cytokines, interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and interferon  $\gamma$  (IFN $\gamma$ ), are cytotoxic to pancreatic islet  $\beta$  cells, possibly by inducing nitric oxide and/or oxygen radical production in the  $\beta$  cells. Peroxynitrite, the reaction product of nitric oxide and the superoxide radical, is a strong oxidant and cytotoxic mediator; therefore, we hypothesized that peroxynitrite might be a mediator of cytokine-induced islet  $\beta$ -cell destruction. To test this hypothesis we incubated islets isolated from human pancreata with the cytokine combination of IL-1 $\beta$ , TNF $\alpha$ , and IFN $\gamma$ . We found that these cytokines induced significant increases in nitrotyrosine, a marker of peroxynitrite, in islet  $\beta$  cells, and the increase in nitrotyrosine preceded islet-cell destruction. Peroxynitrite mimicked the effects of cytokines on nitrotyrosine formation and islet  $\beta$ -cell destruction. L-N<sup>G</sup>-monomethyl arginine, an inhibitor of nitric oxide synthase, prevented cytokine-induced nitric oxide production but not hydrogen peroxide production, nitrotyrosine formation, or islet  $\beta$ -cell destruction. In contrast, guanidinoethylidysulphide, an inhibitor of inducible nitric oxide synthase and scavenger of peroxynitrite, prevented cytokine-induced nitric oxide and hydrogen peroxide production, nitrotyrosine formation, and islet  $\beta$ -cell destruction. These results suggest that cytokine-induced peroxynitrite formation is dependent upon increased generation of superoxide (measured as hydrogen peroxide) and that peroxynitrite is a mediator of cytokine-induced destruction of human pancreatic islet  $\beta$  cells. (*Lab Invest* 2001, 81:1683–1692).

Insulin-dependent (type 1) diabetes mellitus is an autoimmune disease that results from selective destruction of the insulin-producing  $\beta$  cells in the pancreatic islets of Langerhans. The islets are infiltrated by mononuclear cells of the immune system, mostly macrophages and T lymphocytes, and this is followed by destruction of the islet  $\beta$  cells (Bach, 1994).  $\beta$ -cell destruction may result from direct contact with cytotoxic T lymphocytes, as well as from exposure to inflammatory products of activated macrophages and T lymphocytes, such as free radicals and cytokines (Corbett and McDaniel, 1992; Mandrup-Poulsen et al, 1990). The proinflammatory cytokines, interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and interferon  $\gamma$  (IFN $\gamma$ ), acting individually or more potently in combination, are cytotoxic to rodent (Campbell et al, 1988; Mandrup-Poulsen et al, 1987; Pukel et al, 1988) and human (Rabinovitch et al, 1990; Soldevila et al, 1991) islets in vitro. The cytotoxic actions of cytokines on islet  $\beta$  cells are mediated, at least in part, by free radicals generated in the  $\beta$  cells (Rabinovitch and Suarez-Pinzon, 1998). Both oxygen-

based radicals and the nitrogen-based radical, nitric oxide, are produced in human islets incubated with proinflammatory cytokines; however, cytotoxic effects of the cytokines on islet  $\beta$  cells have been related to the production of oxygen free radicals and not nitric oxide (Eizirik et al, 1994; Rabinovitch et al, 1996).

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) (Beckman et al, 1990; Pryor and Squadrito, 1995). Peroxynitrite production has been observed in many inflammatory conditions and current evidence suggests that peroxynitrite is a more potent oxidant and cytotoxic mediator than nitric oxide or superoxide alone (Crow and Beckman, 1995; Szabó, 1996). Also, human islets are more resistant than rodent islets to nitric oxide-induced damage, whereas both human and rodent islets are highly sensitive to peroxynitrite-induced damage (Delaney et al, 1996). The aims of the present study were to determine whether peroxynitrite is generated in human islet  $\beta$  cells exposed to cytokines in vitro and, if so, whether peroxynitrite might be a mediator of the cytotoxic effects of the cytokines on the islet  $\beta$  cells.

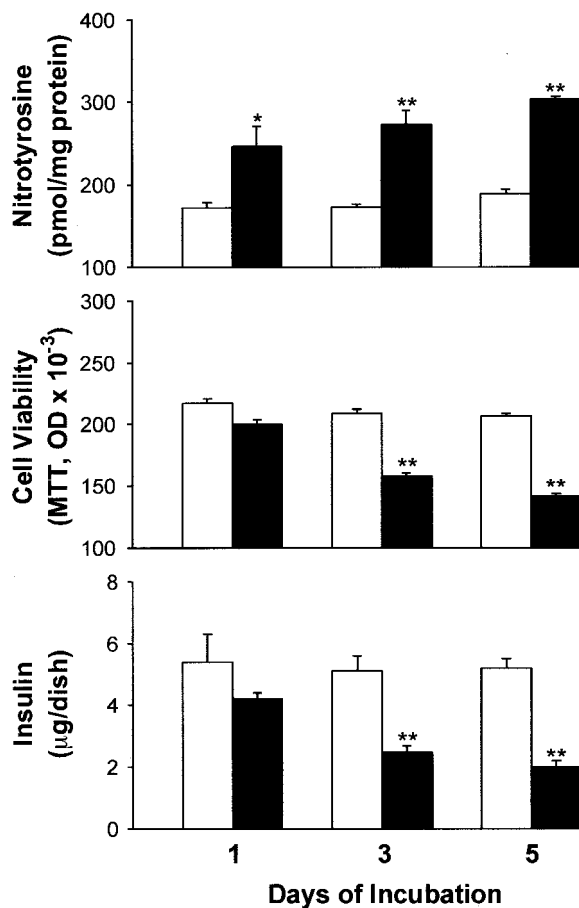
## Results

The cytokine combination of IL-1 $\beta$ , TNF $\alpha$ , and IFN $\gamma$  induced significant increases in nitrotyrosine in human islets after 1 day of incubation, and significant decreases in islet-cell viability and insulin content were detected after 3 days of incubation with cytokines (Fig.

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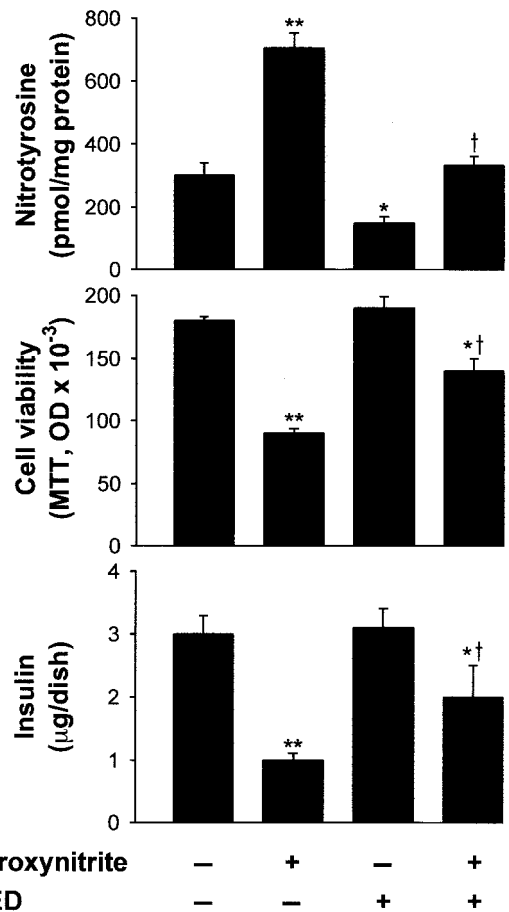
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**Figure 1.** Time course effects of the cytokine combination of interleukin-1β (IL-1β) (30 U/ml), tumor necrosis factor α (TNFα) (10<sup>3</sup> U/ml), and interferon γ (IFNγ) (10<sup>3</sup> U/ml) on nitrotyrosine formation (10<sup>3</sup> islets/dish), cell viability (10<sup>5</sup> cells/well), and insulin content (10<sup>3</sup> islets/dish) in human islets. Islets were incubated in medium without (open bars) and with (solid bars) cytokines for 1, 3, and 5 days. Values are means ± se for four experiments. \**p* < 0.05, \*\**p* < 0.01 vs cytokine-free incubations.

1). Thus, cytokine-induced production of peroxynitrite (measured as nitrotyrosine) preceded islet-cell destruction. To determine whether cytokine-induced peroxynitrite production might be a cause of islet β-cell destruction, first we examined the effects of adding peroxynitrite to islets. We found that peroxynitrite could mimic the effects of cytokines; thus, peroxynitrite induced nitrotyrosine formation in islets and decreased islet cell viability and insulin content (Fig. 2). Next, we examined the effects of guanidinoethyl-disulphide (GED), a peroxynitrite scavenger. GED (100 μM) significantly reduced peroxynitrite-induced nitrotyrosine formation in islet cells, and this was accompanied by significant increases in islet cell viability and insulin content (Fig. 2). Importantly, GED (100 μM) completely prevented cytokine-induced nitrotyrosine formation and decreased insulin content in the islets, and these effects of GED were dose-dependent, from 3 to 100 μM (Fig. 3).

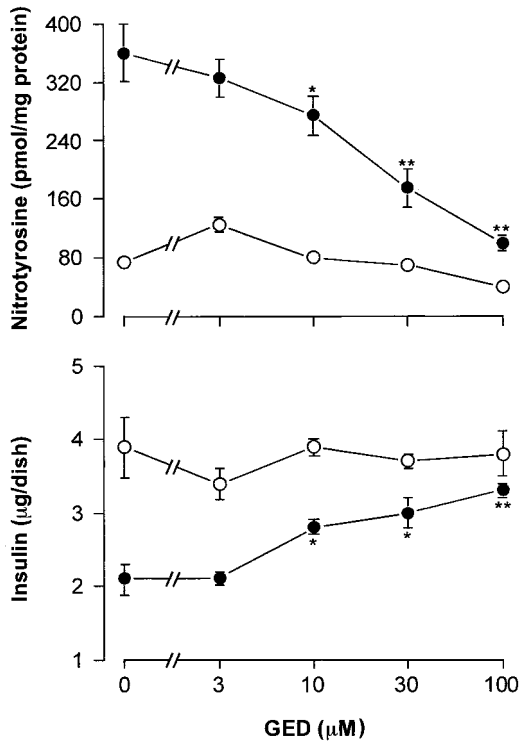
The above studies with GED suggested that cytokine-induced peroxynitrite generation was responsible for islet β-cell destruction; GED, however, inhibits inducible



**Figure 2.** Guanidinoethyl-disulphide (GED) (100 μM) reduces peroxynitrite (500 μM)-induced nitrotyrosine formation (10<sup>3</sup> islets/dish) and decreased cell viability (10<sup>5</sup> cells/well) and recovery of insulin (10<sup>3</sup> islets/dish) in islets after a 14 hour incubation. Values are means ± se for four experiments. \**p* < 0.05, \*\**p* < 0.01 vs peroxynitrite-free incubations; †*p* < 0.05 vs incubations with peroxynitrite alone.

nitric oxide synthase (iNOS) in addition to scavenging peroxynitrite (Cuzzocrea et al, 1998; Szabó et al, 1996). Therefore, we compared the effects of GED with L-N<sup>G</sup>-monomethyl arginine (L-NMMA), a well-known inhibitor of NOS (Gross et al, 1990). L-NMMA prevented cytokine-induced nitric oxide production but did not prevent cytokine-induced nitrotyrosine formation and decreased recovery of insulin and DNA in the islet cultures (Fig. 4). In contrast, GED prevented cytokine-induced production of both nitric oxide and nitrotyrosine, and decreases in insulin and DNA in islets were also prevented (Fig. 4). The finding that L-NMMA prevented cytokine-induced nitric oxide, but not peroxynitrite production, suggested that cytokine-induced peroxynitrite production might be dependent upon increased production of superoxide and not nitric oxide. This interpretation was supported by the findings that cytokines induced a significant increase in the production of hydrogen peroxide (a product of superoxide), and that GED, but not L-NMMA, prevented cytokine-induced hydrogen peroxide production and islet β-cell destruction (Fig. 5).

Finally, we used an immunohistochemical technique to specifically identify β cells in islets and to confirm

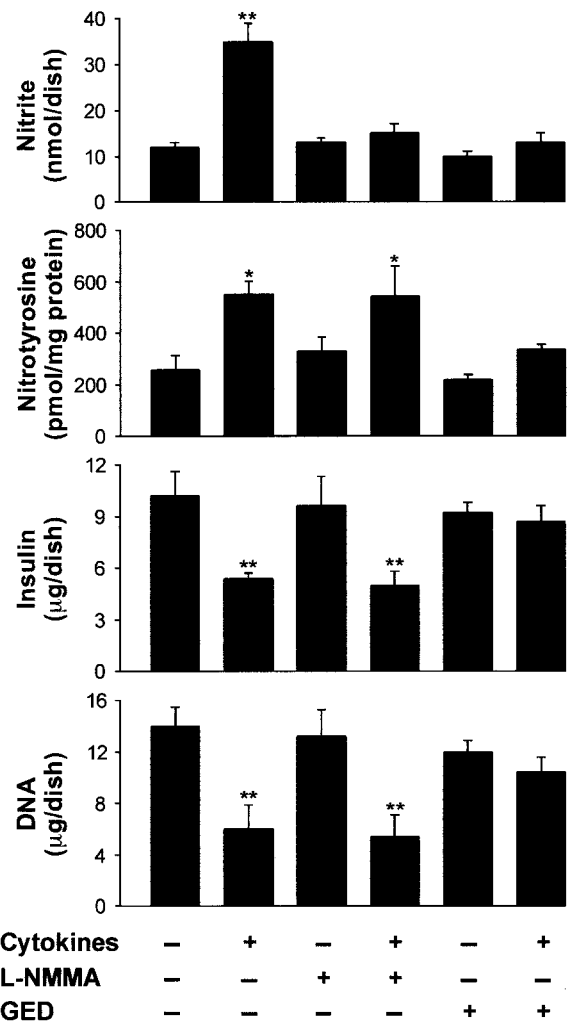


**Figure 3.** Dose-dependent effects of GED (3–100  $\mu$ M) on nitrotyrosine formation and insulin content in islets ( $10^3$ /dish) incubated for 5 days with the cytokine combination of IL-1 $\beta$  (30 U/ml), TNF $\alpha$  ( $10^3$  U/ml), and IFN- $\gamma$  ( $10^3$  U/ml). Islets were incubated in medium without (○) and with (●) cytokines. Values are means  $\pm$  SE for four experiments. \* $p$  < 0.05, \*\* $p$  < 0.01 vs GED-free incubations with cytokines.

that cytokines increased nitrotyrosine formation in  $\beta$  cells (Fig. 6). We found that cytokines significantly increased nitrotyrosine-positive  $\beta$ -cells in the islet cell cultures, and that the percentage of  $\beta$  cells that expressed nitrotyrosine after exposure to cytokines was greatly increased (from  $12 \pm 2\%$  to  $48 \pm 2\%$ ) (Fig. 7). Also, the  $\beta$ -cell composition of islets was significantly decreased after incubation with cytokines (from  $83 \pm 3\%$  to  $65 \pm 2\%$  of total islet cells). Importantly, the peroxynitrite scavenger, GED, significantly reduced the numbers of nitrotyrosine-positive  $\beta$  cells, as well as  $\beta$ -cell losses, that were induced by cytokines (Fig. 7).

**Discussion**

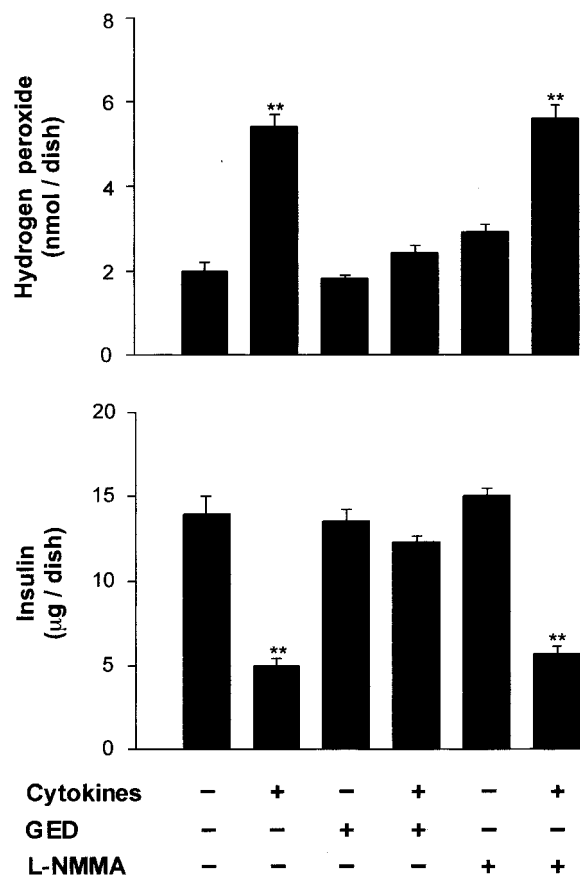
Type 1 diabetes is the result of pancreatic islet  $\beta$ -cell destruction by an autoimmune process. Pancreatic islets are infiltrated by cells of the immune system (macrophages and lymphocytes), and these cells damage and destroy islet  $\beta$  cells by a variety of mechanisms. For example, oxygen free radicals and nitric oxide produced by cytokine-activated macrophages, as well as by  $\beta$  cells exposed to proinflammatory cytokines, have been implicated as mediators of  $\beta$ -cell destruction in autoimmune diabetes (Corbett and McDaniel, 1992; Mandrup-Poulsen et al, 1990; Rabinovitch and Suarez-Pinzon, 1998). Pancreatic is-



**Figure 4.** An inhibitor of nitric oxide synthase (L-N<sup>G</sup>-monomethyl arginine [L-NMMA], 1 mM) prevents cytokine (IL-1 $\beta$ , 30 U/ml; TNF $\alpha$ ,  $10^3$  U/ml; and IFN- $\gamma$ ,  $10^3$  U/ml)-induced nitrite production, but does not prevent cytokine-induced nitrotyrosine formation and decreased insulin and DNA contents in islets ( $10^3$ /dish) after 5 days of incubation. In contrast, GED (100  $\mu$ M) prevents cytokine-induced nitrite and nitrotyrosine production, as well as decreases in insulin and DNA in the islets. Values are means  $\pm$  SE for five experiments. \* $p$  < 0.05, \*\* $p$  < 0.01 vs incubations without cytokines, L-NMMA, or GED.

let  $\beta$  cells are exceptionally vulnerable to the cytotoxic actions of free radicals because of constitutively low expression of antioxidant enzymes (Asayama et al, 1986; Cornelius et al, 1993; Grankvist et al, 1981; Lenzen et al, 1996; Malaisse et al, 1982; Welsh et al, 1995). Hyperexpression of oxygen free radical scavenging enzymes, such as superoxide dismutase, catalase, and glutathione peroxidase, either transgenically (Kubisch et al, 1994, 1997; Xu et al, 1999) or by gene transfection (Benhamou et al, 1998; Hohmeier et al, 1998; Moriscot et al, 2000; Tiedge et al, 1997, 1998, 1999) protects islet  $\beta$  cells against oxidant- and cytokine-induced damage.

Although the aforementioned studies support the hypothesis that free radicals mediate the cytotoxic actions of cytokines on islet  $\beta$  cells, it is not clear which free radicals are the cytotoxic species. Both



**Figure 5.**

The cytokine combination of IL-1 $\beta$  (30 U/ml), TNF $\alpha$  (10<sup>3</sup> U/ml), and IFN $\gamma$  (10<sup>3</sup> U/ml) significantly increases hydrogen peroxide production and decreases insulin content in islets (10<sup>3</sup>/dish) after 5 days of incubation. GED (100  $\mu$ M), but not L-NMMA (1 mM), prevents cytokine-induced hydrogen peroxide production and decreased insulin in the islets. Values are means  $\pm$  se for four experiments. \* $p$  < 0.05, \*\* $p$  < 0.01 vs incubations without cytokines, GED, or L-NMMA.

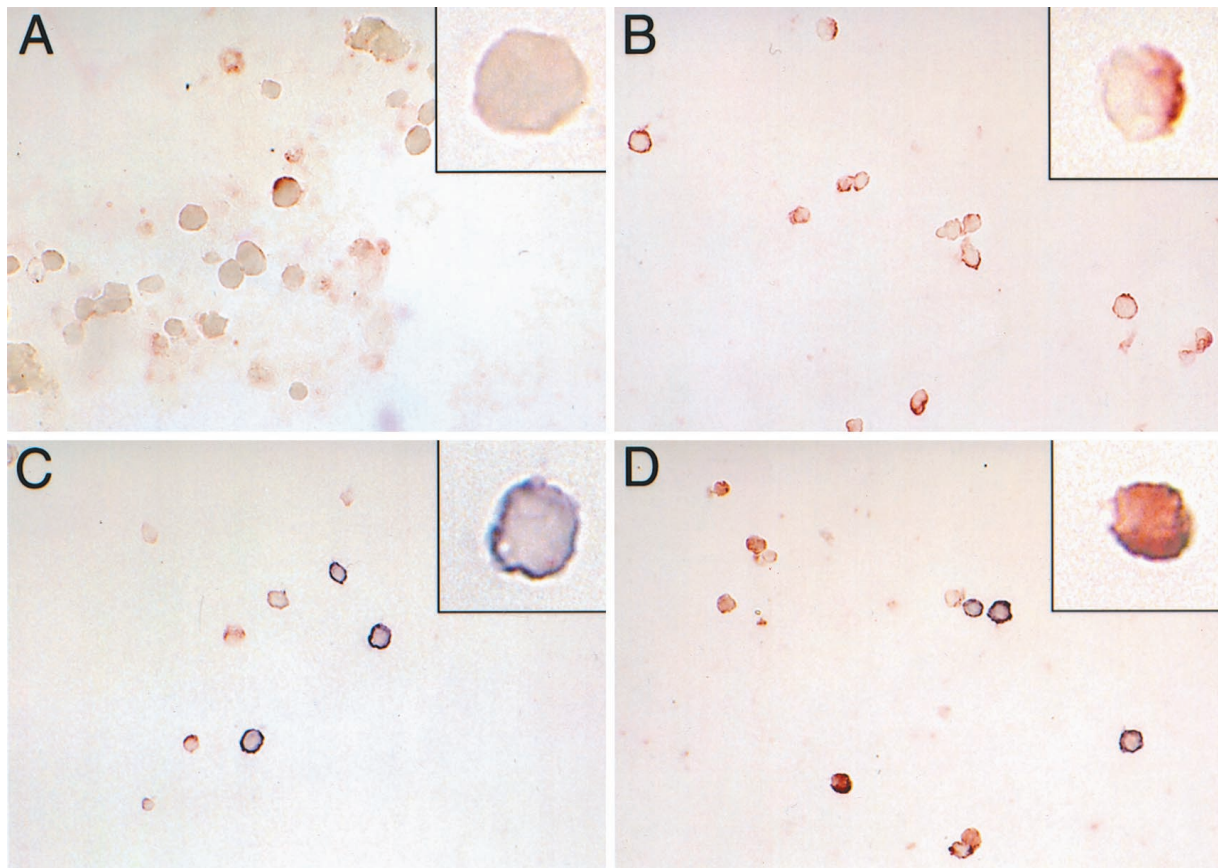
nitric oxide and superoxide have been implicated as free radical mediators of cytokine-induced  $\beta$ -cell destruction (Corbett et al, 1993; Rabinovitch et al, 1996). Peroxynitrite (ONOO<sup>-</sup>) is a highly reactive oxidant species produced by the combination of superoxide (O<sub>2</sub><sup>-</sup>) and nitric oxide (NO $\cdot$ ) (Beckman et al, 1990; Pryor and Squadrito, 1995). Peroxynitrite formation has been reported in a variety of inflammatory and tissue destructive conditions, and current evidence suggests that peroxynitrite is a more potent oxidant and cytotoxic mediator than superoxide or nitric oxide alone (Crow and Beckman, 1995; Szabó, 1996). Peroxynitrite has been reported to be highly cytotoxic to rat and human islet cells in vitro (Delaney et al, 1996). In a previous study, we found that peroxynitrite was formed in pancreatic islets in vivo in conjunction with  $\beta$ -cell destruction and autoimmune diabetes development in nonobese diabetic (NOD) mice (Suarez-Pinzon et al, 1997). In that study, peroxynitrite was detected as nitrotyrosine (formed by peroxynitrite-induced nitration of tyrosine residues on proteins) in islet-infiltrating macrophages as well as in  $\beta$  cells of prediabetic NOD mice, and nitrotyrosine was detected in

the majority of  $\beta$  cells of acutely diabetic NOD mice (Suarez-Pinzon et al, 1997).

In this study, we found that peroxynitrite is formed in human pancreatic islet  $\beta$  cells incubated with proinflammatory cytokines, and peroxynitrite formation correlated with  $\beta$ -cell destruction. Furthermore, our findings provide evidence that peroxynitrite is a mediator of cytokine-induced  $\beta$ -cell destruction. We found that cytokines induced peroxynitrite formation, measured as nitrotyrosine, in intact human pancreatic islets and in islet  $\beta$  cells. The mercaptoalkylguanidine compound, GED, prevented cytokine- and peroxynitrite-induced nitrotyrosine formation in islets, and this was associated with protection against both cytokine- and peroxynitrite-induced  $\beta$ -cell destruction. In addition to scavenging peroxynitrite, GED prevented cytokine-induced nitric oxide formation, consistent with the ability of GED to inhibit iNOS (Szabó et al, 1996). The protective effect of GED against cytokine-induced  $\beta$ -cell destruction was related, however, to prevention of nitrotyrosine and not nitric oxide formation, because another NOS inhibitor, L-NMMA, prevented cytokine-induced nitric oxide formation to the same extent as did GED, but did not prevent cytokine-induced nitrotyrosine formation nor  $\beta$ -cell destruction.

Our finding that prevention of cytokine-induced nitric oxide production by L-NMMA did not prevent  $\beta$ -cell destruction confirms previous observations that cytokine-induced destruction of human islet  $\beta$  cells is nitric oxide-independent (Eizirik et al, 1994; Rabinovitch et al, 1994). The present study demonstrates that cytokine-induced human islet  $\beta$ -cell destruction is dependent on peroxynitrite production. Because peroxynitrite is the product of superoxide and nitric oxide and prevention of cytokine-induced nitric oxide production by L-NMMA did not prevent peroxynitrite production, our findings suggest that cytokines lead to increased peroxynitrite formation in islet  $\beta$  cells mainly by increasing superoxide production. This interpretation was supported by the findings that cytokines induced a significant increase in the production of hydrogen peroxide (a product of superoxide), and that GED, but not L-NMMA, prevented cytokine-induced hydrogen peroxide production and islet  $\beta$ -cell destruction. Although L-NMMA blocked cytokine-induced nitric oxide production, basal nitric oxide production was not blocked; therefore, nitric oxide was still available to combine with superoxide when production of this reactive oxygen species was increased by cytokines, leading to increased peroxynitrite production. Thus, superoxide, not nitric oxide, may be limiting for peroxynitrite production in human islet  $\beta$  cells.

Mitochondria are the main source of superoxide production in cells. Oxidative phosphorylation and ATP production occurs along the electron transport chain localized to the mitochondrial inner membrane, and auto-oxidation of reduced electron transport chain components results in superoxide production. Superoxide may leak out of the electron transport chain if mitochondrial integrity or function is impaired in any way. Mitochondrial perturbations result in the release



**Figure 6.**

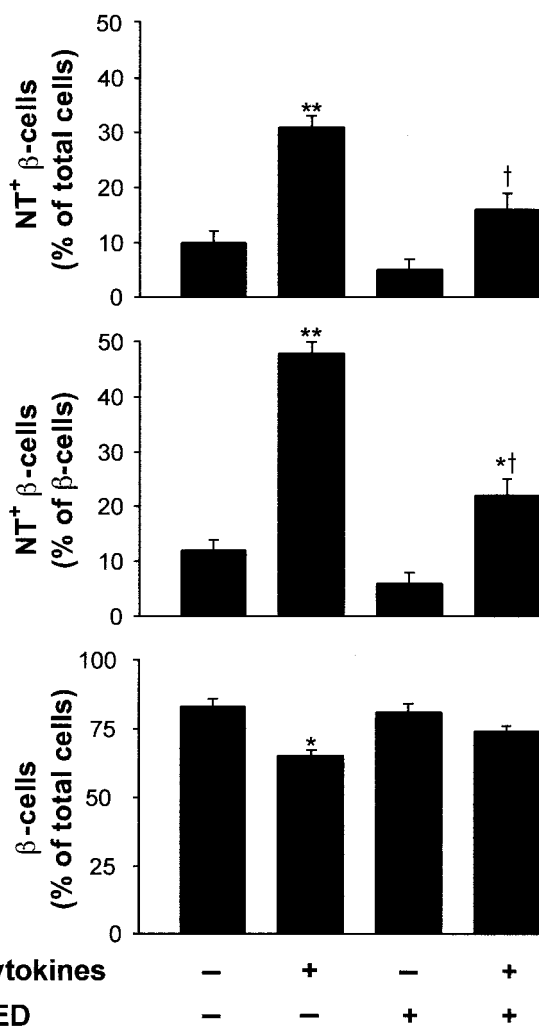
Photomicrographs of human islet cells identified by two-color immunohistochemical staining. Islet cells were incubated without (A and C) and with (B and D) the cytokine combination of IL-1 $\beta$  (30 U/ml), TNF $\alpha$  (10<sup>3</sup> U/ml), and IFN $\gamma$  (10<sup>3</sup> U/ml). Nitrotyrosine-positive islet cells (stained red using an antibody to nitrotyrosine) are more numerous after incubation in medium with cytokines (B) than in control medium (A). Nitrotyrosine-negative cell (A, inset). Nitrotyrosine-positive cell (B, inset). Nitrotyrosine-positive islet  $\beta$  cells (stained blue using an antibody to insulin and red using an antibody to nitrotyrosine) are more numerous after incubation in medium with cytokines (D) than in control medium (C). Nitrotyrosine-negative  $\beta$  cell (C, inset). Nitrotyrosine-positive  $\beta$  cell (D, inset). Magnification,  $\times 440$ , and insets,  $\times 1890$ .

of cytochrome *c*, an electron transport protein, with consequent increased mitochondrial release of superoxide and induction of cellular apoptosis (Cai and Jones, 1998; Green and Reed, 1998). This may be a mechanism by which proinflammatory cytokines induce oxygen free radical production and  $\beta$ -cell destruction. For example, Bcl-2 is an antiapoptotic protein that prevents cytochrome *c* release from mitochondria, and we have previously found that hyperexpression of Bcl-2 in human islet  $\beta$  cells, by gene transfection of islets, prevented cytokine-induced lipid peroxidation and  $\beta$ -cell death (Rabinovitch et al, 1999).

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. For example, peroxynitrite was reported to trigger the development of DNA single-strand breakage and activation of the DNA repair enzyme, poly (ADP-ribose) synthetase in murine thymocytes, leading to NAD<sup>+</sup> depletion and potentiation of the peroxynitrite-induced mitochondrial dysfunction and free radical generation, resulting in cell necrosis (Virág

et al, 1998). This sequence of events may also occur when peroxynitrite is generated in islet  $\beta$  cells. For example, peroxynitrite addition to human islets produced DNA strand breaks in islet cells, and this was accompanied by mitochondrial dysfunction, detected as impaired glucose oxidation, followed by cell necrosis (Delaney et al, 1996). The observation that cytokines induce apoptosis and not necrosis of human islet cells (Delaney et al, 1997) does not exclude peroxynitrite as a mediator of cytokine-induced  $\beta$ -cell destruction, because peroxynitrite may lead to cell death by either apoptosis or necrosis, depending on the intensity and duration of exposure to the oxidant (Bonfoco et al, 1995; Lin et al, 1995). In the present study, we did not determine whether cytokine-induced peroxynitrite production led to  $\beta$ -cell death by apoptosis or necrosis.

In summary, this study demonstrates that proinflammatory cytokines induce peroxynitrite production in human pancreatic islet  $\beta$  cells and that peroxynitrite, not nitric oxide, is the radical associated with the destructive effects of the cytokines on the  $\beta$  cells. In addition, prevention of production and/or scavenging of peroxynitrite prevented cytokine-induced destruction of human islet  $\beta$  cells. In another recent study, we



**Figure 7.** The cytokine combination of IL-1β (30 U/ml), TNFα (10<sup>3</sup> U/ml), and IFNγ (10<sup>3</sup> U/ml) significantly increases numbers of nitrotyrosine-positive β cells and decreases total β cells in islet cells (10<sup>5</sup>/well) incubated for 5 days. GED (100 μM) significantly decreases numbers of cytokine-induced nitrotyrosine-positive β cells. Values are means ± SE for three experiments. \*p < 0.05, \*\*p < 0.01 vs cytokine-free incubations; † p < 0.05 vs incubations with cytokines alone.

found that prevention of peroxynitrite formation in pancreatic islet β cells of autoimmune diabetes-prone NOD mice prevented β-cell destruction and diabetes development (Suarez-Pinzon et al, 2001). Taken together, these findings suggest that therapeutic interventions that target peroxynitrite may prevent autoimmune destruction of pancreatic islet β cells and insulin-dependent diabetes in humans.

## Materials and Methods

### Cytokines and Chemicals

Recombinant human (rHu) cytokines were used: rHu IL-1β (2–4 × 10<sup>7</sup> U/mg) was provided by Upjohn Co. (Kalamazoo, Michigan), and rHu TNFα (4 × 10<sup>7</sup> U/mg) and rHu IFNγ (1.8 × 10<sup>7</sup> U/mg) were provided by Genentech (South San Francisco, California). Guanidi-

noethyldisulphide ·2HCl(GED), a selective inhibitor of iNOS and scavenger of peroxynitrite (Cuzzocrea et al, 1998; Szabó et al, 1996) was provided by Dr. G. J. Southan (Inotek Corp., Beverly, Massachusetts). L-N<sup>G</sup>-monomethyl arginine (L-NMMA) acetate salt was provided by Dr. S. Moncada (Wellcome Research Laboratory, Beckenham, United Kingdom). Peroxynitrite and its decomposition product (negative control) were purchased from Alexis Biochemicals (San Diego, California). Reagents for immunohistochemical staining were obtained from the following sources: Affinity-purified rabbit anti-nitrotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, New York), and rabbit isotype-matched IgG antibody was purchased from Cedarlane Laboratories (Hornby, Ontario, Canada). 3-nitro-L-tyrosine was purchased from Aldrich Chemicals (Milwaukee, Wisconsin). Guinea pig anti-insulin and control antibodies were purchased from Linco Research (St. Louis, Missouri). Biotinylated goat anti-rabbit and anti-guinea pig antibodies, and streptavidin-peroxidase and streptavidin-alkaline phosphatase conjugates were purchased from Zymed Laboratories (South San Francisco, California). Substrate chromogens, 3-amino 9-ethylcarbazole and 4-chloro 1-naphtol, and Crystal Mount were purchased from Biomedica (Foster City, California). RPMI-1640 and OPTI-MEM 1 culture media, bovine serum albumin, fetal calf serum, HEPES, and cell dissociation buffer were purchased from Life Technologies (Burlington, Ontario, Canada). Saponin and other chemicals were purchased from Sigma Chemicals (St. Louis, Missouri).

### Human Islets

Human pancreases were obtained, with informed consent of relatives, from twelve brain-dead organ donors. The human ethics committee of University of Alberta Hospitals approved tissue procurement and experimental protocols. Pancreases were removed from donors after in situ vascular perfusion with University of Wisconsin organ preservation solution, and islets were isolated, as previously described (Lakey et al, 1999; Ricordi et al, 1988). Briefly, islets were isolated by intraductal controlled perfusion and digestion of the pancreases using an enzyme (Liberase human islet, Roche, Montreal, Quebec, Canada) and by gentle mechanical dissociation. Islets were then purified on continuous gradients of Ficoll-diatrizoic acid in a Cobe blood cell processor (model 2991; Cobe Laboratories, Lakewood, Colorado). For some experiments, islets were dissociated into single cells by incubation at 37° C for 10 minutes in Ca<sup>2+</sup>/Mg<sup>2+</sup>-free PBS containing 0.2 mg/ml EDTA (cell dissociation buffer), followed by syringe injection through progressively narrower gauge needles from sizes 16 to 22.

### Islet and Islet Cell Incubations

Islets (10<sup>3</sup>) were incubated in 1.6 ml medium in 35 × 10-mm Falcon tissue culture dishes (Becton Dickinson, Lincoln Park, New Jersey). Islet cells (10<sup>5</sup>) were incubated in 170 μl 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, Naperville, Illinois) and incubated for 30 minutes at 37° C (5% CO<sub>2</sub>) to allow the cells to attach to the slide before adding 200  $\mu$ l medium. Islets and islet cells were incubated for 4 to 6 days at 37° C (5% CO<sub>2</sub>) in RPMI-1640 medium containing 11 mM D-glucose and supplemented with 2 mM L-glutamine, 0.1 mM sodium pyruvate, 10% heat-inactivated fetal calf serum, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.25  $\mu$ g/ml amphotericin B, and 12 mM HEPES, and the medium was changed every 2 days. The islets and islet cells were then washed in phenol red-free OPTI-MEM 1 reduced serum medium containing 0.3 mM L-arginine, 11 mM D-glucose, and supplemented with 2% bovine serum albumin, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin, 0.25  $\mu$ g/ml amphotericin B, and 12 mM HEPES (test medium). In the first set of experiments, islets and islet cells were incubated at 37° C (5% CO<sub>2</sub>) for 1, 3, and 5 days in test medium, with and without the cytokine combination of IL-1 $\beta$  (30 U/ml), TNF $\alpha$  ( $10^3$  U/ml), and IFN $\gamma$  ( $10^3$  U/ml). Islets and islet cells were washed three times in PBS. One group of islets was assayed for nitrotyrosine content and another group for insulin content. The dissociated islet cells were examined for cell viability by 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT, Sigma) assay. In the second set of experiments, islets and islet cells were incubated at 37° C (5% CO<sub>2</sub>) for 14 hours in test medium with peroxynitrite decomposition product (negative control), medium with peroxynitrite (500  $\mu$ M), medium with guanidinoethylidithiophosphate (GED, 100  $\mu$ M), and medium with peroxynitrite and GED. Islets and islet cells were washed three times in PBS, and then one group of islets was assayed for nitrotyrosine content and another group for insulin content. The dissociated islet cells were examined for cell viability by MTT assay.

In the third set of experiments, islets were incubated at 37° C (5% CO<sub>2</sub>) for 5 days in test medium with cytokines, IL-1 $\beta$  (30 U/ml), TNF $\alpha$  ( $10^3$  U/ml), and IFN $\gamma$  ( $10^3$  U/ml) alone and cytokines together with GED (3, 10, 30, and 100  $\mu$ M). The islets were washed three times in PBS, and then one group of islets was assayed for nitrotyrosine content and another group for insulin content. In the fourth set of experiments, islets were incubated at 37° C (5% CO<sub>2</sub>) for 5 days in test medium alone, medium with cytokines IL-1 $\beta$  (30 U/ml), TNF $\alpha$  ( $10^3$  U/ml), and IFN $\gamma$  ( $10^3$  U/ml), medium with L-N<sup>G</sup>-monomethyl arginine (L-NMMA, 1 mM), medium with cytokines and L-NMMA, medium with GED (100  $\mu$ M), and medium with cytokines and GED. Media from one group of islets were assayed for contents of nitrite and hydrogen peroxide. Islets were washed three times in PBS. One group of islets was assayed for nitrotyrosine content, one group for insulin content, and a third group for DNA content. In the fifth set of experiments, islet cells were incubated at 37° C (5% CO<sub>2</sub>) for 5 days in test medium alone, medium with cytokines, IL-1 $\beta$  (30 U/ml), TNF $\alpha$  ( $10^3$  U/ml), and

IFN $\gamma$  ( $10^3$  U/ml), medium with GED (100  $\mu$ M), and medium with cytokines and GED. Islet cells were washed three times in PBS, and then processed for immunohistochemical studies to identify and quantify nitrotyrosine-positive islet  $\beta$ -cells.

### **Nitrite Assay**

Nitrite, the stable end product of nitric oxide in aqueous solution, was measured in islet incubation media by an on-line semiautomated procedure using HPLC (Green et al, 1982), modified as previously reported (Rabinovitch et al, 1996).

### **Nitrotyrosine Assay**

Sample preparation was as previously described (Hensley et al, 1997). Islets were briefly sonicated in 400  $\mu$ l sodium acetate (10 mM, pH 6.5), and then rapidly vortexed for 1 hour and centrifuged for 10 minutes at 12,000  $\times$  g. A 50- $\mu$ l aliquot of the supernatant was removed for protein assay by the bicinchoninic acid method (Pierce, Rockford, Illinois). To 150  $\mu$ l of the supernatant was added 25  $\mu$ l sodium acetate buffer and 50  $\mu$ l pronase (1 mg/ml in acetate buffer). The solution was then heated at 50° C for 18 hours 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 minutes. The supernatant was frozen at -20° C until derivatization and quantitation by HPLC, as previously described (Kamisaki et al, 1996). Derivatization of nitrotyrosine was performed by adding 10  $\mu$ l sodium borate, 0.1 M, pH 8.7 and 10  $\mu$ l 4-fluoro-7-nitrobenzo-2-oxa-1,3-diazole (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 minutes. The reaction was terminated by the addition of 15  $\mu$ l 0.1 M HCl, and an aliquot (50–80  $\mu$ l) was injected into the HPLC column. The chromatography procedure was as previously described (Kamisaki et al, 1996). The detection limit for nitrotyrosine was approximately 1 pmol at a signal-to-noise ratio of 5.

### **Hydrogen Peroxide Assay**

Hydrogen peroxide in islet incubation media was measured by spectrophotometry using an assay based on the peroxidase-oxidase reaction and able to measure hydrogen peroxide concentrations as low as the nanomolar range (Demmano et al, 1996).

### **MTT Assay**

Islet cell viability was determined by a colorimetric assay that detects the reduction of MTT into soluble blue colored formazan crystals (Mosmann, 1983; Sladowski et al, 1993).

### **Insulin Assay**

Insulin was extracted from islets by incubation in acidified ethanol (75% ethanol, 1.5% 12 mM HCl, and

23.5% H<sub>2</sub>O) for 18 hours 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) for human insulin.

### DNA Assay

DNA in islets was assayed by a modification of the method of Duke and Sellins (Duke and Sellins, 1989), as previously described (Rabinovitch et al, 1996).

### Immunohistochemical Studies

Islet cells attached to tissue culture chamber glass slides were fixed with 4% paraformaldehyde in PBS for 10 minutes and washed twice in PBS. The slides were stored at -70° C until cell staining was performed, as previously described (Suarez-Pinzon et al, 1997). Briefly, the fixed cells were permeabilized with 1.5% saponin in PBS (PBS-saponin). Endogenous cell peroxidase was blocked by incubation in PBS-saponin containing 1% H<sub>2</sub>O<sub>2</sub>, followed by 20% normal goat serum. The cells were incubated first with rabbit anti-nitrotyrosine antibody, 10 µg/ml. Control incubations were performed with rabbit isotype-matched IgG and a mixture of anti-nitrotyrosine antibody and 3-nitro-L-tyrosine, 10 mM, to neutralize the anti-nitrotyrosine antibody. Next, the cells were incubated with a secondary antibody, biotinylated goat anti-rabbit IgG, and then with streptavidin-peroxidase conjugate and substrate chromogen, 3-amino 9-ethylcarbazole, which stained nitrotyrosine-containing cells red. β-cells were then identified by incubating the islet cells with guinea pig anti-insulin antibody, diluted 1:30 in PBS-saponin or guinea pig isotype control antibody. Secondary antibody was biotinylated goat antiguinea pig Ig, then streptavidin-alkaline phosphatase conjugate and substrate chromogen, 4 chloro 1-naphtol, which stained the insulin-containing β cells blue. β cells containing nitrotyrosine were identified by a deep blue granular staining (insulin) against a more diffuse intense red cytoplasmic staining (nitrotyrosine). Stained cells were mounted on the slides using Crystal Mount. A total of 3000 cells was scored blindly by two independent observers who each scanned 60 different microscopic fields (oil immersion, ×100).

### Statistical Analysis

Islets and islet cells from individual donor pancreases were incubated in triplicate for each incubation condition, and mean values were calculated for each incubation condition for a given pancreas (one experiment). Data are expressed as means ± SE for three to five different experiments (one donor pancreas per experiment). Differences between group means were analyzed by Student's unpaired *t* test, or by ANOVA and Bonferroni's multiple comparisons posthoc test, as appropriate. Values for *p* < 0.05 were considered statistically significant.

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