Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans

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ABSTRACT Cytokines have been implicated as immunological effector molecules that mediate beta cell destruction associated with insulin-dependent diabetes mellitus. In this report we demonstrate that the cytokine combination of human recombinant interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), and interferon γ (IFN-γ) induces the formation of nitric oxide by human islets. This combination of cytokines stimulates both the formation of the nitric oxide derivative, nitrite, and the accumulation of cGMP by human islets. The nitric oxide synthase inhibitor N(G)-monomethyl-L-arginine prevents formation of both cGMP and nitrite. IL-1β and IFN-γ are sufficient to induce nitric oxide formation by human islets, whereas TNF-α potentiates nitrite production. This combination of cytokines (IL-1β, TNF-α, and IFN-γ) also influences insulin secretion by human islets. Pretreatment of human islets with low concentrations of this cytokine combination (IL-1β at 15 units/ml, 0.7 nM TNF-α, and IFN-γ at 150 units/ml) appears to slightly stimulate insulin secretion. Higher concentrations (IL-1β at 75 units/ml, 3.5 nM TNF-α, and IFN-γ at 750 units/ml) inhibit insulin secretion from human islets, and the inhibitory effect is prevented by N(G)-monomethyl-L-arginine. This higher concentration of cytokines also induces the formation of an electron paramagnetic resonance-detectable g = 2.04 axial feature by human islets that is characteristic of the formation of an iron-dithio-dinitrosyl complex. The formation of this complex is prevented by N(G)-monomethyl-L-arginine, thus confirming that this cytokine combination induces the formation of nitric oxide by human islets. These results indicate that nitric oxide mediates the inhibitory effects of cytokines on glucose-stimulated insulin secretion by human islets and suggest that nitric oxide may participate in beta-cell dysfunction associated with insulin-dependent diabetes mellitus.

Insulin-dependent diabetes mellitus (IDDM) is an autoimmune disease characterized by specific destruction of the pancreatic islet beta cell (1). The destruction of beta cells is believed to be mediated by infiltrating lymphocytes. The ability of T cells to adoptively transfer diabetes in diabetes-prone BB rats (2) and in the NOD mouse indicates that T cells participate in beta-cell destruction (3). Cytokines, released by infiltrating lymphocytes, have also been implicated as possible mediators of beta-cell destruction. Pretreatment of isolated rat islets with the cytokine human recombinant interleukin 1β (IL-1β) results in a concentration- and time-dependent inhibition of glucose-stimulated insulin secretion that is followed by islet destruction after prolonged exposures to this cytokine (4, 5).

The free-radical nitric oxide has been implicated as the cellular effector molecule that mediates the inhibitory and cytotoxic effects of IL-1β on rat islets (6). Pretreatment of rat islets for 18–24 hr with IL-1β results in nearly complete inhibition of glucose-stimulated insulin secretion that is prevented by the nitric oxide synthase inhibitors, N(G)-nitro-L-arginine methyl ester and N(G)-monomethyl-L-arginine (NMMA) (7–9). These inhibitors also prevent IL-1β-induced nitrite production, cGMP accumulation, and the formation of electron paramagnetic resonance (EPR)-detectable iron-nitrosyl complexes by rat islets (9, 10). Nitric oxide appears to mediate the inhibitory effects of IL-1β by targeting intra-cellular iron-sulfur centers, resulting in inhibition of the enzymatic activity of mitochondrial iron-sulfur-containing enzymes (8–10).

Nitric oxide is the product of the oxidation of L-arginine to L-citrulline by nitric oxide synthase. Two isoforms of nitric oxide synthase have been characterized (11, 12). Cytokines and endotoxin induce the expression of one isoform of nitric oxide synthase, which generates high levels of nitric oxide that is cytotoxic or cytostatic to target cells (13, 14). Expression of the other isoform is constitutive, and the low levels of nitric oxide produced by this isoform function as a signaling molecule (15, 16). Although the constitutive isoform of nitric oxide synthase has been purified from human brain (17), only a limited number of studies have provided evidence for the presence of the inducible isoform in human tissue. Isolated human monocytes have been shown to produce nitrite in response to lipopolysaccharide (18), and isolated human hepatocytes produce nitric oxide after pretreatment with a mixture of cytokines and endotoxin (19). Also, Hibbs et al. (20), have shown increased levels of nitrate in both serum and urine in patients after IL-2 therapy, and Ochoa et al. (21, 22) have shown increased nitrogen oxide levels in patients after trama, during sepsis, and after human tumor immunotherapy.

The presence of both isoforms of nitric oxide synthase has been confirmed in rat islets (7–10, 23, 24), but neither isoform has been demonstrated in human islets. The effects of cytokines on nitric oxide formation and the effects of nitric oxide on insulin secretion by human islets have also not been characterized. In this report evidence is presented that implicates cytokine-induced nitric oxide formation by human islets as an effector molecule that may mediate islet dysfunction associated with IDDM.

MATERIALS AND METHODS

Materials. Recombinant human interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) were obtained from Boehringer Mannheim and IL-1β was from Cistron Biotechnology (Pine Brook, NJ). CMRL-1066 tissue culture medium was from Gibco. NMMA acetate was obtained from Calbio.

Abbreviations: IL-1β, human recombinant interleukin 1β; IFN-γ, interferon γ; TNF-α, tumor necrosis factor; NMMA, N(G)-monomethyl-L-arginine; IDDM, insulin-dependent diabetes mellitus; EPR, electron paramagnetic resonance.

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chem. All other chemicals were from commercially available sources.

**Human Islet Isolation and Cytokine Exposure.** Human islets, provided by David Scharp (Washington University School of Medicine), were isolated by collagenase digestion, as described (25). Human islets were either cultured for 7 days in complete CMRL-1066 tissue culture medium (CMRL-1066/2 mM L-glutamine/10% heat-inactivated fetal bovine serum, penicillin at 100 units/ml/streptomycin at 100 µg/ml/25 mM Hepes, pH 7.4) at 37°C, cryopreserved, or used freshly after isolation; CMRL-1066 contains 0.3 mM L-arginine. No apparent differences in nitric oxide production by human islets were seen under any of these culture conditions.

Human islets were incubated for 24–48 hr at 37°C in complete CMRL-1066 before the initiation of experiments. After this culture period, human islets were washed in 2.5 ml of complete CMRL-1066 three times and then counted into Petri dishes containing 1 ml or 2.5 ml of complete CMRL-1066. Cytokines were added to final concentrations as indicated in figure legends, and the islets were incubated for various periods under an atmosphere of 95% air/5% CO₂ at 37°C.

**Glucose-Induced Insulin Secretion.** After the culture period with cytokines human islets were washed three times (3 ml per wash) in Krebs–Ringer bicarbonate buffer (KRB: 25 mM Hepes/115 mM NaCl/24 mM NaHCO₃/5 mM KCl/1 mM MgCl₂/2.5 mM CaCl₂, pH 7.4) containing 3 mM d-glucose and 0.1% bovine serum albumin. Groups of 30 human islets were counted into 10 × 75 mm siliconized borosilicate tubes and preincubated for 30 min in 200 µl of the same buffer. The preincubation buffer was removed, and glucose-stimulated insulin secretion was initiated by adding 200 µl of fresh KRB containing either 3 mM or 20 mM d-glucose, followed by a 30-min incubation. Both preincubation and incubation were done under 95% air/5% CO₂ at 37°C with shaking. Insulin secretion was determined on the incubation buffer by insulin RIA.

**Measurement of cGMP Levels.** Isolated human islets (100) were incubated in complete CMRL-1066 for various periods at 37°C under 95% air/5% CO₂ in 1-ml Petri dishes containing the indicated concentrations of cytokines or NMMA. Iso-butylmethylxanthine was added to a final concentration of 1 mM, and the islets were incubated for 30 additional min at 37°C. The human islets were isolated and incubated in 1 ml of 6% trichloracetic acid for 1 hr at 4°C and then sonicated for 15 sec on setting 3 of a Branson sonifier, model 250. The cell lysate was centrifuged at 2500 × g for 15 min at 4°C, and the supernatant was extracted three times with 4-ml portions of water-saturated ether. Samples were lyophilized to dryness, and cGMP was determined using a commercially available cGMP RIA kit (DuPont/NEN).

**Nitrite Determination.** Groups of 160 islets were cultured for 24 or 48 hr in 200 µl of complete CMRL-1066. One hundred microliters of the supernatant, obtained by centrifugation, was mixed with 100 µl of Griess reagent (26), and the formation of nitrite was determined by measuring the absorbance at 540 nm with a Titertek Multiskan MCC/340 plate reader.

**EPR Spectroscopy.** At least 7000 human islets cultured in 8 ml of complete CMRL-1066 or CMRL-1066 containing IL-1β at 75 units/ml, 3.5 nM TNF-α, and IFN-γ at 750 units/ml, or 0.5 mM NMMA, or this cytokine and NMMA combination for 18 hr at 37°C were isolated and frozen at −70°C. EPR spectroscopy was done on the islets at 77 K with a Bruker 300 spectrometer at a frequency of 9.44 GHz and a receiver gain of 3.2 × 10³. The power was 1 mW, the modulation frequency was 9.44 GHz.

**RESULTS AND DISCUSSION**

Because IL-1β-induced nitric oxide production has been implicated as the cellular effector molecule that mediates rat islet dysfunction, we examined the effects of IL-1β on nitrite production by human islets. At a concentration (15 units/ml) that induces islet dysfunction and nitric oxide production by rat islets, IL-1β does not appear to induce the formation of nitrite by human islets (Fig. 1). At this concentration IL-1β also has no effect on glucose-stimulated insulin secretion by human islets (data not shown). However, the combination IL-1β and IFN-γ induces the formation of nitrite by human islets after a 24-hr exposure. TNF-α potentiates IL-1β- and IFN-γ-induced nitric oxide production by human islets. Individually, IL-1β, TNF-α, or IFN-γ each fails to induce the formation of nitrite by human islets. The requirement for two cytokine signals is similar to that for cytokine-induced nitric oxide formation by several cell types including macrophages, hepatocytes, and endothelial cells (11), but it is in contrast to the individual effects of IL-1β on nitric oxide production by rat islets (6).

To further confirm that cytokines induce the formation of nitric oxide, we examined the effects of IL-1β, TNF-α, and IFN-γ on nitrite formation and the accumulation of cGMP by human islets. cGMP accumulation increases in response to nitric oxide activation of soluble guanylate cyclase (16). Table 1 shows that pretreatment of human islets for 24 and 48 hr with IL-1β at 15 units/ml, 0.7 nM TNF-α, and IFN-γ at 150 units/ml results in the production of nitrite and in the accumulation of cGMP, both of which are prevented by NMMA. Aminoguanidine, a recently identified selective inhibitor of the inducible isofrom of nitric oxide synthase (27), also prevents nitrite formation by human islets.

Because nitric oxide has been implicated as an effector molecule that mediates the inhibitory effects of IL-1β on glucose-stimulated insulin secretion by rat islets, we examined the effects of cytokine-induced nitric oxide production on insulin secretion by human islets (Fig. 2). Pretreatment of human islets for 24 hr with IL-1β at 15 units/ml, 0.7 nM TNF-α, and IFN-γ at 150 units/ml slightly stimulates glucose-induced insulin secretion. Although this stimulation is not statistically significant, it is similar to the effects produced by low concentrations of IL-1β on glucose-induced
Table 1. Cytokine-induced nitrite formation and cGMP accumulation by human islets

<table>
<thead>
<tr>
<th>Condition</th>
<th>Nitrite, pmol/islet</th>
<th>cGMP, fmol/islet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pretreatment for 24 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.7 ± 0.2</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>NMMA</td>
<td>&lt;0.2</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>Cytokines</td>
<td>2.0 ± 0.1</td>
<td>16.0 ± 3.4</td>
</tr>
<tr>
<td>Cytokines + NMMA</td>
<td>0.6 ± 0.3</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Cytokines + AG</td>
<td>0.4 ± 0.09</td>
<td>ND</td>
</tr>
<tr>
<td>Pretreatment for 48 hr</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.9 ± 0.5</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>NMMA</td>
<td>1.8 ± 0.5</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Cytokines</td>
<td>8.5 ± 1.6</td>
<td>25.2 ± 1.1</td>
</tr>
<tr>
<td>Cytokines + NMMA</td>
<td>1.6 ± 0.5</td>
<td>8.7 ± 0.1</td>
</tr>
</tbody>
</table>

Isolated human islets were pretreated with cytokines (IL-1β at 15 units/ml, IFN-γ at 150 units/ml, and 0.7 nM TNF-α), 0.5 mM NMMA, 0.5 mM aminoguanidine (AG), or both this cytokine combination and NMMA, or aminoguanidine for the indicated times in complete CMRL-1066 tissue culture medium. Medium nitrite and islet cGMP levels were determined as described in text. Nitrite values are the average ± SEM of three individual experiments. cGMP values are from an individual experiment that is representative of three experiments. The effects of aminoguanidine on cGMP levels were not determined (ND).

Insulin secretion from rat islets. Pretreatment of rat islets with IL-1β at 0.5 unit/ml for 15–18 hr potentiates glucose-stimulated insulin secretion, whereas pretreatment with IL-1β at 5 units/ml results in a potent inhibition of glucose-stimulated insulin secretion (28). The ability of this cytokine combination to stimulate insulin secretion at these low concentrations does not appear to be related to the production of nitric oxide, as NMMA has little effect on this stimulation. NMMA also has no effect on IL-1-induced stimulation of insulin secretion by rat islets, further suggesting that nitric oxide formation does not participate in this stimulation (8, 9). Evidence suggests that IL-1β-induced stimulation of insulin secretion by rat islets may be related to an increase in the level of proinsulin biosynthesis (29).

The inability of nitric oxide to inhibit insulin secretion at these cytokine concentrations (IL-1β at 15 units/ml, 0.7 nM TNF-α, and IFN-γ at 150 units/ml) may reflect the possibility that a threshold level of nitric oxide is required to inhibit insulin secretion by human islets. To determine a concentration of these cytokines that causes the maximum production of nitric oxide, human islets were incubated for 24 hr with increased concentrations of IL-1β, TNF-α, and IFN-γ. A 1× concentration of these cytokines is equal to the concentra-

![Fig. 2](image2.png)

Fig. 2. Effects of IL-1β, TNF-α, and IFN-γ on glucose-stimulated insulin secretion by human islets. Human islets were pretreated for 24 hr with IL-1β at 15 units/ml, 0.7 nM TNF-α, IFN-γ at 150 units/ml, or 0.5 mM NMMA in complete CMRL-1066 tissue culture medium, as indicated. The islets were then washed three times in KRB buffer/3 mM D-glucose and counted into tubes (30 islets per tube); insulin secretion was then induced. Results are the average ± SEM of two individual experiments containing three replicates per condition.

![Fig. 3](image3.png)

Fig. 3. Effect of cytokine concentration on nitrite formation by human islets. Human islets (160 islets per 200 μl of complete CMRL-1066 medium) were incubated for 24 hr at 37°C in the presence of increased concentrations of the cytokine combination of IL-1β, TNF-α, and IFN-γ, where a 1× concentration is equal to IL-1β at 15 units/ml, 0.7 nM TNF-α, and IFN-γ at 150 units/ml. The medium was removed, and formation of nitrite was determined. Results are the average ± SEM of an individual experiment containing three replicates per condition and are representative of three experiments.

![Fig. 4](image4.png)

Fig. 4. Pretreatment of human islets for 24 hr with this cytokine combination results in a potent inhibition of glucose-stimulated insulin secretion. Nitric oxide appears to participate in cytokine-induced inhibition of insulin secretion because NMMA partially prevents the inhibitory effects of these cytokines on insulin secretion by human islets. However, NMMA does not completely prevent cytokine-induced inhibition of insulin secretion by human islets, suggesting that other factors may also be involved. These factors may include oxygen free radicals that have been implicated as destructive agents in cytokine-induced islet-cell lysis (30).
The individual effects of these cytokines on nitrite formation, at concentrations required to inhibit glucose-stimulated insulin secretion by human islets, were also examined. Fig. 5 shows that individually neither IL-1β at 75 units/ml, 3.5 nM TNF-α, nor IFN-γ at 750 units/ml induced the formation of nitrite after a 24-hr culture period. However, IL-1β and IFN-γ in combination induced a 5-fold increase in the level of nitrite produced, as compared with untreated control islets. TNF-α further stimulates the production of nitrite by these two cytokines. These results are completely consistent with the individual effects of these cytokines at 5-fold lower concentrations (Fig. 1).

To confirm that IL-1β at 75 units/ml, 3.5 nM TNF-α, and IFN-γ at 750 units/ml induce the formation of nitric oxide by human islets, the effect of this cytokine combination on the formation of EPR-detectable iron–nitrosyl complexes was examined. Fig. 6 shows that pretreatment of human islets for 18 hr with IL-1β at 75 units/ml, 3.5 nM TNF-α, and IFN-γ at 750 units/ml induces the formation of an axial g = 2.04 feature that is characteristic of an iron–nitrosyl complex. The formation of an EPR-detectable g = 2.04 iron–nitrosyl complex has been used to confirm the formation of nitric oxide by activated macrophages (31, 32), macrophage target cells (33), rat islets (9, 10), and the insulinoma cell line RINm5F (34). NMMA prevents the formation of this axial feature, demonstrating the requirement for nitric oxide synthase activity. The simultaneous formation of nitrite from the same islets used for EPR spectroscopy was also examined. Cytokines stimulated an increase in nitrite production from an untreated control value of 0.3 ± 0.2 pmol per islet to 2.05 ± 0.1 pmol per islet, and NMMA blocked this increase in nitrite formation. The formation of this iron–nitrosyl complex and the simultaneous formation of nitrite further confirm that cytokines induced the production of nitric oxide by human islets.

Data presented in this study indicate that human islets respond to cytokines in a concentration-dependent manner. Low concentrations of cytokines appear to slightly stimulate insulin secretion, whereas higher concentrations inhibit glucose-induced insulin secretion by human islets. This result is similar to the effects of IL-1β on insulin secretion by isolated rat islets (4, 5, 28). Nitric oxide appears to participate in cytokine-induced inhibition of insulin secretion by human islets, because NMMA partially prevents these effects. These results provide evidence for the involvement of the free-radical nitric oxide in cytokine-induced human beta-cell dysfunction that occurs early in IDDM.
The cellular mechanism by which nitric oxide mediates cytokine-induced inhibition of glucose-stimulated insulin secretion appears to be the result of iron–sulfur-center destruction of iron–sulfur-containing enzymes. The formation of EPR-detectable iron–nitosyl complexes has been demonstrated to occur in macrophages, hepatocytes, and islets, under conditions in which mitochondrial electron transport at complexes 1 and 2, and mitochondrial aconitase activity are impaired (6, 14). Also glucose-stimulated insulin secretion is specifically sensitive to impaired mitochondrial function, as glucose metabolism is absolutely required for insulin secretion. These results implicate nitric oxide-mediated mitochondrial iron–sulfur protein destruction as the mechanism of cytokine-induced impairment of the insulin secretory response by human islets.

IDDM is characterized by lymphocytic infiltration into the islet. This cellular infiltrate contains macrophages and monocytes that produce IL-1 and TNF, and T cells that produce IFN-γ. The release of these cytokines may induce the expression of nitric oxide synthase by human islets, as well as by macrophages. We have recently demonstrated that IL-1β induces the formation of nitric oxide by the rat islet beta cell (34), and many laboratories have reported that TNF and IFN-γ induce the formation of nitric oxide by macrophages. In addition, activated rat macrophages have been shown to kill syngeneic islet cells by α-arginine-dependent nitric oxide formation (35), and NMMA appears to partially prevent low-dose streptozotocin-induced diabetes in male CBA mice (36, 37). Because nitric oxide appears to mediate both cytokine-induced inhibition of insulin secretion by human and rat islets and rat islet cell lysis, it is tempting to implicate nitric oxide as an effector molecule that may participate in beta-cell dysfunction and destruction observed in IDDM. Cytokine-induced nitric oxide formation does not preclude the involvement of cytotoxic T cells that are believed to be required for autoimmune beta-cell destruction. The production of cytotoxic amounts of nitric oxide by beta cells, and possibly macrophages, may function in concert with T cells, resulting in beta-cell destruction.

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