Perspectives in Diabetes

Does Nitric Oxide Mediate Autoimmune Destruction of β-Cells?
Possible Therapeutic Interventions in IDDM

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Cytokines have been implicated as immunological effector molecules that induce dysfunction and destruction of the pancreatic β-cell. The mechanisms of cytokine action on the β-cell are unknown; however, nitric oxide, resulting from cytokine-induced expression of nitric oxide synthase, has been implicated as the cellular effector molecule mediating β-cell dysfunction. Nitric oxide is a free radical that targets intracellular iron-containing enzymes, which results in the loss of their function. The cytokine IL-1β induces the formation of nitric oxide in isolated rat islets and the insulinoma cell line, RINm5F. NMMA and NAME, both inhibitors of nitric oxide synthase, completely protect islets from the deleterious effects of IL-1β. These inhibitors are competitive in nature and inhibit both the cytokine-inducible and constitutive isoforms of nitric oxide synthase with nearly identical kinetics. This may preclude their use as therapeutic agents because of increases in blood pressure which result from the inhibition of constitutive nitric oxide synthase activity. Aminoguanidine, an inhibitor of nonenzymatic glycoxidation of cellular and extracellular constituents associated with diabetic complications, recently has been reported to inhibit nitric oxide synthase. Aminoguanidine is 40-fold more effective in inhibiting the inducible isofrom of nitric oxide synthase, suggesting that aminoguanidine or analogues may serve as potential therapeutic agents to block diseases associated with nitric oxide production by the inducible isofrom of nitric oxide synthase. In vivo administration of TNF IL-1 has been shown to induce antidiabetogenic effects in the NOD mouse. This antidiabetogenic effect of cytokines appears to conflict with evidence suggesting that cytokines mediate β-cell dysfunction. The role of nitric oxide in both cytokine-mediated β-cell dysfunction, and the antidiabetogenic effects of cytokines, as well as the potential therapeutic use of aminoguanidine, are evaluated in this study. Diabetes 41:897–903, 1992

Cytokines have been implicated as immunological effector molecules that mediate β-cell dysfunction and destruction associated with IDDM. IDDM is characterized by lymphocytic infiltration of the pancreatic islet, followed by β-cell death (1). In animal models of immune-mediated diabetes, macrophages have been demonstrated to be among the first inflammatory cells to infiltrate the islet (2,3). These macrophages express class II MHC antigens and secrete cytokines, including IL-1 and TNF. Using a crude cytokine preparation isolated from human peripheral blood mononuclear cells, Mandrup-Poulsen et al. (4) demonstrated that such a preparation affected rat islet function and viability in a concentration- and time-dependent fashion (5). IL-1 antibodies or removal of IL-1 from the crude cytokine preparation by affinity chromatography prevented these inhibitory effects, suggesting that IL-1 was required for cytokine-mediated inhibition of insulin secretion from rat islets (6). Pretreatment of isolated rat islets for 18–24 h with human recombinant IL-1 resulted in an inhibition of glucose-stimulated insulin secretion, protein biosynthesis, and islet-cell replication (7–11). Under these conditions, IL-1-induced impairment of β-cell function was followed by β-cell death in a concentration- and time-dependent fashion (10). These results led Mandrup-Poulsen et al. (10) to propose a model that suggests a triggering event occurs in a single islet and that it requires the presence of both macrophages and helper T-cells. This triggering event leads to the release, processing, and presentation of β-cell antigens, resulting
in the initiation of the immune response. The immune response results in an increase in the local concentration of cytokines IL-1, TNF, and IFN-γ, which leads to the destruction of the β-cell via the inhibition of mitochondrial function. This is possibly caused by the production of oxygen free radicals, which function as secondary mediators. The process continues until the islet is devoid of β-cells. In this study, we examine the effects of cytokines on β-cell function and viability, and we propose a cellular effector mechanism by which cytokine-induced β-cell death requires the induction of the enzyme nitric oxide synthase, which catalyzes the formation of the free radical nitric oxide. We also propose novel strategies for the prevention of or intervention in disease states (such as IDDM) associated with nitric oxide production.

**CYTOKINES**

Cytokines are small antigen-nonspecific glycoproteins (10–30 kDa) that are synthesized and rapidly secreted by a variety of different cells in response to numerous stimuli. Cytokines produce many effects on their target cells, including the promotion of cell proliferation and gene induction. The specific cytokines required to induce β-cell dysfunction and destruction depend on the species being studied. IL-1 alone appears to be sufficient to induce β-cell dysfunction and destruction in the rat (10–12), whereas TNF, which alone has no apparent effect on islet function or viability, functions in a synergistic fashion with IL-1 (10–12). The synergistic effect of TNF and IL-1 appears to be a common theme in many cytokine-mediated effector systems. In the mouse, IFN-γ and TNF appear to produce β-cell death (13); this same cytokine combination also appears to be cytotoxic to human islet cells (14,15). These results are striking because they illustrate the different cytokine requirements among the mouse, rat, and human species in the mechanism of β-cell destruction.

Paradoxically, in vivo injections of TNF or IL-1 also produce antidiabetogenic effects in the NOD mouse (16). The mechanism by which these cytokines produce such variable effects is unknown. We propose that the antidiabetogenic effects observed with in vivo administration of this cytokine may be the result of macrophage-mediated nitric oxide formation. L-arginine metabolism through the nitric oxide synthase pathway appears to mediate suppressor macrophage activity of mitogen-induced rat or mouse lymphocyte proliferation in vitro (17). In these studies, Mills (17) also has shown that inhibitors of nitric oxide synthase augment mitogen-induced lymphocyte proliferation. TNF induces nitric oxide formation by macrophages in vitro, suggesting that the antidiabetogenic effects of TNF in the NOD mouse may be caused by TNF-induced nitric oxide generation by macrophages, which results in the inhibition of lymphocyte proliferation. Furthermore, CFA also has been shown recently to prevent insulitis and diabetes in the NOD mouse (18). The prevention of diabetes was associated with an inhibition of lymphocyte proliferation. CFA contains endotoxins known to stimulate nitric oxide production by macrophages, further suggesting that nitric oxide production may inhibit lymphocyte proliferation and thereby prevent diabetes. However, other defects in the NOD mouse (e.g., cytokine release) also might participate in the process.

**BIOCHEMICAL MECHANISM OF IL-1 MEDIATED β-CELL DYSFUNCTION**

**Oxygen radicals.** Highly reactive oxygen free radicals have been suggested to function as secondary effector molecules that may mediate β-cell dysfunction and destruction (10,19). It has been proposed that the oxygen free radicals, superoxide and hydroxyl radical, are liberated either by infiltrating macrophages or that cytokines secreted by infiltrating lymphocytic cells induce the formation of oxygen free radicals within the β-cell mitochondria (10). Islets appear to be sensitive to oxygen radical formation because of low concentrations of mitochondrial Mn superoxide dismutase (20) and glutathione peroxidase (21)—endogenous enzymatic scavengers of oxygen free radicals that convert the highly reactive superoxide anion to hydrogen peroxide, water, and molecular oxygen. Desferrioxamine, an agent that binds iron, which prevents the formation of oxygen free radicals via the Fenton reaction, reduces the incidence of diabetes associated with multiple low dose STZ injections (22). Free radical scavengers also have been used to prevent the inhibitory effects of cytokines on insulin secretion in vitro (23). Although controversial, nicotinamide, which at high concentrations functions as an oxygen free radical scavenger, has been reported to partially prevent or delay the onset of IDDM in children predisposed to diabetes based on the presence of islet-cell antibodies (24). In vitro free radical scavengers have been used to prevent the inhibitory effects of cytokines on β-cell function; however, complete protection has never been achieved by any of these types of interventions (10,19).

**Nitric oxide.** Nitric oxide is the product of the mixed functional oxidation of one of the guanidino nitrogens of L-arginine to L-citrulline by nitric oxide synthase (Fig. 1; 25,26). At least two different isoforms of nitric oxide synthase (constitutive and cytokine inducible) have been characterized. These isoforms are differentiated by gene expression and cofactor requirements. The constitutive isoform of nitric oxide synthase is a Ca2+ and calmodulin-dependent enzyme (27,28), while expression of the

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**FIG. 1.** L-arginine-dependent nitric oxide pathway.
other isomorph is inducible by cytokines, and the activity of this isomorph is Ca\(^{2+}\) and calmodulin independent (29,30). Both enzymes are flavoproteins containing bound FAD and FMN, are NADPH dependent, and require tetrahydrobiopterin as an additional cofactor (28,29). The constitutive enzyme is present in endothelium, brain, platelets, and recently has been shown to exist in islets (28,31,31a). Endothelin, TNF, IFN-\(\gamma\), and IL-1 have been shown to induce the expression of the inducible isomorph of nitric oxide synthase in macrophages, smooth muscle, microglial cells, mesangial cells, hepatocytes, and \(\beta\)-cells of pancreatic islets (28,30,32).

The biological functions of nitric oxide differ depending on the isomorph of nitric oxide synthase. The constitutive enzyme releases low levels of nitric oxide in response to physiologic stimuli or receptor activation. Nitric oxide released under these conditions functions as a signaling molecule that mediates several physiologic responses, including endothelium-dependent relaxation and the inhibition of platelet aggregation—it may also participate in glucose-stimulated insulin secretion (28,31,31a). These responses are believed to be mediated by the direct activation of guanylate cyclase by nitric oxide, resulting in the formation of cGMP. Nitric oxide is produced in much larger quantities by the cytokine-inducible isomorph of nitric oxide synthase and appears to function as an effector molecule that mediates cytostatic and cytotoxic effects (30). These effects apparently are caused by nitric oxide-mediated destruction of iron-sulfur centers of iron-containing enzymes and result in an impairment of mitochondrial function and DNA synthesis (33,34).

Analogue of \(L\)-arginine, in which one of the guanidino nitrogen is either methylated or nitrosylated, have been shown to inhibit nitric oxide synthase. NMMA and NAME are two commonly used compounds that block nitric oxide formation. Both of these inhibitors appear to be competitive in nature and inhibit both the inducible and constitutive isoforms of nitric oxide synthase with nearly identical kinetics. Also, \(D\)-arginine does not serve as a substrate for nitric oxide synthase, which demonstrates that the stereochemistry of the anomeric carbon of \(L\)-arginine must be preserved for nitric oxide synthase activity (28,30).

**Formation of nitric oxide by pancreatic islet \(\beta\)-cells.**

The formation of nitric oxide by islets pretreated with IL-1 was initially demonstrated by the ability of NAME and NMMA to block the inhibitory effects of IL-1 on glucose-stimulated insulin secretion (Fig. 2; 32,35). Importantly, NMMA completely prevents IL-1-induced inhibition of glucose-stimulated insulin secretion. NAME provided partial protection against the inhibitory effects of IL-1 on insulin secretion by islets (35) at a concentration of this competitive inhibitor (1 mM) that is identical to that of the substrate \(L\)-arginine (1 mM). In these studies, IL-1 also was shown to induce the formation of nitrite (an oxidative product of nitric oxide) and both NAME and NMMA block nitrite formation. The formation of nitric oxide by islets was confirmed by the detection of an IL-1-induced iron-nitrosyl complex using electron paramagnetic resonance spectroscopy by our laboratory (Fig. 3; 32). The generation of this iron-nitrosyl complex was prevented by pretreatment of islets with NMMA in addition to IL-1, thus demonstrating the requirement for nitric oxide synthase activity. Furthermore, the protein synthesis inhibitor cycloheximide is known to prevent IL-1-induced inhibition of glucose-stimulated insulin secretion (36). Cycloheximide also blocked the formation of nitric oxide by islets (36a). These studies provided the first evidence that IL-1 induces the expression of nitric oxide synthase by islets and that nitric oxide may be the cellular effector molecule that mediates IL-1-induced impairment of islet function.

**CELLULAR MECHANISM OF IL-1’S ACTIONS**

Inhibition of mitochondrial function has been proposed as the mechanism by which IL-1 mediates an impairment in the insulin-secretory response of the \(\beta\)-cell. Pretreatment of islets with IL-1 results in an inhibition of glucose oxidation (9,37,37a), which appears to be mediated by nitric oxide because NMMA and cycloheximide compete.
This is suggested by our recent observation that IL-1 suggests that the cellular targets for nitric oxide are iron-sulfur centers of iron-containing enzymes (32,38). The ability of IL-1 to induce the formation of nitric oxide by dispersed islet cells, and that this inhibition is prevented by NMMA (unpublished observations). Aconitase is a mitochondrial enzyme that contains iron–sulfur clusters known to be sensitive to nitric oxide (33,34).

The formation of nitric oxide by the islet also may explain the ability of oxygen free radical scavengers to partially protect against the inhibitory effects of IL-1 on β-cell function. Nitric oxide appears capable of interacting with the superoxide anion to form hydroxyl radicals (39). Hydroxyl radicals may produce deleterious effects on mitochondrial function and DNA synthesis. Because the β-cell is unique in that it contains low levels of endogenous oxygen free radical scavenging enzymes (20,21), oxygen radicals generated by the interaction of superoxide with nitric oxide may further participate in β-cell destruction. Oxygen free radical scavengers under these conditions might be expected to partially protect against the inhibitory effects of IL-1 on β-cell function by removing the highly reactive hydroxyl radical.

We have proposed a schematic model by which IL-1 mediates β-cell dysfunction and destruction (Fig. 4). In this model, IL-1 is produced by activated macrophages present during islet infiltration. IL-1 interacts with its receptors on the β-cell triggering a series of signaling events that include the induction of mRNA transcription and protein translation. Receptors for IL-1 have been demonstrated on both the Rin-m5F and HIT insulinoma cell lines (40,41), and transcriptional and translational inhibitors have been shown to prevent IL-1–induced inhibition of glucose-stimulated insulin secretion by isolated islets (36). We propose that IL-1 induces the expression of nitric oxide synthase, which results in the generation of the free radical nitric oxide. Nitric oxide targets iron–sulfur containing enzymes (aconitase, ribonucleotide reductase, and possibly electron transport chain enzymes; 30) resulting in an inhibition of mitochondrial function and DNA synthesis. Nitric oxide mediated inhibition of these enzymes ultimately results in β-cell death. Macrophages infiltrating the islet also may produce nitric oxide and further damage the islet β-cell. Syngeneic activated peritoneal macrophages recently have been demonstrated to kill islet cells by an L-arginine dependent pathway (42).

**Mechanism of β-cell destruction.** The pancreatic islet is a complex microorgan that contains both endocrine cells (β-, α-, δ-, and polypeptide) and nonendocrine cells (macrophages, fibroblasts, endothelial, and dendritic). The β-cell is selectively destroyed in IDDM by a process believed to be mediated by nitric oxide. Although, IL-1–induced formation of nitric oxide by isolated islets has been demonstrated, the islet endocrine cell type responsible for nitric oxide formation is unknown (i.e., does IL-1 induce nitric oxide production by the β-cell?). This question is further complicated by the nonendocrine cells of the islet, which contain the cytokine-inducible isoform of nitric oxide synthase. Using purified populations of β-cells and α-cells isolated by fluorescence-activated cell sorting, we recently demonstrated that IL-1 induces the formation of nitric oxide by purified β-cells. In contrast, IL-1 does not appear to induce the formation of nitric oxide by purified α-cells (unpublished observations). The formation of nitric oxide by purified β-cells is believed to mediate IL-1–induced inhibition of insulin secretion because NMMA prevents the inhibitory effects of this cytokine. IL-1 also has been demonstrated to induce the formation of nitric oxide by the insulinoma cell line Rin-m5F as evidenced by IL-1–induced nitrite and iron-nitrosyl complex formation, both of which are prevented by NMMA (unpublished observations). Because the β-cell is a source of nitric oxide and because macrophages do not appear to produce nitric oxide in response to IL-1 (43), it appears that IL-1 directly induces the expression of nitric oxide synthase by the endocrine β-cell. We currently are examining whether IL-1–induced nitric oxide formation by the β-cell is sufficient to induce β-cell death, or if nitric oxide production by macrophages is also required for β-cell death in vitro.

**INTERVENTIONS**

An understanding of cellular mechanisms involved in the etiology of IDDM may lead to the development of therapeutic agents to intervene in the disease process. Early efforts directed at the use of oxygen free radical scavengers have been disappointing. Immunomodulation studies, such as the previously described antidiabetogenic effects of TNF or IL-1 in the NOD mouse, have proven somewhat more successful. If our hypothesis that nitric oxide mediates β-cell death is correct, then inhibition of nitric oxide generation should prevent the onset of IDDM. This appears to be the case with the multiple low-dose STZ-induced model of autoimmune diabetes. Treatment of male CBA mice with multiple low doses of STZ for 5 days induces hyperglycemia and lymphocytic infiltration into the islet. However, NMMA treatment for 5 days...
following STZ injections partially prevents hyperglycemia and reduces the extent of lymphocytic infiltration into the islet (44). These results provide the first indication that prevention of nitric oxide formation in vivo may block the onset of diabetes, however the cellular mechanism by which NMMA prevents STZ-induced diabetes is unknown. While NMMA is a potent inhibitor of nitric oxide synthase, it may not be suitable for clinical interventions because it is not selective for the inducible isoform of nitric oxide synthase and it also inhibits the constitutive isoenzyme. Inhibition of constitutive nitric oxide synthase by NMMA may prove undesirable because of impaired cell-cell communication and increases in blood pressure.

The current challenge is to identify selective and potent inhibitors for the two isoforms of nitric oxide synthase. Corticosteroids, including dexamethasone, have been shown to block the induction of the inducible isoform of nitric oxide synthase without affecting the activity of the constitutive enzyme (28). We recently demonstrated that dexamethasone also blocks IL-1-induced inhibition of glucose-stimulated insulin secretion and nitric-oxide formation by islets (unpublished observations). These results suggest a potential promise for the use of corticosteroids to block the inducible isoform of nitric oxide synthase, although there are numerous disadvantages to these nonspecific approaches.

In collaboration with Drs. Joseph Williamson and Ronald Tilton at Washington University School of Medicine, we recently discovered an inhibitor (aminoguanidine) that is selective for the inducible isoform of nitric oxide synthase (45). Fig. 5 demonstrates that aminoguanidine blocks IL-1-induced nitrite formation from Rin-m5F cells (IC₅₀ -10 μM) in a concentration-dependent manner similar to the effects of NMMA. In contrast, aminoguanidine is 40-fold less effective than NMMA in increasing mean arterial blood pressure of rats. The selective properties of aminoguanidine on the inducible isoform of nitric oxide synthase have been confirmed using purified constitutive and inducible nitric oxide synthase isoenzymes isolated from rat brain and macrophage, respectively (unpublished observations). Aminoguanidine is an analogue of L-arginine in that it contains two chemically equivalent guanido nitrogen groups in addition to a hydrazine moiety (Fig. 5). This hydrazine moiety is believed to confer selectivity for the inducible isoform of nitric oxide synthase because replacement with a methyl group (methylguanidine) results in the loss of selectivity and reduced potency (unpublished observations). These studies suggest that aminoguanidine may represent a selective and potent inhibitor of the inducible isoform of nitric oxide synthase and warrants examination as a potential therapeutic agent to prevent diseases associated with the inducible isoform of nitric oxide synthase.

The remarkable ability of aminoguanidine to block nitric oxide formation also suggests that nitric oxide may mediate some of the complications associated with diabetes. Brownlee et al. (46) have shown that aminoguanidine is a potent inhibitor of nonglycemic advanced glycation of cellular and extracellular constituents, a process believed to be associated with vascular and neural complications of diabetes. NMMA also recently has been shown to block vascular dysfunction using the skin chamber granulation tissue model (45), suggesting that nitric oxide may participate in vascular dysfunction associated with diabetic complications.

**FUTURE STUDIES**

Important questions that remain to be answered are whether human islets produce nitric oxide and whether nitric oxide mediates β-cell dysfunction and destruction in humans. These questions are complicated by the difficulty in demonstrating nitric oxide production by human tissue in vitro, although indirect evidence of nitric oxide production by humans (based on urinary nitrite levels) has been obtained (47). We recently demonstrated that the cytokine combination of IL-1 and IFN-γ induces nitric oxide production by human islets in vitro; and when presented alone, IL-1, TNF, and IFN-γ have no effect on nitric oxide generation by human islets. The production of nitric oxide by human islets was demonstrated by IL-1 and IFN-γ-induced nitrite formation and cGMP accumulation, both of which were blocked by NMMA (unpublished observations).

The recent identification of nitric oxide as a cellular effector molecule that mediates cytokine-induced β-cell dysfunction and destruction offers promise in the imple-
mentation of new strategies to unravel the cellular mechanisms involved in IDDM. This experimental approach is further enhanced by the discovery of a selective and potent inhibitor of the inducible isozyme of nitric oxide synthase (aminoguanidine). It will be of interest to determine whether aminoguanidine prevents spontaneous diabetes in both the NOD mouse and the BB rat, and if nitric oxide mediates the antidiabeticogenic effects of TNF or IL-1 in vivo. These approaches are important as they may explain both the antidiabetogenic and cytotoxic effects of cytokines, and resolve the paradox involving the protective and cytotoxic role of cytokines in β-cell destruction associated with IDDM.

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