Nitric oxide (NO), initially described as endothelium-derived relaxation factor (EDRF) in blood vessels, has emerged as a fundamental messenger molecule in a variety of cells with a wide spectrum of biological actions. In addition to its pivotal role in the cardiovascular system, NO acts as a neurotransmitter and inhibits platelet aggregation and proliferation of smooth muscle cells (9). These physiological functions are catalyzed by a constitutively expressed NO synthase (NOS) in the vasculature (eNOS) and in the neurons (bNOS), an enzyme that is calcium/calmodulin dependent and releases picomolar amounts of NO in response to receptor stimulation (5).

In addition to conveying these physiological processes, excess NO production by a cytokine-inducible NOS (iNOS) participates in host defense and immunological reactions and may play a role as effector molecule in autoimmune diseases. Thus, since the observation by Southern et al. that pancreatic islet cells produce NO in response to cytokines and that inhibition of NO production protects these from cytokine-induced suppression of insulin release, considerable interest has focused on NO as a mediator of β-cell injury in insulin-dependent diabetes mellitus (11). β-Cells are particularly sensitive to damage by NO and free radicals because of their low levels of free radical-scavenging enzymes such as Mn superoxide dismutase, catalase, and glutathione peroxidase.

NO released in response to exposure of pancreatic islets to cytokines may be formed either in the β-cells themselves, leading to self-destruction of these, or, alternatively, in intraislet macrophages or endothelial cells from which it diffuses into β-cells (Fig. 1A). Evidence for the β-cell as the source and site of action of NO has been provided by Corbett and co-workers (2), who demonstrated that interleukin (IL)-1β was able to induce large amounts of NO in rodent β-cells and β-cell lines but not in α-cells. Furthermore, transgenic mice overexpressing iNOS in pancreatic β-cells develop insulin-dependent diabetes (14). Alternatively, NO could be produced by residual macrophages or endothelial cells within the islets or by infiltrating macrophages (1). There is in vitro evidence that rat islets are lysed by activated rat macrophages, a process that is l-arginine dependent and preventable by blockade of the NOS with N\(^\text{G}\)-monomethyl-l-arginine (L-NMMA) (6).

As pancreatic islets are densely capillarized, the endocrine cells are surrounded by numerous endothelial cells. Endothelial lining cells can, on stimulation by cytokines, be transformed into cytotoxic effector cells and thus contribute to local tissue destruction. In fact, primary cultures of rat islet capillary endothelial cells were found to respond with a marked increase of iNOS mRNA expression and subsequent NO production on exposure to IL-1β, tumor necrosis factor (TNF)−α, and interferon-γ (13).

NO is the first gas known to act as a biological messenger in mammals. It has a very short half-life in vivo and rapidly decomposes by condensation with molecular oxygen to form nitrite and nitrate. Before its decomposition, the highly reactive NO molecule reacts with specific molecular targets, thereby exerting its messenger and cytotoxic effects. Preferential molecular targets of NO are proteins containing iron-sulfur clusters or heme-iron prosthetic groups such as, e.g., guanylyl cyclase. By binding to the iron atom of this...
enzyme, guanosine 3',5'-cyclic monophosphate (cGMP) is synthesized. cGMP is an important second messenger in endothelial and neuronal cells. Also in insulin-producing cells, NO augments cGMP formation (2). On the other hand, by binding to the iron-containing Krebs cycle enzyme aconitase (which converts citrate to isocitrate), NO inhibits enzyme activity leading to decreased glucose oxidation, oxygen consumption, and adenosine 5'-triphosphate (ATP) generation in rat islets (15). Apart from binding to susceptible iron groups in enzymes, NO inhibits ribonucleotide reductase, activates cyclooxygenase and stimulates prostaglandin E\textsubscript{2} synthesis, induces adenosine 5'-diphosphate-ribosylation of glyceraldehyde-3-phosphate dehydrogenase resulting in decreased glycolysis, and directly damages islet cell DNA. DNA strand breaks were observed in islet cells incubated with activated macrophages and sodium nitroprusside. In addition to directly reacting with protein prosthetic groups, NO can also combine with oxygen to eventually produce potent cellular killers such as the highly toxic hydroxyl radical, OH, and peroxynitrate (ONOO\textsuperscript{-}), which is a powerful oxidant. Studies with rat islet cells and monolayer cultures of human islets exposed to various cytokines have, indeed, revealed that NO and oxygen free radicals may act in concert to kill the β-cell.

Although NO appears to play a major role in β-cell destruction in rodent autoimmune diabetes, its importance as an effector molecule of β-cell injury in human insulin-dependent diabetes mellitus is less evident. Human islets and human β-cells are clearly more resistant to the inhibitory effect of NO than rodent islets (4). Thus various combinations of cytokines are required for the induction of iNOS, and the effects on islet function are less dramatic. Furthermore, blockade of NOS does not protect human islets from cytokine-mediated impairment of β-cell function. The increased resistance to damage by NO, free oxygen radicals, and cytokines is caused by higher expression of the antioxidant enzymes catalase and superoxide dismutase and of the heat shock protein HSP 70 in human islets (3). In addition, human islets possess a higher capacity for DNA repair compared with rodent cells, emphasizing the importance of β-cell repair and defense mechanisms for the development of autoimmune diabetes in different species.

Unlike in rodents, evidence is still lacking whether human β-cells are able to produce NO on stimulation with cytokines, nor has it convincingly been shown whether human macrophages can be induced to synthesize and release sufficient amounts of NO to exert toxic effects on β-cells. Thus, although NO is considered to play an important part as an effector molecule in β-cell destruction in rodents, any putative role of NO in the pathogenesis of human autoimmune diabetes needs to be clarified.

Besides its role as an effector molecule of β-cell destruction, several lines of evidence indicate that NO derived from breakdown of L-arginine may be involved in the signal transduction...
pathway of physiological insulin secretion. More than 30 years ago it was shown that L-arginine mediates protein-induced insulin secretion and potentiates D-glucose-induced insulin release. Furthermore, L-arginine deficiency is associated with insulinopenia and a failure to secrete insulin in response to glucose. Evidence that NO could be involved in the signal transduction pathway mediating insulin release, it was argued that the insulinoma-derived HIT-T15 β-cell line may not represent a valid model of β-cells and that the presence of NADPH activity in the islets does not convincingly prove the constitutive expression of NOS in islets and islet β-cells, respectively.

Thus the role of NO in physiological insulin secretion remained controversial. A series of studies looking into accumulated insulin release in cultured islets or using perfused rat pancreas argued against a stimulatory effect of NO on insulin release. Moreover, incubation of islets with a NO inhibitor or perfusion of rat pancreata with NG-nitro-L-arginine methyl ester (L-NAME) and chronic administration of L-NAME to anesthetized rats did not result in a detectable inhibition of insulin release. However, because NO is likely to act rapidly and affect the early phase of insulin secretion, a possible stimulatory effect may be masked in experimental settings with cultured islets or perfused pancreata, because inhibition of NO exerts additional systemic effects on vasoconstriction, increases capillary blood flow, and interferes with neuronal mechanisms.

To circumvent these problems and to study the direct effects of NO on β-cell function, we perfused rat islets in the presence or absence of L-NMMA (12). Ambient glucose was raised from nonstimulatory levels (1.6 mM) to 16 mM, and insulin was determined at 1-min intervals in the perfusate. In the presence of L-NMMA, but not of cGMP release (8). These data suggested a role for NO in the regulation of cGMP levels in β-cells. Along the same line, Schmidt et al. (10) found that β-cell-derived HIT-T15 cells constitutively expressed NOS and produced NO and insulin in the presence of D-glucose. Insulin production was preventable by L-NMMA and NG-nitro-L-arginine, two potent inhibitors of the enzymatic conversion of L-arginine to NO. In these same experiments, NO formation was paralleled by an increase in cGMP, again supporting the notion that L-arginine-derived NO acts principally by stimulating guanylate cyclase. Blockade of the enzyme resulted in reduced glucose-stimulated insulin secretion both in vivo and in vitro (10). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and protein immunoblot analysis of HIT-T15 cells with antisera to purified NOS from rat cerebellum revealed the presence of a constitutive NOS in these β-cell-derived cells. Furthermore, isolated islets of Langerhans displayed reduced nicotinamide adenine dinucleotide phosphate (NADPH) diaphorase, which was merely located at the islet periphery. Although these data were suggestive of an involvement of L-arginine-derived NO in the signal transduction pathway mediating insulin release, it was argued that the insulinoma-derived HIT-T15 β-cell line may not represent a valid model of β-cells and that the presence of NADPH activity in the islets does not convincingly prove the constitutive expression of NOS in islets and islet β-cells, respectively.

FIGURE 2. Effects of NG-monomethyl-L-arginine (L-NMMA; A), NG-monomethyl-D-arginine (D-NMMA; B), and carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide PTIO (C) on glucose-stimulated insulin secretion from perfused rat islets. Batches of 50 islets were perfused in the presence or absence of 0.5 mM L-NMMA or D-NMMA or 10 mM carboxy-PTIO. Arrows indicate the beginning and end of the first phase. Traces show control (Ο), L-NMMA (■), D-NMMA (●), and carboxy-PTIO (□)-exposed islets. Reproduced from Ref. 12 with permission. Copyright Springer-Verlag.
its inactive D-enantiomer, the early phase of glucose-stimulated insulin secretion was blunted (Fig. 2. A and B), suggesting that endogenously produced NO is involved in the secretagogue-induced insulin secretion under physiological conditions. This was further supported by the finding that addition of the NO scavenger carboxy-2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO) to the incubation medium also resulted in a blunted early insulin peak (Fig. 2C). The inhibitory effect was only seen during the first 10 min after glucose stimulation and was strongly dependent on the concentration of the NOS inhibitor, with 0.5–1 mM being most effective. This illustrates that the stimulatory effect of NO on insulin secretion is subtle and pertains mainly to the early phase of glucose-stimulated insulin release. Such a narrow time/concentration window is consistent with a physiological role of NO as mediator of secretagogue-induced signal transduction in rat islets and explains why the stimulatory effect may be masked in experimental settings measuring accumulated insulin or applying higher concentrations of the inhibitor.

If endogenously produced (L-arginine derived) NO participates in the signal transduction pathway of insulin secretion, the question arises whether NO is produced in the β-cell itself or in other cell types of the islet organ. Data are still conflicting as to whether pancreatic islets, particularly the endocrine cells, constitutively express NOS. NOS immunoreactivity and/or NADPH diaphorase staining in rat and mouse islet cells has been described by some investigators, whereas others failed to demonstrate constitutive expression of NOS in human and rat islets. We found that α (glucagon producing)-islet cells, and to some extent δ (somatostatin producing)-cells, express NOS (Fig. 3A) but could not detect NOS in the insulin-producing islet β-cells. At the subcellular level, NOS immunoreactivity was confined to the secretory granules of the α- and δ-cells (Fig. 3B). This is in keeping with reports demonstrating NOS in secretory granules of gastric D-cells and synaptic vesicles of enteric neurons. The presence of NOS in the secretory granules of α- and δ-cells suggests that NO is produced in these cells and subsequently diffuses to β-cells. Therefore, the effect of NO on glucose-stimulated insulin release under physiological conditions could be exerted in a paracrine manner by neighboring α- and/or δ-cells or by endothelial cells (Fig. 1B).

The mechanism by which NO triggers insulin release remains to be elucidated. Different possibilities may be envisaged. One putative mechanism could be that NO exerts its action via increasing intracellular calcium through mobilization of calcium from intracellular pools such as the endoplasmic reticulum or from mitochondria. We have provided experimental evidence that such a mechanism may be operative, i.e.,
that the rapid NO-mediated insulin secretion could be triggered by an increase of intracellular calcium caused by a release of calcium from the mitochondrial calcium pool (7). NO inhibits the mitochondrial respiratory chain by binding to cytochrome c and/or cytochrome oxidase. As a consequence, the mitochondrial membrane potential decreases and Ca\(^{2+}\) leaves the mitochondria. This is followed by restoration of the mitochondrial membrane potential and Ca\(^{2+}\) reuptake by mitochondria. To assess the mechanism whereby NO stimulates insulin secretion, we used INS-1 cells. These rat insulinoma-derived β-cells are considered a valid model of native β-cells because they have retained many morphological, enzymatic, and secretory characteristics of normal β-cells, including responsiveness to physiological glucose stimuli. The addition of 2 µM NO gas to INS-1 cells resulted in a rapid increase in insulin release, which was paralleled by a reversible decrease of the mitochondrial membrane potential and by an intermittent rise of cytosolic Ca\(^{2+}\), increasing from 115 nM before NO was added to 160 nM thereafter. Mitochondrial calcium depletion by uncoupling and blockade of the respiratory chain with carbonyl cyanide \(m\)-chlorophenylhydrazone (CCCP) and antimycin A before addition of NO abolished the NO-induced insulin release (Fig. 4). Conversely, the NO-induced insulin release could be mimicked by pretreating the cells with the mitochondrial poisons. Chelation of intracellular, but not extracellular, calcium prevented the NO-induced insulin release, as did preincubation with epinephrine, which inhibits insulin exocytosis. An intermittent rise in the cytosolic calcium caused by mobilization from the mitochondrial calcium pool could thus explain the stimulatory effect of NO observed during the early phase of glucose-stimulated insulin release, which is then followed by the classical glycolytic pathway operating via closure of ATP-sensitive potassium channels, cell depolarization, and calcium influx. Besides acting via the redistribution of intracellular calcium, NO may target the insulin vesicle and modulate synaptic vesicle docking fusion reactions, as has been observed in synaptosomes, for example, or act via cell surface receptors or ion channels of the β-cells.

References


