Exogenous Nitric Oxide and Endogenous Glucose-Stimulated β-Cell Nitric Oxide Augment Insulin Release

Simon R. Smukler,1 Lan Tang,1 Michael B. Wheeler,1,2 and Anne Marie F. Salapatek1

The role nitric oxide (NO) plays in physiological insulin secretion has been controversial. Here we present evidence that exogenous NO stimulates insulin secretion, and that endogenous NO production occurs and is involved in the regulation of insulin release. Radioimmunoassay measurement of insulin release and a dynamic assay of exocytosis using the dye FM1–43 demonstrated that three different NO donors—hydroxyxamine (HA), sodium nitroprusside, and 3-morpholinosydnonimine (SIN-1)—each stimulated a marked increase in insulin secretion from INS-1 cells. Pharmacological manipulation of the guanylate cyclase/guanosine 3′,5′-cyclic monophosphate pathway indicated that this pathway was involved in mediating the effect of the intracellular NO donor, HA, which was used to simulate endogenous NO production. This effect was further characterized as involving membrane depolarization and intracellular Ca2+ ([Ca2+]i) elevation. SIN-1 application enhanced glucose-induced [Ca2+]i responses in primary β-cells and augmented insulin release from islets in a glucose-dependent manner. Real-time monitoring of NO using the NO-sensitive fluorescent dye, diaminofluorescein, was used to provide direct and dynamic imaging of NO generation within living β-cells. This showed that endogenous NO production could be stimulated by elevation of [Ca2+]i levels and by glucose in both INS-1 and primary rat β-cells. Scavenging endogenously produced NO-attenuated glucose-stimulated insulin release from INS-1 cells and rat islets. Thus, the results indicated that applied NO is able to exert an insulinotropic effect, and implicated endogenously produced NO in the physiological regulation of insulin release. Diabetes 51: 3450–3460, 2002
effect of applied NO, functionally demonstrating the involvement of a signaling pathway, and identifying features of an underlying mechanism. A NO-sensitive dye was used to provide direct and dynamic visualization of NO generation within living insulin-secreting cells, both insulinoma and primary β-cells. We demonstrated that endogenous NO production can be stimulated by glucose and implicated this glucose-stimulated NO production in physiological glucose-stimulated insulin secretion.

**RESEARCH DESIGN AND METHODS**

**(Reagents and materials.** Culture media and supplements were purchased from Gibco (Grand Island, NY). 3-Morpholinosydnonimine (SIN-1), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl) pyridinium dibromide (FM1–43), bis-(1,3-dihydroxybutyric acid)trimethine oxonol (DiBAC 4(3)), and Pura2-acetoxyethyl ester (Pura2-AM) were obtained from Molecular Probes (Eugene, OR). H-1[2,4](oxadiazolo[4,3-a]quinazolin-1-one (ODQ) and ionomycin were obtained from Calbiochem (San Diego, CA). The Rat Insulin Radioimmunoassay (RIA) Kit was purchased from Linco (St. Charles, MO). All other chemicals and reagents were from Sigma (St. Louis, MO). Hydroxylamine marginally reduced the pHi of solutions, which was then adjusted to pH 7.5 with NaOH. All incubations were performed for a 20 min duration prior to the experiment. For experiments with NOS inhibition, cells were preincubated for 1 h with the inhibitor Nω-monomethyl-l-arginine (l-NMMA; 1 mmol/l), and l-NMMA was included in all solutions. Total osmolarity was maintained at its original level in all high extracellular KCl/H11001 solutions by increasing KCl and decreasing NaCl in equimolar amounts.

**Cell culture and islet isolation.** INS-1 cells (a generous gift from C. Wollheim; passages 30–45) were cultured with the media and by the method of Asfari et al. (29). Islets were isolated from male SD rats and cultured as previously described (29).

**Insulin secretion studies.** INS-1 insulin secretion experiments were performed in Krebs-Ringer bicarbonate buffer (KRBB), and islet secretion studies were performed in islet culture media for a 1-h experimental incubation, as previously described (29). All experimental agents were mixed together in experimental solutions (KRBB or media) and then added to the cells at the start of the experimental incubation. Insulin concentration in experimental samples was determined using the Linco Rat Insulin RIA Kit. Each experiment was performed with n ≥ 9, results were averaged from at least three independent experiments, and data were normalized to controls.

**Fluorescent imaging studies.** Epifluorescence imaging experiments were performed using equipment described elsewhere (30). The standard extracellular solution used for all imaging experiments consisted of the following (in mmol/l): 140 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, and 10 HEPES at pH 7.3. Solutions were continuously perfused at a rate of ~6 ml/min at room temperature. For FM1–43 studies, cells were incubated with 2 μmol/l FM1–43 for 4 min until cell membranes were fully stained. FM1–43 was maintained in all solutions throughout the course of the experiment. Fluorescence images were obtained with 485-nm excitation and a 570-nm long-pass emission filter (Chroma Technology, Brattleboro, VT) using an Olympus 100×, 1.35 NA oil immersion objective. Exposures lasted 0.4 s and images were acquired at ~0.3 Hz. The experimental recordings obtained were analyzed with Merlin software (LSR, Cambridge, U.K.) by designating a region of interest to include only the plasma membrane, yielding average fluorescence intensity values for the cell membrane throughout the course of the experiment. Background changes in fluorescence were monitored and subtracted from changes in cell membrane fluorescence. Each experiment was performed with two to five cells (n describes the number of cells).

For Ca2+ imaging studies, cells were loaded with 4 μmol/l Pura2-AM for 40 min at 37°C in standard extracellular solution. Fluorescence images were obtained with 340- and 380-nm excitation and a 530-nm long-pass emission filter (Omega Optical, Brattleboro, VT) using an Olympus 100×, 1.35 NA oil immersion objective. Exposures lasted 0.2 s for each wavelength, and images were acquired at ~0.2 Hz. Images obtained were analyzed using the Merlin software with intracellular free Ca2+ ([Ca2+]i) concentrations calculated using the Grynkwicz equation: 

\[
[Ca^{2+}]_i = K_d \times \frac{x_i \times (R - R_{min})}{(R_{max} - R)},
\]

where \(K_d\) is the dissociation constant for Pura2, \(x_i\) is the ratio of saturated to free fluorescence at 380-nm excitation, \(R\) is the ratio of fluorescence to 340 nm/350 nm, \(R_{max}\) is the ratio of fluorescence when the dye is completely free of Ca2+, \(R_{min}\) is the ratio of fluorescence when the dye is completely saturated with Ca2+. Pura2 fluorescence was calibrated to the Ca2+ concentration using 10 μmol/l ionomycin in the presence of 5 mmol/l CaCl2 for maximal fluorescence ratio, or with no added Ca2+ and 10 mmol/l EGTA for minimal fluorescence ratio.

For membrane potential imaging, cells preloaded with Pura2 were incubated in 100 mmol/l DBAC (3) was maintained in all experimental solutions throughout the experiment. For NO imaging studies, cells were loaded in 4 μmol/l 4,5-diaminofluorescein diacetate (DaF-DA) or DaFz-DA with or without 4 μmol/l DaFz-AM for 40 min at 37°C in standard extracellular solution. DaFz-2 fluorescence images were obtained as above, and 485-nm excitation was used for imaging DBAC (3) or DaFz-2, with filters and optics as described for Ca2+ imaging. Representative traces are shown from at least five similar experiments.

**Plate reader fluorimetry.** Plate reader fluorimetry experiments were performed with ~50,000 cells/well in a 96-well plate using a Fluocount Microplate Fluorimeter (Packard Biosciences, Meriden, CT). Cells were loaded for 40 min in 10 mmol/l DaFz-DA, which was then replaced with experimental solutions. Fluorescence readings were 0.5 s in length and taken every 2.5 min for 1 h with a Fluorocount filter set with 485-nm excitation and 530-nm emission. The last four readings of the hour were averaged and divided by the initial reading at the beginning of the hour to obtain percent changes over basal. Each experiment was performed with n ≥ 9, results were averaged from at least four independent experiments, and data were normalized to controls.

**Statistics.** Data are expressed as means ± SE, unless otherwise specified. Statistical comparisons were performed by ANOVA with Dunnett’s post test for comparing groups to control or the Bonferroni post test for comparisons between groups where an acceptable level of significance was considered at \(P < 0.05\).

**RESULTS**

**NO stimulates insulin secretion**

**Measurement of insulin release.** The INS-1 β-cell line was chosen because it possesses important physiological characteristics of primary β-cells (28) and because a recent study demonstrated the presence of a cNOS enzyme (11). To exclude effects attributable to differences in NO delivery, donor by-products, or other nontarget effects of NO donor agents, three chemically distinct NO donors were used: hydroxylamine (HA; 2 mmol/l), sodium nitroprusside (SNP; 100 μmol/l), and SIN-1 (500 μmol/l), each with a different mechanism of NO generation (31,32). Each NO donor compound was able to stimulate insulin release under basal (0 mmol/l) and stimulatory glucose (15 mmol/l) conditions (Fig. 1A and B, respectively). Although it is known that HA can be metabolized to NO by intracellular enzymes (31), we confirmed that HA exerted its stimulatory effect through the production of NO by showing that addition of the NO scavenger cPTIO (200 μmol/l) blocked its insulinoergic effect and was unable to prevent insulin release stimulated by 30 mmol/l KCl (Fig. 1C), verifying that cPTIO was not exerting nonspecific inhibition of secretion. Scavenging NO with cPTIO was also found to block stimulation by SNP and SIN-1 (data not shown). Staining with the vital dye, propidium iodide, was performed after each incubation, as well as 5 and 24 h after a 1-h incubation with NO donors, and confirmed that there was no change in cell viability. Since the effect of NO was observed even in the absence of glucose, subsequent experiments characterizing this effect were performed under 0 mmol/l glucose conditions to allow examination of the insulinoergic effect of NO in isolation.

**Measurement of NO-stimulated exocytosis.** NO is highly reactive in biological systems and can freely diffuse into cells, and thus is likely to exert rapid effects (1). To examine these rapid responses in a dynamic manner, single-cell exocytosis was monitored using the styryl dye, FM1–43. This cell-impermeable dye is virtually nonfluorescent in solution and partitions into the plasma membrane,

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Figure 2C shows averaged responses to each of the NO donors from many cells (n > 30 for each condition). Each donor produced a stimulation profile similar to that of increased [K+]o, with a rapid onset (significantly different than control within 20 s of exposure), followed by a rise to a steady-state plateau. A time point 3 min after addition of the stimulatory agent was chosen to represent this steady state (Fig. 2D). This steady-state point was used for statistical analysis and when displaying the remainder of the FM1-43 data. Using this single-cell assay of exocytosis, we verified the ability of NO to exert a rapid stimulatory effect on secretion from INS-1 cells.

**NO acts via the NO/guanylate cyclase/cGMP pathway.** We next examined whether the observed effect of NO was mediated through the most well-characterized NO signaling pathway: the NO/guanylate cyclase (GC)/guanosine 3',5'-cyclic monophosphate (cGMP) pathway (36). HA was used in the subsequent studies as it releases NO intracellularly (31) and so most closely reproduces endogenous NO production. Figure 3A shows that the stimulatory effect of HA (2 mmol/l) was abolished by GC inhibition with ODQ (10 μmol/l); however, this did not prevent stimulation provided by 30 mmol/l [K+]o, thereby demonstrating that ODQ was not exerting nonspecific inhibition. The stimulatory ability of NO was mimicked by GC activation with 3-(5′-hydroxymethyl-3′-furyl)-1-benzylindazole (YC-1; 50 μmol/l) and by the membrane-permeable cGMP analog, 8-(4-chlorophenylthio)-cGMP (CPT-cGMP; 10 μmol/l). As expected, the stimulatory effect produced by CPT-cGMP was not affected by inhibition of the upstream GC with ODQ. Insulin secretion experiments confirmed results obtained with FM1-43, showing that YC-1 (50 μmol/l) was able to stimulate insulin release and that ODQ (10 μmol/l) was able to block release stimulated by 2 mmol/l HA (Fig. 3B) while having no effect on 30 mmol/l [K+]o-stimulated insulin release. (Secretion with K+ and ODQ was 110 ± 4% of K+ alone; n = 9.) These results suggest that the NO/GC/cGMP signaling pathway is involved in mediating the stimulatory effect of HA.

**Membrane depolarization and [Ca2+]i elevation mediate the effect of NO.** Normal physiological stimulation of insulin release by glucose involves membrane depolarization followed by Ca2+ influx through voltage-gated Ca2+ channels. Application of diazoxide (DZ; 250 μmol/l), a specific activator of ATP-sensitive K+ (KATP) channels, was able to inhibit insulin release stimulated by 2 mmol/l HA (Fig. 3B), thereby suggesting that NO may induce membrane depolarization as a necessary part of its stimulatory effect.

Calcium imaging with Fura2 was then performed to determine whether the mechanism of the observed HA effect involved elevation of [Ca2+]i. Figure 4A shows that HA (2 mmol/l) stimulated a spiking, oscillatory elevation of [Ca2+]i, similar to that seen with glucose (15 mmol/l), but unlike the single [Ca2+]i spike produced by high [K+]o (30 mmol/l) (Fig. 4B). To assess membrane potential concurrently with measurement of [Ca2+]i, simultaneous imaging of the membrane potential dye, DiBAC4(3), and Fura2 were performed. Figure 4C confirms the utility of these dyes by demonstrating the [Ca2+]i and membrane potential responses to glucose, effects that were augmented by tetraethylammonium (TEA; 10 mmol/l), which
is known to enhance glucose-induced depolarization and Ca\(^{2+}\) entry (37). As expected, DZ (250 \(\mu\)mol/l) was found to repolarize the cell and halt the \([\text{Ca}^{2+}]_i\) response, whereas 30 mmol/l \([\text{K}^{+}]_o\) was still able to directly depolarize the cell and cause \([\text{Ca}^{2+}]_i\) influx. Using Fura2 and DiBAC\(_4\)(3), the \([\text{Ca}^{2+}]_i\) response to HA was shown to occur simultaneously with membrane depolarization, and DZ was able to repolarize the cell and halt the \([\text{Ca}^{2+}]_i\) response (Fig. 4D). These results demonstrated that HA is able to elicit an increase in \([\text{Ca}^{2+}]_i\), likely because of membrane depolarization.

**Endogenous NO production in INS-1 cells and involvement in glucose-stimulated insulin release.** Although the presence of a cNOS enzyme has been indicated in \(\beta\)-cells, as of yet there has been only indirect measurement of its functional activity in producing NO. We used imaging of the NO-sensitive dye, Daf2 (38), to provide direct and dynamic visualization of NO production within living insulin-secreting cells. Exogenous application of the spontaneous NO donor SNP (50 \(\mu\)mol/l) to Daf2-loaded INS-1 cells resulted in an increase in fluorescence fairly uniformly throughout the entire cell that was attenuated by NO scavenging with cPTIO (200 \(\mu\)mol/l) (Fig. 5A). To stimulate endogenous cNOS activity, we elevated \([\text{Ca}^{2+}]_i\), with the application of the \(\text{Ca}^{2+}\) ionophore, ionomycin (5 \(\mu\)mol/l), after which we added the NOS substrate, l- arg (1 \(\mu\)mol/l). Ionomycin alone stimulated a modest increase in NO, which was greatly increased by l-arg (Fig. 5B). Notably, endogenous NO production was not uniformly produced throughout the cell and often appeared localized in a punctate manner (Fig. 5B, top).

To further characterize Daf2 measurement of exogenous NO donation and endogenous cNOS activity and to conduct cell population studies, plate reader fluorimetry experiments with INS-1 cells were performed. Each NO donor was able to stimulate an increase in Daf2 fluorescence, with SIN-1 being the most potent (Fig. 6A). Furthermore, the effect of SNP was shown to be dosage dependent (at 100 and 500 \(\mu\)mol/l) and was blocked by NO scavenging with cPTIO (200 \(\mu\)mol/l). Dosage dependency was observed, and NO scavenging could be accomplished with cPTIO or hemoglobin for each donor used (data not shown). Figure 6B displays the measurement of endogenous NO production. Even a low concentration of l-arg (100 \(\mu\)mol/l) was able to stimulate NO production, an effect that was greater with increased l-arg concentrations (data not shown). Importantly, we observed that glucose (15 mmol/l) alone stimulated a significant increase in NO production and that exogenous l-arg augmented this effect. The NOS inhibitor L-NMMA (1 mmol/l) was able to prevent endogenous NO production stimulated by l-arg and glucose. These results further validated the use of Daf2 to measure exogenous and endogenous NO in living cells and demonstrated that glucose is able to stimulate endogenous NO production in INS-1 cells.

To determine whether this endogenous NO production plays a role in physiological insulin release, as is suggested by the insulinotropic effect of exogenous NO, we used an
Effect of exogenous NO on normal glucose-stimulated insulin release. We demonstrated that glucose dependence was observed for both the ability of exogenous NO to elevate [Ca\(^{2+}\)]\(_i\) in dispersed β-cells and the insulinotropic effect of NO on intact islets. 

**Endogenous NO production in primary β-cells and involvement in glucose-stimulated insulin release.** The capability of primary β-cells for endogenous NO production was demonstrated by elevating [Ca\(^{2+}\)]\(_i\), with ionomycin (5 μmol/l) and adding L-arg (1 mmol/l) (Fig. 8A). As with INS-1 cells, [Ca\(^{2+}\)]\(_i\) elevation alone induced some NO production, which was further increased by L-arg. This stimulation was observed whether glucose (to identify β-cells) was applied before or after ionomycin. Most importantly, it was found that glucose (15 mmol/l) alone stimulated an increase in endogenous NO production concurrent with its stimulation of a [Ca\(^{2+}\)]\(_i\) response (Fig. 8B). To verify the functional significance of this glucose-stimulated NO generation, we demonstrated that scavenging of NO with cPTIO (200 μmol/l) was able to reduce glucose (16.67 mmol/l)-stimulated insulin secretion from rat islets (Fig. 8C). These results demonstrated the ability of primary β-cells to produce NO in response to [Ca\(^{2+}\)]\(_i\) elevation and glucose and implicated this endogenous NO production as having a role in normal glucose-stimulated insulin release.

A role for NO in the regulation of insulin release is supported by the demonstration of a cNOS enzyme in insulinoma and primary β-cells (5–11) and the insulinotropic action of the NOS substrate L-arg (12). In the present study, we established that NO is able to exert an insulinotropic effect, using measurement of insulin release from INS-1 cells and intact rat islets, as well as dynamic measurement of single-cell exocytosis. The effect of applied NO was characterized to involve intracellular signaling via the NO/GC/cGMP pathway, and the cellular mechanism underlying this effect was found to involve cell membrane depolarization and elevation of [Ca\(^{2+}\)]\(_i\). Monitoring NO in INS-1 and primary β-cells demonstrated the existence of endogenous NO production, showed that NO generation could be stimulated by glucose, and implicated this endogenous NO in glucose-stimulated insulin release.

The insulinotropic effect of NO on INS-1 cells and the pathway involved was demonstrated with two different techniques. We used standard RIA measurement of insulin release as well as measurement of exocytosis using the fluorescent dye, FM1-43 (30,33–35). Using FM1–43 allows for dynamic, real-time measurement of exocytosis with high temporal resolution (in the order of seconds). This may prove to be important as NO effects are likely to be exerted very rapidly because of NO’s high reactivity and freedom of movement in biological systems. Although we
limited our analysis to averaged fluorescence changes over the cell membrane, we did note that different regions of the cell membrane increased in fluorescence intensity preferentially over others. These areas may correspond to "secretory addresses" along the cell membrane where various molecules and channels involved in exocytosis are co-localized, as has been reported by others in the literature (30,35,39,40).

Our demonstration of a stimulatory effect of NO on insulin release is in agreement with previously published reports (5,13–15,21,22); however, a number of studies argue against a stimulatory role for NO (6,9,16–18,23–27). Since we used the intracellular NO donor HA to simulate physiological endogenous NO production (and demonstrated this production using Daf2) and because we used a ß-cell line, we produced an experimental model in which we were able to observe the effect of NO produced within the ß-cell acting on the ß-cell. This model may provide more specific information about the existence and activity of NO signaling in ß-cells as compared to studies using islets or the whole pancreas, in which pharmacological manipulation of the NO pathway may affect any or all of the cell types present, with resultant effects on the ß-cells originating in any of these. We chose to characterize this HA effect in isolation (i.e., without glucose stimulation) to avoid possible interpretive challenges for two reasons. First, it is difficult to discern the specific stimulatory effects of NO when there is accompanying stimulation by glucose. Second, the presence of additional stimuli may actually alter the apparent effect of NO. For example, we demonstrated that under conditions of high glucose and elevated arginine, endogenous NO production is increased. It is also known that the precise level of NO is crucial in determining its resultant effect, with low levels being involved in physiological signaling and higher levels becoming cytotoxic (1,41). Therefore, the supraphysiological elevation of NOS substrate, or the application of exogenous NO donors under these conditions of already

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FIG. 4. Effect of HA on [Ca2+]i and membrane potential in INS-1 cells. A: HA (2 mmol/l) stimulated oscillations in [Ca2+]i, similar to that of glucose (15 mmol/l), whereas stimulation by 30 mmol/l [K+]o, B) produced a typical single-spike response. C: Simultaneous imaging of DiBAC4(3) to assess membrane potential (top) and Fura2 to measure [Ca2+]i (bottom) demonstrated the stimulation by glucose (15 mmol/l), which was potentiated by TEA (10 mmol/l). DZ (250 μmol/l) repolarized the cell and halted the [Ca2+]i response, and subsequent 30 mmol/l [K+]o addition depolarized the cell and caused Ca2+ influx. D: HA induced cell depolarization (top) concurrently with a [Ca2+]i response (bottom), and DZ repolarized the cell and halted the [Ca2+]i response. Shown are representative traces from at least five similar experiments.
elevated NO, may result in excessive NO production, yielding cytotoxic effects. It should be noted that propidium iodide staining showed that cell viability was not altered by our NO application.

Although the endogenous substrate in NO formation, the amino acid L-arg, has long been known to be a potent insulinotropic agent (12), we did not use it to identify the effect of NO in insulin secretion because its mechanism of action remains controversial. In addition to its role in NO formation, L-arg may also affect insulin release via its metabolism and metabolic by-products (42) or biophysically by its actions in directly depolarizing the plasma membrane via entry of this cationic amino acid (43). As the insulinotropic effect of L-arg may be caused by any combination of these events, it is difficult to distinguish the specific role of NO production in L-arg stimulated insulin release. It is therefore judicious to make use of NO donors to gain information about the possible physiological role of endogenous NO production, and, moreover, to use an intracellular NO donor to more closely simulate endogenous NO production.

Our studies, without using a strategy of NOS inhibition, have consistently demonstrated that NO is stimulatory to insulin release. It is interesting to note that many of the studies reporting NO as a negative modulator of insulin release have based their conclusions on the observation

FIG. 5. Measurement of exogenous and endogenous NO production in INS-1 cells with Daf2. A: INS-1 cells preloaded with Daf2 were exposed to the NO donor SNP (50 μmol/l), which increased Daf2 fluorescence, after which the NO scavenger cPTIO (200 μmol/l) was added, which attenuated the fluorescence increase. Inset shows a trace of the Daf2 fluorescence increase with SNP application (red bar) without addition of cPTIO. B: Cells were exposed to the Ca²⁺ ionophore, ionomycin (5 μmol/l), which modestly increased Daf2 fluorescence, after which the NOS substrate L-arg (1 mmol/l) was added, which stimulated a larger increase. Fluorescence intensity levels within images are pseudocolored according to the bar scale shown at the right, with low intensity being blue and high being red. Fluorescence trace shown is the averaged fluorescence for all cells pictured. Lettered pictures are images obtained at the time point indicated by the corresponding letters on the fluorescence trace. Shown are representative results from at least five similar experiments.
that NOS inhibition, using \( \text{N}^\text{G}\)-nitro-L-arginine methyl ester (L-NAME) or L-NMMA, was able to stimulate insulin release. However, use of these NOS inhibitors to specifically identify the effect of NO on insulin release has numerous complications. First, they are both able to exert nonspecific effects, unrelated to the NOS enzyme, which may stimulate insulin release. These include biophysical (depolarizing) effects of the cationic L-NMMA (43), and the direct effects of \( \text{L}\)-NAME to inhibit \( K_{\text{ATP}} \) channels and voltage-dependent \( K^+ \) (\( K_v \)) channels (44), to name two. Studies attempting to control for \( K_{\text{ATP}} \) channel inhibition (i.e., by application of high \( K^+ \), to depolarize the cell and inclusion of DZ) neglect to address the inhibition of the \( K_v \) channels, which we have previously shown to enhance insulin release (29). Second, even if NOS inhibition is specifically obtained with these or other NOS inhibitors, any effect on insulin release may not be attributable to reduced NO production, but rather to additional, alternate, accompanying effects of NOS inhibition. This is exemplified in studies that have shown that NO supplementation is unable to reverse the effects of NOS inhibition (45), which has led to two main proposals. The first is based on the fact that the NO synthesis intermediate \( \text{N}^\text{G}\)-hydroxy-L-argin (L-OH-arg) and the co-product L-citrulline (L-cit) (2) are able to inhibit activity of the enzyme arginase, which converts \( \text{L}\)-argin to \( \text{L}\)-ornithine (L-orn) and urea (46). NOS inhibition reduces \( \text{L}\)-OH-arg and \( \text{L}\)-cit levels, which re-
moves arginase inhibition, resulting in increased L-orn levels, and L-orn has been shown to stimulate insulin release (42,45). In this scenario, increased L-orn generation, rather than decreased NO production, underlies the insulinotropic effect of NOS inhibition. A second mechanism, recently proposed by Lajoix et al. (11), involves the capacity of the β-cell NOS enzyme to effect cytochrome c reductase activity, which would be enhanced by inhibition of NO synthesis by NOS. Lajoix and colleagues proposed that the resultant increase in respiratory chain activity would produce a rise in ATP production and a subsequent stimulation of insulin release. Thus, overall, when interpreting findings obtained using NOS inhibitors, it is difficult to discern whether any observed changes are attributable to nonspecific effects, unrelated to the NOS enzyme, and furthermore, whether an effect produced from NOS inhibition may be unrelated to a reduction in NO synthesis.

We have provided functional evidence demonstrating the involvement of the NO/GC/cGMP pathway in the observed insulinotropic effect of HA on INS-1 cells through the use of established pharmacological agents that target key points in this pathway. This finding is consistent with data reporting that endogenously produced NO stimulates increased cGMP within rat islets and β-cell lines (5,13), although others have reported no effect on cGMP levels using mouse islets (18). This disparity may be attributable to differences in experimental design, such as the time frame of NO stimulation (i.e., minutes or an hour) or whether a phosphodiesterase inhibitor was included. Our finding that NO is able to induce an elevation of β-cell [Ca2+]i is in agreement with previous studies in rat β-cells and cell lines (7,21,22,47). Interestingly, it was found that this [Ca2+]i response displayed an oscillatory profile, similar to that seen with glucose stimulation and unlike that of high [K+]o depolarization. We have further shown that NO caused a concurrent depolarization of the cell membrane, and that DZ was able to repolarize the cell and halt the [Ca2+]i response. This observation at the single-cell level is consistent with the observed inhibition of NO-stimulated insulin release by DZ and suggests that depolarization by NO is responsible for the [Ca2+]i response.

Although much evidence points to the existence of a cNOS enzyme in β-cells, the functional NO-synthesizing activity of this cNOS under physiological conditions remains controversial. To directly confirm β-cell NOS func-

![FIG. 8. Endogenous NO production in primary β-cells and involvement in islet glucose-stimulated insulin secretion. Simultaneous imaging of Fura2 to measure [Ca2+], (top) and Daf2 to monitor NO (bottom) was performed. A: Ionomycin (5 μmol/l) elevated [Ca2+], and increased NO, with the addition of L-arg (1 mmol/l) augmenting the increase in NO. A subsequent glucose (15 mmol/l)-induced [Ca2+], response identified the cell as a β-cell. B: Glucose (15 mmol/l) alone stimulated a concurrent increase in NO and [Ca2+]. Shown are representative traces from at least five similar experiments. C: Measurement of insulin release from intact islets showed that NO scavenging with cPTIO (200 μmol/l) was able to reduce the stimulation of release by 16.67 mmol/l glucose (HG). Low glucose (LG) was 2.78 mmol/l. Data are means ± SE (n = 15). *P < 0.001 vs. LG; †P < 0.001 vs. HG.](image_url)
tionality, we used the NO-sensitive fluorescent dye, Daf2 (38), to image NO production in living β-cells. To our knowledge, this is the first study to use Daf2 to directly monitor and provide real-time, dynamic visualization of endogenous NO production or generation from exogenous donors in living insulin-secreting cells. Using Daf2, we confirmed the NO-producing ability of the NO donors used and demonstrated NO scavenging. We showed that endogenous NO production under high [Ca\textsuperscript{2+}]i conditions often appeared in a punctate manner, suggesting that there exists subcellular locales of NO generation, which may be important in selective delivery of the highly reactive NO to target molecules. This observation is consistent with recent data reporting subcellular localization of the β-cell NOS enzyme (e.g., to secretory vesicles, the mitochondria, the nucleus) (11). Not only has the capacity for NO generation in β-cells been demonstrated, but we have also reported that endogenous NO production can be stimulated by glucose, and that this stimulation can be blocked by NOS inhibition. The ability of glucose to stimulate endogenous NO generation likely stems from its ability to increase cellular levels of the NOS co-substrate NADPH (48) and Ca\textsuperscript{2+}, effects that are likely to increase cNOS activity (2). This finding is in agreement with studies using indirect measurement of NO (via nitrate conversion to nitrite and the Griess reaction) (5,14). Considering that applied NO exerted an insulinotropic effect, it is tempting to speculate that the observed glucose-stimulated NO production may be involved in normal physiological glucose-stimulated insulin secretion. Our results suggested that this is indeed the case, as scavenging of endogenously produced NO was able to reduce glucose-stimulated insulin release. Although the NOS substrate l-arg was not provided in our experimental solutions for these secretion experiments (because of the nonspecificity of its insulinotropic action, as detailed above), the β-cell expresses both recycling enzymes, argininosuccinate synthase and argininosuccinate lyase, which recycle the NOS co-product l-cit back to l-arg, thereby acting to maintain a supply of l-arg required for NO production (7). This was evident in our Daf2 experiments in which glucose or ionomycin alone, without exogenous l-arg, stimulated NO generation.

Experiments using dispersed primary rat β-cells and intact islets were performed in this study. Interestingly, NO donation by SIN-1 did not elicit a β-cell [Ca\textsuperscript{2+}]i response by itself, but was able to potentiate a glucose-induced [Ca\textsuperscript{2+}]i response. Notably, glucose-unresponsive cells, most likely non-β-cells, displayed little or no alterations in [Ca\textsuperscript{2+}]i after NO administration in the presence or absence of glucose. This finding would suggest some cellular specificity to β-cells in the ability of NO to alter [Ca\textsuperscript{2+}]i, an observation that may be relevant in the functional delineation of this diffusible, seemingly ubiquitous molecule. Consistent with these single-cell observations, NO augmented islet insulin release in a glucose-dependent manner. It is not yet clear why glucose dependence of the NO effect was observed in β-cells as compared to INS-1 cells, but this may be due to the inherent differences between these cells with respect to the NO-stimulated pathway(s) or more general differences in physiological function leading to altered excitability. For example, in the basal state under zero glucose conditions, INS-1 cells are generally more “excited” than primary β-cells, in that there is often occasional spontaneous electrical activity and [Ca\textsuperscript{2+}]i spikes (49; S.R.S. and A.M.F.S., unpublished observations). Furthermore, INS-1 cells are more “excitable” compared to primary cells, as is evidenced by their secretory response to lower glucose levels. As such, the increased “excitability” of INS-1 cells may allow for endogenous NO to trigger insulin release, whereas NO is unable to trigger, but is able to enhance, release from primary β-cells. The inability of HA to stimulate insulin release from islets, even in the presence of glucose, may be caused by the poor penetration of HA into the inner islet area (i.e., where most β-cells are located) and/or the very low islet β-cell expression level of enzymes that are required to metabolize HA to NO (e.g., catalase) (50). The latter hypothesis is supported by the observation that HA was unable to effectively elevate NO or stimulate a [Ca\textsuperscript{2+}]i response in dispersed single primary β-cells. As with INS-1 cells, endogenous β-cell NO production was observed in response to [Ca\textsuperscript{2+}]i elevation and glucose stimulation. Further, this NO generation was implicated in glucose-stimulated insulin secretion, as NO scavenging reduced glucose-stimulated insulin release from islets.

Studies within the field point to an intimate and complex role for NO in the β-cell. In our study, we demonstrated the ability of applied NO to exert an insulinotropic effect, and proceeded to characterize this effect. We then extended this work to demonstrate endogenous NO production and implicate it in glucose-stimulated insulin secretion. Our study suggests that the physiological stimulation of endogenous β-cell NO synthesis occurs after a glucose-stimulated elevation of [Ca\textsuperscript{2+}]i, with subsequent activation of the cNOS enzyme. The resultant NO then plays an amplifying role in glucose-stimulated insulin release. Our findings contribute to a more complete understanding of β-cell physiology by demonstrating that the NO system is operational and physiologically relevant to the regulation of insulin release. Whether dysfunction or dysregulation of the NO system is involved in β-cell pathology, or whether it can be targeted for therapeutic treatment strategies, warrants further study.

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