Glucose-induced release of nitric oxide from mouse pancreatic islets as detected with nitric oxide-selective glass microelectrodes

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Nunemaker CS, Buerk DG, Zhang M, Satin LS. Glucose-induced release of nitric oxide from mouse pancreatic islets as detected with nitric oxide-selective glass microelectrodes. Am J Physiol Endocrinol Metab 292: E907–E912, 2007. First published November 22, 2006; doi:10.1152/ajpendo.00518.2006.—Nitric oxide (NO) is believed to play an important role in pancreatic islet physiology and pathophysiology. Research in this area has been hampered, however, by the use of indirect methods to measure islet NO. To investigate the role of NO in islet function, we positioned NO-sensitive, recessed-tip microelectrodes in close proximity to individual islets and monitored oxidation current to detect subnanomolar NO in the bath. NO release from islets consisted of a series of rapid bursts lasting several seconds and/or slow oscillations with a period of ∼100–300 s. Average baseline NO near the islets in 2.8 mM glucose was 524 ± 59 nM (n = 12). Raising glucose from 2.8 to 11.1 mM augmented NO release by 429 ± 133 nM (n = 12, P < 0.05), an effect blocked by the NO synthase inhibitor L-NAME (n = 3). We also observed that glucose-stimulated increases in NO release were contemporaneous with changes in NAD(P)H and O2 but occurred well before increases in calcium associated with glucose-stimulated insulin secretion. In summary, we demonstrate that NO release from islets is oscillatory and rapidly augmented by glucose, suggesting that NO release occurs early following an increase in glucose metabolism and may contribute to the stimulated insulin secretion triggered by supraphreshold glucose.

Calcium; oscillations; N02-nitro-L-arginine methyl ester; oxygen; reduced forms of islet nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate

Nitric oxide (NO) is an important regulator of many physiological functions, including neurotransmission, hormone secretion, regulation of blood flow, and immune responses (8). In pancreatic islets, which secrete insulin in response to a rise in plasma glucose, NO can be generated by several isoforms of NO synthase (NOS), including an inducible form, iNOS (1), and the constitutive forms neuronal (nNOS) (21) and endothelial NOS (eNOS) (37). Under pathophysiological circumstances, cytokine-induced NO production has been linked to β-cell dysfunction and cell death (29, 36), which is thought to contrbute to the autoimmune cascade responsible for type 1 diabetes (9, 14).

NO also appears to play a physiological role in islet function as well, as evidenced by reports that exogenous sources of NO either stimulate (22, 32, 35, 42) or inhibit (10, 34, 38) secretagogue-induced insulin secretion. Other studies have shown dual effects, either stimulatory or inhibitory depending on the dose (17–20), or no effect (19, 30). Opposing effects of NO have also been observed on KATP channel activity (11, 39), an important regulator of β-cell membrane potential and islet excitability. Similarly, intracellular calcium ([Ca2+]i), which is involved with insulin secretion and a number of important cellular processes, appears to be increased by low and blunted by high doses of NO (20). Although these studies establish that NO modulates islet secretory processes, taken together they present a complex portrait of the role of NO in islet stimulus-secretion coupling.

However, in examining the effects of NO on islet function, to our knowledge relatively few studies have directly measured NO produced endogenously by islets (26, 27, 35). To this end, we used NO-sensitive, recessed-tip microelectrodes (5, 7) fabricated to detect dissolved NO released from mouse pancreatic islets. This enabled us to perform NO measurements with high spatial and temporal resolution under physiological conditions to determine whether NO is released from islets and, if so, to determine its pattern of release and sensitivity to glucose. We report that endogenous NO release detected with NO electrodes appears to be oscillatory and may be involved in the early stages of stimulus-secretion coupling. These results support the use of recessed NO microelectrodes as a potentially valuable tool to further elucidate the physiological role of NO in islet function.

MATERIALS AND METHODS

Islet isolation and culturing. Islets were isolated from male Swiss-Webster mice (25–35 g), using protocols in accordance with the Institutional Animal Care and Use Committee of Virginia Commonwealth University, as previously described (44). Briefly, mice were anesthetized by cervical dislocation, and their pancreases were rapidly removed. Collagenase (Crescent Chemical, Islandia, NY) was then injected at 2 mg/ml into the pancreas via the bile duct or through direct injection into pancreatic tissue, and islets were incubated for 10–20 min to free them from the exocrine pancreas. Islets were then hand picked twice under a dissecting microscope (Olympus, Tokyo, Japan) and placed in RPMI medium supplemented with 10% FBS and penicillin and streptomycin and incubated at 37°C in a 95/5% air/CO2 mixture for ~24 h before experimentation.

NO measurements. The design and use of NO electrodes has been described previously (5, 7). Briefly, recessed, gold-plated NO microsensors fabricated from glass micropipettes with tip diameters between 5 and 10 μm and recesses between 10 and 15 μm deep were used to measure NO near individual islets. Tips were coated with Nafion polymer to reduce interference from other chemical species. NO microsensors were held at 850 mV relative to a grounded Ag/AgCl reference electrode in the bath, using a sensitive electrometer (Model 610C; Keithley Instruments, Cleveland, OH) to amplify the resulting current. The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
NO oxidation currents. Two-point calibrations were performed before and after the experiments in saline equilibrated with compressed gases (100% N₂ and 1,800 ppm NO, balance N₂) in a temperature-controlled chamber at 35°C (see Fig. 1). Current output from the electrometer was digitized and then converted to nM (10⁻⁹ mol/l) units of NO on the basis of calibration curves obtained for individual NO microelectrodes (156.5 nM/pA for the electrode used in this study) after baseline drift was removed. NO concentrations were reported as changes in NO concentration (ΔNO).

To measure NO release from islets, we transferred each islet to a small volume perifusion chamber (Warner Instruments, Hamden, CT) mounted on the stage of an Olympus IX50 inverted fluorescence microscope. Only one islet was placed in the chamber for each experiment to eliminate the possibility of NO signals being detected from other islets. A Minipulse II peristaltic pump (Gilson, Paris, France) was used to deliver solutions at a rate of 0.5 ml/min. Islets were perifused with a Krebs-Ringer bicarbonate (KRB) buffered solution containing 11.1 mM glucose (NaCl was adjusted to 130.5 mM to maintain osmolarity) for an additional 10 – 15 min. The NO electrode was positioned to within 10 μm of the glass microelectrode with recessed tip and photograph (Fig. 1A). The NO electrode was positioned to within 10 μm of the islet surface in the direction of the flow before the recording was initiated so that solutions flowed past the tip of the electrode and then past the islet. After monitoring NO release for 5 – 10 min, islets were then perifused with KRB solution containing 11.1 mM glucose (NaCl was adjusted to 130.5 mM to maintain osmolarity) for an additional 10 – 15 min. In a subset of recordings, 100 μM N⁵-nitro-l-arginine methyl ester (l-NAME) was included in each of these solutions.

Intracellular calcium measurements. [Ca²⁺], was measured using the ratimetric dye fura-2 AM (fura-2) as previously described (44). Briefly, islets were loaded with 2 μM fura-2 for 20 min, washed, and then incubated for an additional 10 min. Individual islets were then transferred to the perifusion chamber of the same Olympus inverted epifluorescence microscope equipped with fura-2 optics that was used for the NO recordings described above. Excitation light was supplied to the preparation via a light pipe transmitting light from a Xenon burner. A galvanometer-driven mirror and dichroic cube were used to produce alternating excitation at 340 and 380 nm ("HyperSwitch"); IonOptix, Milton, MA). Ratios were then collected at 510 nm using a photomultiplier (Electron Tubes, Middlesex, UK) and were analyzed using IonWizard software (IonOptix).

NAD(P)H autofluorescence measurements. Measurements of the reduced forms of islet NAD and NADP, referred to as NAD(P)H, were made using the same Olympus IX50 inverted fluorescence microscope used for the other experiments. Instead of loading islets with fura-2, however, islets were placed directly into the recording chamber or, record NAD(P)H autofluorescence using 340 nm excitation and 510 nm emission. Although the peak excitation and emission wavelengths of NAD(P)H are ideally 360 and 450 nm, respectively, our recording settings were sufficiently sensitive to detect glucose-induced changes in fluorescence that were consistent with previous reports of islet NAD(P)H (4, 15, 23, 28, 31).

Oxygen consumption measurements. Custom-made, Whalen-type recessed cathode Po₂ microelectrodes (41) with ~5-μm tips were used. Details of the operational principles and methods for fabricating recessed cathode Po₂ microelectrodes are reviewed in Buerk (6). Briefly, polarographic currents were generated by the electrochemical reduction of O₂ at a constant potential of ~0.7 V relative to an Ag/AgCl reference in the superfusion chamber and were then amplified with a sensitive electrometer. Po₂ microelectrode currents measured during the islet study were digitized by computer. O₂ diffusion fields are almost completely confined within the recess [length (L)] when it is relatively deep compared with the cathode diameter (d). Computer modeling predicts that >95% of the diffusion field is contained within the recess when L/d = 10 (33). On the basis of these design criteria, good current sensitivity, rapid time response, lack of stirring artifact, and minimal calibration errors are possible with tip diameters <5 μm.

Data analysis and statistics. Glucose-induced changes in relative amounts of NO were calculated by subtracting the average baseline ΔNO signal recorded for 5 min in low glucose (2.8 mM) from the average ΔNO value during the first 10 min in high glucose (11.1 mM). A two-tailed t-test was used to assess differences in NO release between low and high glucose conditions for individual recordings; a Mann-Whitney U-test was used to compare all 12 records collectively. A two-tailed t-test assuming unequal variance was used to assess the effects of l-NAME on glucose-induced NO release and to assess differences in the latencies between calcium and NO measured within the same recording. A one-way ANOVA was performed to assess differences in the latencies among calcium, O₂, NAD(P)H, and NO responses to increased glucose. A P value <0.05 was considered statistically significant.

RESULTS

Measuring NO release with recessed-tip microelectrodes. As described in detail in MATERIALS AND METHODS, a schematic and a photograph of a recessed NO-sensitive microelectrode is shown in Fig. 1A. Figure 1B shows the limits of NO detection used to calibrate each microelectrode, obtained by bubbling known concentrations of NO into the experimental chamber. Current swings having amplitudes of a few pA were seen under calibration conditions and were reproducible. Deflections in microelectrode current were ~0.05 pA peak to peak when the
electrode was placed in a phosphate-buffered saline solution without islets present, as shown in Fig. 1C, top. However, when the microelectrode was positioned to within 10 microns of an islet, as shown in Fig. 1C, bottom, large transient changes in the microelectrode current were observed. Thus the changes in electrode current noted in the vicinity of islets were well above the level of detection of the electrodes, and the fluctuations observed were not due to electrode instability or noise, which was of much lower amplitude.

Patterns of islet NO release in 2.8 and 11.1 mM glucose. Current fluctuations indicative of transient NO release were observed in islets bathed in saline containing 2.8 mM glucose, which is substimulatory for insulin secretion. As shown in Fig. 2, A and B, NO release occurred in brief “bursts” in 8 of 12 islets tested. These bursts were typically 10–30 s in duration and could be observed throughout the duration of the recording whether 2.8 or 11.1 mM glucose was used. Each burst of NO release consisted of several sharp “transients,” and each transient was typically <2 s in duration. An example of a recording in which NO bursts and transients were less prominent is shown in Fig. 2C. Figure 2, A–C, right, displays an expanded segment of each recording to highlight the kinetic properties of islet NO release. In several cases, we observed regularly occurring slow oscillations in the NO baseline that had a period on the order of 2–5 min (e.g., note baseline of Fig. 2A).

Glucose increases baseline NO release. Although NO release was clearly detectable in 2.8 mM glucose, raising glucose to 11.1 mM significantly increased baseline levels of NO release, as shown in Fig. 2, although the effect was somewhat obscured by the large transients and bursts of transients seen in most islets (see Fig. 2, A and B). As shown in Fig. 3A, which depicts the average of the NO recordings of 12 islets with basal NO release normalized to zero, raising glucose from 2.8 to 11.1 mM caused a mean increase in NO release. The increase in NO following glucose elevation was statistically significant in 10 of 12 islets when comparing NO concentrations in 2.8 vs. 11.1 mM glucose.

To confirm that the rise in NO release seen in response to glucose was not an artifact, NO measurements were compared in the presence (n = 3) or absence (n = 12) of 25 or 100 μM L-NAME. In Fig. 3B, we compare the average NO release during 2.8 mM glucose with NO release during 11 mM glucose or 11 mM glucose plus L-NAME. The glucose-induced increase in NO release was inhibited by L-NAME such that ∆NO observed on raising glucose from 2.8 to 11.1 mM was +429 ± 133 nM without L-NAME (n = 12, P < 0.02) vs. −33 ± 43 nM in the presence of L-NAME (n = 3). Thus the elevation in NO due to increased glucose required NOS activity.

Glucose-stimulated increases in [Ca2+]i follow increased islet metabolism. Using a fluorescent dye to monitor NO synthesis within cells, previous studies (20, 35) have linked NO synthesis to changes in [Ca2+]i. Using NO-sensitive electrodes and fura-2 imaging in the present study, we were able to simultaneously measure [Ca2+]i and NO release, rather than NO intracellular concentration, to determine a possible temporal relationship. As shown in Fig. 4A, increases in NO consis-

Fig. 2. NO is released from islets in complex patterns. A–C: patterns of NO release were measured every 0.1 s by NO-sensitive microelectrode. “Bursts” and “transients” of NO release were frequently observed in A and B (labeled in A, right), which is representative of 8 of 12 NO recordings, whereas C is representative of 4 of 12 recordings in which NO transients were not frequently observed. Vertical dotted lines indicate the switch from a 2.8 to 11.1 mM glucose saline solution. Boxes surround a portion of each trace that is expanded in A–C, right.
Not only did the rise in NO precede the first-phase \[\text{Ca}^{2+}\] rise, it also appeared to precede the "phase 0" drop in \[\text{Ca}^{2+}\] associated with calcium uptake into the endoplasmic reticulum as ATP/ADP increases (43, 44).

In parallel studies, we also examined the time course of glucose-stimulated changes in oxygen and NAD(P)H, two indicators of glucose metabolism. As shown in Fig. 4B, an increase in NAD(P)H was observed 61 ± 5 s \((n = 4)\) after glucose stimulation. Similarly, a rapid decrease in oxygen electrode voltage, indicative of increased oxygen consumption near the islet surface, followed glucose stimulation by 60 ± 8 s \((n = 4)\), as shown in Fig. 4C. These measures of glucose metabolism demonstrated that glucose stimulated metabolic changes began with approximately the same latency as for NO but preceded elevation in islet \[\text{Ca}^{2+}\].

In Fig. 5, we compare the latencies for NO, \[\text{Ca}^{2+}\], O2, and NAD(P)H. These studies indicate that increases in \[\text{Ca}^{2+}\] thus appear to be secondary to two different measures of islet metabolism \([\text{O}_2\) and NAD(P)H]. It should be noted that, although these studies were performed under similar conditions with the same equipment on separate islets in parallel rather than simultaneously, we cannot be entirely certain of the precise temporal relationship among the various measures. These data nevertheless suggest that increases in NO release occur early and reflect activation of islet glucose metabolism at approximately the same time that changes in NAD(P)H production and oxygen consumption occur. Metabolic changes are well known to precede the elevations in islet \[\text{Ca}^{2+}\] and insulin secretion that require the closure of KATP channels in the β-cell membrane (3, 12).

**DISCUSSION**

By adapting recessed NO-sensitive microelectrodes to the study of pancreatic islets for the first time, we demonstrate in the present study that NO is released from islets in complex rhythmic patterns across multiple time domains. The most rapid type of NO release that we detected was in the form of brief, sharp transients or spikes that were typically <2 s in duration and tended to be clustered together into bursts lasting ~10–30 s in duration. These events were evident in 2.8 mM glucose, which is well below the threshold of activation for
islet electrical activity and glucose-stimulated secretion [~5–7 mM (2)]. Similar events were observed in 11.1 mM glucose as well. Although NO release thus occurs in substimulatory glucose concentrations, it is also augmented by increased glucose concentrations, confirming previous studies (18, 26, 35, 40).

What are the implications of endogenous NO release for stimulus-secretion coupling and islet physiology? NO release is thought to modulate insulin secretion, as confirmed in several secretory studies (10, 17, 20, 22, 32, 34, 35, 38, 42). This would suggest that the NO release we observed at low glucose might play a role in governing constitutive or basal insulin secretion observed in vivo and in vitro. Furthermore, our finding that NO release increases with glucose stimulation, combined with earlier observations that blocking endogenous NO production reduces glucose-stimulated insulin secretion (18, 26, 40), suggests that NO has a stimulatory effect on insulin secretion. Because NO is also involved in islet physiology, some of the controversy over the stimulatory vs. inhibitory effects of NO may be related to islet health (13, 24, 25). Additional studies are clearly necessary to more fully elucidate these possibilities.

We also investigated the temporal relationship between NO, [Ca\(^{2+}\)], and other islet metabolic variables following glucose stimulation. In our simultaneous measurements of NO and [Ca\(^{2+}\)], NO release in response to glucose stimulation clearly increased well before [Ca\(^{2+}\)] increased. In fact, the glucose-stimulated increases in NO even preceded the [Ca\(^{2+}\)] decrease (phase 0) associated with calcium uptake into the endoplasmic reticulum as ATP/ADP increases (43, 44). In parallel studies of glucose stimulation, changes in oxygen consumption and NAD(P)H occurred with roughly the same latency as the glucose-induced changes in NO and thus also preceded the rise in islet [Ca\(^{2+}\)]. These findings thus suggest that glucose-induced increases in NO release may be closely linked to very early stages of glucose metabolism that are related to stimulus-secretion coupling in the islet.

Our finding that glucose-stimulated NO release precedes increases in [Ca\(^{2+}\)] appears to contradict another report of glucose-stimulated NO release (35). Although we are not certain why similar studies produced different time courses, several possibilities exist. First, diaminofluorescein 2 fluorescence is a marker of intracellular NO, so changes in dye fluorescence indicate changes in NO production with this method. In contrast, NO-sensitive electrodes detect net NO release at the surface of the islet. Second, whereas most of the cultured primary islet cells were likely β-cells in the Smukler et al. study (35), in our study the location of the electrode is an important determinant of the signal recorded, and we cannot be certain whether the electrode is near a β-cell, an α-cell, or another endocrine cell type. When positioning electrodes for electrophysiological recordings from mouse islets in previous studies, however, our methods sampled β-cells a majority of the time (44). Third, muscle cell lines and rat islet cells may function differently from intact mouse islets (16, 44). These differences may at least partly account for the observed differences in the properties of NO release observed in different studies.

Different isoforms of NOS may provide an additional explanation for the finding that NO release appeared to be temporally correlated to islet metabolism rather than [Ca\(^{2+}\)]. Although cNOS is known to be calcium-dependent, it is possible that β-cell [Ca\(^{2+}\)] was sufficiently elevated under our experimental conditions to reveal metabolically driven cNOS activity. Alternatively, our observations could reflect NO produced by eNOS or another NOS isoform. Future studies examining the effects of inhibitors of glucose transport and metabolism on islet NO production will be required to more fully elucidate the detailed metabolic basis of islet NO production. It will also be important to test whether directly depolarizing islets (e.g., using KCl, whereas glucose was used in the present studies) similarly triggers NO release and to repeat the experiments in the absence of external calcium. These studies should provide evidence as to whether NO can be regulated by multiple mechanisms (both calcium-dependent and -independent) in islets or whether multiple forms of NO may be active. Finally, we hope to inhibit specific NOS isoforms to determine which are most prominent in stimulating NO synthesis under conditions of glucose stimulation and islet depolarization.

In conclusion, we found that NO is spontaneously released in complex rhythmic patterns from islets in low glucose conditions and that NO release increased with glucose stimulation. Furthermore, simultaneous recordings of released NO and [Ca\(^{2+}\)], show that increased NO release occurs before the changes in islet [Ca\(^{2+}\)], in response to glucose stimulation. These findings thus suggest that glucose-stimulated NO release appears to precede the changes in ion channel activity that result in calcium influx and insulin secretion and further suggest that a glucose-sensitive or metabolically sensitive form of NOS exists in islets.

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