Mechanisms of Time-Dependent Potentiation of Insulin Release

Involvement of Nitric Oxide Synthase

Subhadra C. Gunawardana, Jonathan V. Rocheleau, W. Steven Head, and David W. Piston

Time-dependent potentiation (TDP) of insulin release is normally absent in mice. However, we recently demonstrated that TDP occurs in mouse islets under conditions of forced decrease of intracellular pH (pHi) associated with elevated NADPH+H⁺ (NADPH) levels. Hence, TDP in mouse islets may be kept suppressed by neuronal nitric oxide (NO) synthase (nNOS), an NADPH-utilizing enzyme with alkaline pH optimum. To determine the role of nNOS in the suppression of TDP in mouse islets, glucose-induced TDP was monitored in mouse islets in which nNOS activity had been genetically removed or chemically inhibited and compared with the TDP response in wild-type mouse islets with and without forced intracellular acidification. Genetic deletion of nNOS was provided by an nNOS knockout (NOS-KO) mouse model, B6–129S4-Nos1tm1Plh/J. To explore how nNOS inhibits TDP, we compared pHi and NADPH levels in wild-type and NOS-KO islets and monitored TDP with various components of the nNOS reaction added. Glucose normally does not produce TDP in wild-type mouse islets except under forced intracellular acidification. Remarkably, glucose produced strong TDP in NOS-KO islets and in wild-type islets treated with nNOS inhibitors. TDP in NOS-KO islets was not inhibited by the addition of NO, and NOS-KO islets exhibited a lower pH, than wild-type islets. The addition of arginine to wild-type islets also enabled glucose to induce TDP. Our results show that nNOS activity contributes to the absence of TDP in mouse islets putatively through depletion of intracellular arginine. Diabetes 55:1029–1033, 2006

From the Department of Molecular Physiology and Biophysics, Vanderbilt University, Nashville, Tennessee.

Address correspondence and reprint requests to David W. Piston, Department of Molecular Physiology and Biophysics, 702 Light Hall, Vanderbilt University, Nashville, TN 37232. E-mail: dave.piston@vanderbilt.edu.

Received for publication 24 November 2005 and accepted in revised form 21 December 2005.

DMA, dimethylamiloride; HBSS, Hanks’ balanced salt solution; KRBH, Krebs–Ringer bicarbonate HEPES buffer; nNOS, neuronal nitric oxide synthase; pHi, intracellular pH; TDI, time-dependent inhibition; TDP, time-dependent potentiation; TPEM, two-photon excitation microscopy.

© 2006 by the American Diabetes Association.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
This implicates nNOS activity as a major factor responsible for the absence of TDP in mouse islets. We also found that NOS-KO islets maintain a lower pHᵢ than wild-type islets and that the addition of NO does not inhibit the TDP normally present in NOS-KO islets. Furthermore, addition of arginine, the substrate of nNOS, enabled glucose to induce TDP in wild-type mouse islets.

Thus, this study demonstrates that nNOS activity plays a major role in keeping TDP inhibited in normal mouse islets. The major mechanism behind this inhibition is likely the depletion of arginine by nNOS, while altering islet pHᵢ may also play a role.

**RESEARCH DESIGN AND METHODS**

nNOS knockout (NOS-KO) mice were strain B6–129S4-Nos1<sup>tm11002</sup>/J (The Jackson Laboratories, Bar Harbor, ME) (26). C57BL<sub>6</sub> mice (Harlan Laboratories, Indianapolis, IN) were used as wild-type controls. All mice used were males aged 6–10 weeks. The animals were cared for according to the guidelines of the Vanderbilt Institutional Animal Care and Use Committee.

**Media.** Islets were isolated in Hank’s balanced salt solution (HBSS), and Krebs-Ringer bicarbonate HEPES buffer (KRBH) was used for the static incubations for insulin secretion measurements. The components of KRBH vary as follows: 115 mmol/l NaCl, 2.5 mmol/l CaCl<sub>2</sub>, 5 mmol/l NaHCO<sub>3</sub>, 2.8 mmol/l MgSO<sub>4</sub>, 2.5 mmol/l CaCl<sub>2</sub>, 5 mmol/l NaHCO<sub>3</sub>, 10 mmol/l HEPES, and 0.1% BSA. The medium pH was maintained at 7.4. Basal KRBH used for preincubation and nonstimulated controls contained 2.8 mmol/l glucose, while the stimulating media contained 16.7 mmol/l glucose. In the pHᵢ alteration experiments, 40 mmol/l DMA was added to the medium to produce intracellular acidification. One of two chemical inhibitors of NO synthase, i.e., l-NAME (l-N<sup>ω</sup>-nitro-arginine methyl ester) or l-NMMA (l-N<sup>ω</sup>-monomethyl-l-arginine), was added to KRBH where indicated. In the experiments with added NO, SNAP (S-nitroso-N-acetylpenicillamine), a short-term NO donor, or DETA-NO (diethylenetriamine NONOate), a longer-lasting NO donor, was added where indicated. In preparation for imaging experiments for pHᵢ and NADPH autofluorescence for each condition is expressed as a percentage of wild-type control in basal glucose. The number of islets imaged for each condition is denoted by n.

**Statistical Analysis.** Values are expressed as mean ± SE. Groups were compared using paired Student’s t test, and P values are indicated in the figures.

**RESULTS**

To determine whether nNOS activity is responsible for the lack of TDP in mouse islets, we monitored glucose-stimulated insulin secretion in islets previously primed by high glucose 20 min earlier. These experiments were performed in wild-type islets in the presence/absence of nNOS inhibitors during the priming period and in islets from NOS-KO mice. In wild-type mouse islets, which normally do not exhibit TDP, glucose can induce TDP provided there is forced decrease of pHᵢ during the priming period (19) (Fig. 1A). Remarkably, glucose exhibited strong TDP in NOS-KO islets even in the absence of DMA-induced intracellular acidification (Fig. 1A). The presence of DMA did not influence the magnitude of TDP. Glucose-induced TDP was also observed in wild-type islets previously treated with chemical inhibitors of nNOS (Fig. 1B). These results implicate nNOS as a major factor that inhibits/masks TDP in wild-type mouse islets.

In addition to suppressing TDP, nNOS also partially inhibits direct insulin secretion during the priming period. The magnitude of glucose-induced direct insulin release is slightly but significantly larger in NOS-KO islets than in wild-type islets (data not shown). However, since insulin release per se does not affect the induction of TDP (2), the mechanisms whereby nNOS inhibits TDP are more likely to involve the chemical reactions catalyzed by nNOS.

nNOS catalyzes the conversion of arginine to citrulline and NO, utilizing NADPH as a cofactor. Therefore, the inhibitory effect of nNOS on TDP is likely to be mediated by one of three mechanisms: depletion of arginine, depletion of NADPH, and generation of NO. Depletion of arginine or NADPH would putatively remove signaling molecules of TDP from the system, while the NO generated by nNOS could be an inhibitor of TDP. To determine which of these factors were involved, we monitored TDP in NOS-KO islets in the presence and absence of NO donors and in wild-type islets in the presence of exogenous arginine. We also compared cellular NADPH levels in wild-type and NOS-KO islets to determine whether removal of nNOS would prevent the depletion of NADPH.

Addition of exogenous NO using two different NO donors did not inhibit the TDP normally present in NOS-KO islets (Fig. 2), and NADPH levels in NOS-KO islets were equivalent to those observed in wild-type islets (Fig.
3). Together, these data suggest that the inhibition of TDP by nNOS is not mediated by generation of NO or depletion of NADPH. In contrast, the addition of exogenous arginine to wild-type islets enabled glucose to induce TDP (Fig. 4), indicating that depletion of arginine is a mechanism contributing to the inhibition of TDP by nNOS. These data implicate arginine as a signaling molecule in TDP and the utilization of arginine by nNOS as a limiting factor for TDP in mouse islets.

To determine whether nNOS also affects islet pH, we monitored pH in wild-type and NOS-KO islets under basal and glucose-stimulated conditions. NOS-KO islets consistently exhibited a slightly lower pH than wild-type islets (Table 1). Thus, in addition to depleting arginine, nNOS activity helps maintain a higher islet pH, that also may contribute to the masking of TDP in mouse islets.

**DISCUSSION**

As evidenced by several studies, induction of TDP is a promising therapeutic approach to correct the secretory defect in type 2 diabetes (10–14). Inducing TDP in the presence of a secretory defect requires knowledge of the underlying mechanisms of TDP. The limited information available on this subject include that TDP requires the metabolism of glucose (2–4); is not dependent on insulin biosynthesis, elevation of cAMP, or ATP-sensitive K+ channel function (2–4); and may involve cellular phosphoinositide metabolism (5,6). Previous studies in rat islets have demonstrated that TDP is independent of Ca2+ but critically dependent on pH (21).

The importance of pH for TDP is further evidenced by our recent finding that a forced decrease of pH enables glucose to induce TDP in mouse islets where this function is normally absent. The present study documents a plausible mechanism for this effect and the reasons behind the absence of TDP in normal mouse islets. We have demonstrated that TDP can be enabled in mouse islets by removal or inhibition of nNOS activity, an enzyme with a strongly alkaline pH optimum. Glucose induces strong TDP in mouse islets provided nNOS activity is removed or inhibited, indicating that nNOS activity plays a major role in keeping TDP suppressed in normal mouse islets.
As is widely known, nNOS converts arginine to citrulline and NO, utilizing NADPH as a cofactor. The mechanism whereby nNOS inhibits TDP could involve generation of NO, a possible inhibitor of TDP, or removal of NADPH and/or arginine (possible signaling molecules in TDP). To determine which of these mechanisms are involved, we monitored TDP in wild-type islets with added arginine and in NOS-KO islets in the presence of compounds that generate NO.

If nNOS suppresses TDP through the inhibitory action of NO, addition of exogenous NO should inhibit TDP naturally present in NOS-KO islets. However, both NO donors we used failed to prevent TDP, indicating that NO generation is not the mechanism whereby nNOS suppresses TDP. We next explored the depletion of substrates as a possible mechanism of inhibition of TDP. Arginine and NADPH, the major substrate and cofactor of nNOS, may act as signaling molecules in nutrient-induced TDP. To determine whether removal of nNOS prevents the depletion of NADPH, we compared cellular NADPH autofluorescence in wild-type and NOS-KO islets by TPEM. If nNOS causes considerable depletion of NADPH, the levels of NADPH in NOS-KO islets should be significantly larger than those of wild-type islets. This was not the case, as the NADPH autofluorescence in NOS-KO islets was slightly lower than in wild-type islets (Fig. 3). These data indicate that the inhibition of TDP by nNOS is not mediated through depletion of NADPH. In contrast, addition of exogenous arginine during the priming period enabled glucose to induce TDP in wild-type mouse islets (Fig. 4). This implicates arginine as a possible signaling molecule in TDP and arginine depletion as a plausible mechanism for the suppression of TDP by nNOS. Arginine is an insulin secretagogue that stimulates monophasic insulin release. Previous studies in rat islets have reported arginine to induce time-dependent inhibition (TDI) rather than potentiation (30,31). However, TDI by arginine was demonstrated at lower doses (0.5–5 mmol/l) in the absence of a stimulatory concentration of glucose (30). The present study shows arginine to behave differently in mouse islets in the presence of high glucose. This may partially result from species differences. Unlike in rat islets, a previous exposure to arginine did not induce TDI in mouse islets (Fig. 4, condition 2 [2.8 mmol/l glucose plus 20 mmol/l arginine]), and the combination of arginine and high glucose induced significant TDP (Fig. 4, condition 4 [16.7 mmol/l glucose plus 20 mmol/l arginine]). Thus, the ability of arginine to restore glucose-induced TDP in mouse islets may result from species differences, the higher dose of arginine used, and the ability of arginine to enhance metabolic priming signals produced by glucose.

In addition to a direct effect through the depletion of its own substrate, nNOS may exert an indirect effect on TDP through altering islet pH. To explore this possibility, we compared pH in wild-type and NOS-KO islets. Interestingly, NOS-KO islets consistently maintained a lower pH than wild-type islets under both basal and glucose-stimulated conditions (Table 1). The difference in pH is small, but significant, in comparison to the

![FIG. 3. Cellular NADPH autofluorescence in NOS-KO islets is not higher than that of wild-type (WT) islets. NADPH autofluorescence was measured in cultured islets using TPEM, in response to basal and high glucose (G). High glucose increases NADPH autofluorescence in both cytosol and mitochondria. NADPH autofluorescence in NOS-KO islets (□) is slightly but significantly lower than that of wild-type islets (○) (n = 20).](image1)

![FIG. 4. Arginine enables glucose to induce TDP in wild-type islets. Insulin secretion in response to 16.7 mmol/l glucose (G) in wild-type islets previously exposed to glucose in the presence (■) or absence (□) of exogenous arginine (20 mmol/l) (n = 11). High glucose induces TDP in the presence of arginine. The corresponding absolute amounts (ng/ml) for each condition from left to right are 4.5 ± 0.82, 4.7 ± 0.91, 5.6 ± 0.84, and 8.0 ± 0.75.](image2)

| TABLE 1 |
| Comparison of pH in wild-type and NOS-KO islets |
| In basal glucose | In high glucose |
| Wild type | 7.11 ± 0.03 | 7.26 ± 0.03 |
| NOS-KO | 6.93 ± 0.02* | 7.02 ± 0.03* |

Data are means ± SE (n = 20 for each condition). Islets loaded with SNARF5-AM were placed in KRKH medium of either basal (2.8 mmol/l) or high (16.7 mmol/l) glucose. Islets were excited at 514 nm and emission fluorescence recorded at 580 and 630 nm. Average pH was calculated over a stable region of each recording, and these values from n recordings were averaged to obtain the pH values shown. *P < 0.001 vs. same condition in wild-type islets.
effect of DMA. As suggested in previous work (32), this slight intracellular acidification may also contribute to the observed unmasking of TDP through enhanced anaplerotic activity.

Regardless of the mechanism, it is clear that nNOS activity plays a major role in keeping TDP suppressed in wild-type mouse islets. In human islets where TDP is normally present, the secretory defect in type 2 diabetes may be associated with impairment of TDP due to excessive activity of nNOS. Thus, it would be worthwhile to compare islet nNOS activity in the healthy and diabetic situations. If there is overactivity of nNOS in diabetes, treatment with exogenous arginine may prove to be a simple technique to strengthen the secretory capacity by restoring impaired TDP. Induction of TDP with nonglucose secretagogues combined with amiloride derivatives or nNOS inhibitors is also a potential therapeutic approach to enhance insulin release in human diabetes.

ACKNOWLEDGMENTS

This work was supported by the National Institutes of Health grants DK67821 (to S.C.G.) and DK53434 (to D.W.P.).

Some assays and data analysis were performed in part through the use of the Diabetes Research and Training Center Hormone Core Resource facility.

REFERENCES