Original Article

Mechanisms of Time-Dependent Potentiation of Insulin Release

Involvement of Nitric Oxide Synthase

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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 (pH_i) 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-*Nos1*^{tm1Plh}/J. To explore how nNOS inhibits TDP, we compared pH_i 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_i 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

Time-dependent potentiation (TDP) is an important aspect of insulin secretion from the pancreatic β-cell. TDP is defined as an enhancement of the insulin response resulting from a "memory" induced by previous exposure to certain secretagogues. TDP was first documented by Grodsky et al. (1). While TDP is reported in different experimental systems, including rat islets, isolated rat pancreas, and in vivo studies in humans (2–10), the underlying mechanisms are largely unknown. Once induced, TDP lasts for at least 1 h and magnifies subsequent insulin responses to all secretagogues (2–4,9).

In certain diabetic patients with impaired direct insulin secretory response, the potentiating function of glucose remains intact. In such subjects, the impaired insulin response can be restored to normal by inducing TDP (7,10,11). In other cases, both the direct insulin response and TDP are impaired (12–14), and restoring the TDP response may automatically repair the defect in direct insulin secretion. Thus, induction of TDP without raising blood glucose would be a useful therapeutic technique in type 2 diabetes. Such an approach requires knowledge of the underlying mechanisms of TDP.

TDP occurs only in certain species such as human (7–10), rat (2–6), rabbit (15), and spiny mouse (11). It is widely believed that TDP is absent in mice. Several previous studies (16–18) and our preliminary work have shown that mouse islets do not normally exhibit TDP. However, we recently demonstrated that TDP can be enabled/unmasked in mouse islets under certain conditions (19,20). Such conditions are provided by prior treatment with dimethylamiloride (DMA), which is associated with a sustained decrease in islet intracellular pH (pH_i) and an increase in the levels of cellular reducing equivalents (19). DMA treatment enables glucose to induce TDP in mouse islets in which it is normally absent (19,20) and magnifies the TDP normally present in rat islets (21). Since DMA alone does not stimulate insulin release, we postulated that the DMA-induced increase of reducing equivalents is more likely to represent $NADPH+H^+$ (NADPH) rather than $NADH+H^+$ (NADH) (22). In the context of this model, the DMA-induced decrease of pH_i enables/unmasks TDP either by activating an enzyme that generates NADPH or by inhibiting an enzyme that utilizes NADPH. The few islet enzymes that utilize $NADP^+$/NADPH as a cofactor (as opposed to NAD^+/NADH) include glutamate dehydrogenase, isocitrate dehydorgenase, and neuronal nitric oxide (NO) synthase (nNOS). Although all of these enzymes are pH sensitive, nNOS is the most likely candidate due to its strongly alkaline pH optimum, which ranges from 7.5 to 9.5 (23–25).

To test the involvement of nNOS in the suppression of TDP, we monitored TDP in mouse islets in which nNOS had been knocked out or chemically inhibited. Remarkably, in situations where nNOS was absent or inhibited, glucose could induce strong TDP independently of pH_i.

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

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 pH_i 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 pH_i may also play a role.

RESEARCH DESIGN AND METHODS

nNOS knockout (NOS-KO) mice were strain B6–129S4-*Nos1*^{tm1Plh}/J (The Jackson Laboratories, Bar Harbor, ME) (26). C57BL6 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 Hanks' 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 are as follows: 128.8 mmol/l NaCl, 4.8 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4$, 2.5 mmol/l $CaCl_2$, 5 mmol/l $NaHCO_3^-$, 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 pH_i alteration experiments, 40 μmol/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^G-nitro-L-arginine methyl ester) or L-NMMA (N^G-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 (diethylenetriamineNONOate), a longer-lasting NO donor, was added where indicated. In preparation for imaging experiments for pH_i and NADPH measurements, islets were cultured in RPMI-1640 culture medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 0.1 g/l streptomycin, and 11 mmol/l glucose.

Isolation of islets. A modified version of the collagenase digestion method described by Lacy and Kostianovsky (27) was used. Mice were anesthetized by injection of ketamine/xylazine (80/20 mg/kg i.p.). Pancreata were removed, placed in ice-cold HBSS, and minced with scissors. Collagenase (3 mg/ml) was added and the mixture shaken in a 37°C water bath until the tissue was adequately digested. The mixture was then centrifuged, supernatant removed, and the pellet resuspended in HBSS. Centrifugation and resuspension were repeated several times to remove exocrine tissue. The final pellet was resuspended either in basal KRBH medium for secretion measurements or in RPMI-1640 medium for islet culture. Islets were handpicked under a stereo microscope.

Secretion measurements. All incubations were done with freshly isolated islets in a 37°C water bath. Groups of islets were exposed to four different incubation periods, i.e., preincubation, first exposure to high glucose (priming or memory-inducing period), rest in basal glucose, and final stimulation with high glucose (memory-manifesting period). Fresh islets were first preincubated for 40 min in basal KRBH containing 2.8 mmol/l glucose. Islets were then divided into groups and exposed to different conditions for 40 min, as indicated in the RESULTS section, for possible induction of TDP. Control groups were maintained in basal glucose. Subsequently, all groups were washed and rested in basal KRBH for 20 min and then stimulated with 16.7 mmol/l glucose for 40 min. At the end of this final stimulation period, samples were collected for insulin measurement by radioimmunoassay (performed by the Diabetes Research and Training Center Hormone Core Resource facility at Vanderbilt University). Islet insulin content was measured after freezing islets overnight in 1% Triton-X. Insulin secretion is expressed as fractional release, i.e., the percentage of total insulin content released over the period of stimulation. The corresponding absolute values of insulin secreted into the medium (in nanograms per milliliter) under each experimental condition are listed at the end of each figure legend. The number of times each experiment was repeated with islets from different mice is shown as *n*.

Culture of islets. For imaging experiments, islets were cultured using methods developed in our lab, as previously described (19,20). Culture dishes (35 mm) with glass-bottomed wells (Mat-Tek) were used. The dishes were preprepared by coating the wells with human extracellular matrix (BD Biosciences). Freshly isolated islets were carefully placed in each well, covered with RPMI-1640 medium containing 11 mmol/l glucose, and cultured at 37°C in 95% air and 5% CO_2. Under these conditions, the cells in the islet spread out over the glass surface within 14 days, greatly reducing the islet thickness and making them particularly suitable for imaging with confocal microscopy.

pH_i measurements. As described in detail previously (19,20), pH_i in cultured islets was monitored by confocal microscopy using SNARF5 (carboxy-seminaphthorhodofluor-5F-AM), a pH-sensitive fluorescent indicator with single excitation and dual emission (28). Before imaging, cultured islets were maintained in RPMI-1640 medium containing 5 mmol/l glucose for at least 48 h. Loading was done in basal KRBH, and islet pH_i was measured in KRBH containing basal (2.8 mmol/l) or high (16.7 mmol/l) glucose. The number of islets imaged for each condition is denoted by *n*.

Imaging of NADPH. NADPH autofluorescence was measured in cultured islets using two-photon excitation microscopy (TPEM) combined with techniques developed in our lab, as described previously (19,29). Before imaging, cultured islets were maintained in RPMI-1640 medium containing 5 mmol/l glucose for at least 48 h. Imaging was done in KRBH medium. Wild-type and NOS-KO islets were imaged in basal (2.8 mmol/l) and high (16.7 mmol/l) glucose, 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 pH_i during the priming period (19) (Fig. 1*A*). Remarkably, glucose exhibited strong TDP in NOS-KO islets even in the absence of DMA-induced intracellular acidification (Fig. 1*A*). 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. 1*B*). 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.

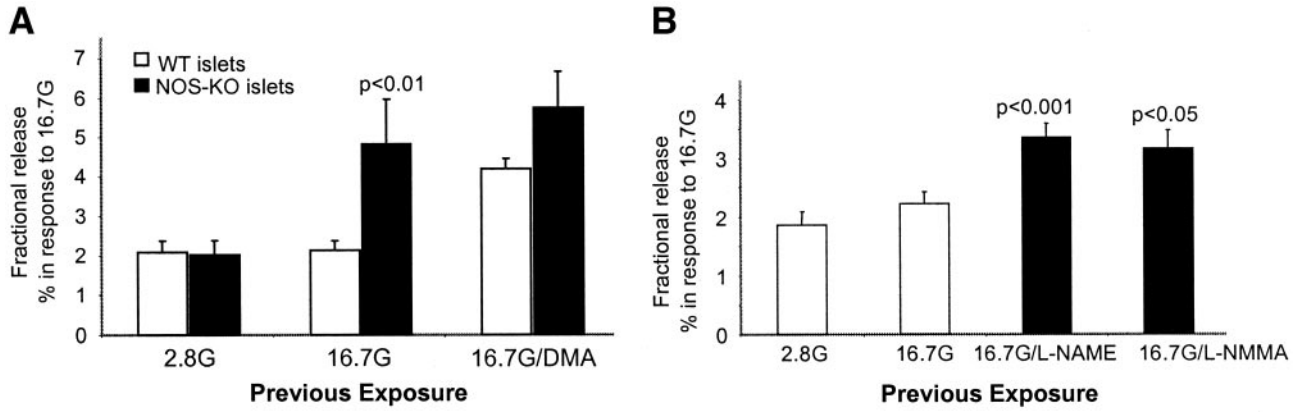

FIG. 1. Glucose induces TDP in the absence of nNOS activity. *A*: Insulin secretion in response to 16.7 mmol/l glucose (G) in islets from wild-type (WT) and NOS-KO mice. Islets were previously exposed to 16.7 mmol/l glucose in the presence or absence of DMA (40 μmol/l), as indicated on the *x*-axis, with controls maintained in basal glucose. Glucose-induced TDP is observed in wild-type islets (□) in the presence of DMA only and in NOS-KO islets (■) in both the presence and absence of DMA ($n = 7$; $P < 0.01$ vs. same condition in wild-type islets and/or both unprimed controls). *B*: Insulin secretion in response to 16.7 mmol/l glucose in islets from wild-type mice only. As indicated on the *x*-axis, islets were previously exposed to 16.7 mmol/l glucose in the presence or absence L-NAME or L-NMMA (5 mmol/l each), with control maintained in basal glucose. Glucose-induced TDP is observed in the presence of either inhibitor (■) (*t* test compares high glucose with and without each inhibitor; $n = 4$ for 16.7G/L-NMMA, $n = 8$ for all other conditions). The corresponding absolute amounts (ng/ml) for each condition from left to right are as follows: *A*: 4.2 ± 0.1, 4.3 ± 0.94, 5.5 ± 0.73, 10.8 ± 1.13, 10.3 ± 0.91, and 13.1 ± 1.7; *B*: 6.1 ± 0.83, 7.8 ± 1.25, 10.5 ± 1.98, and 5.6 ± 0.59.

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_i, we monitored pH_i in wild-type and NOS-KO islets under basal and glucose-stimulated conditions. NOS-KO islets consistently exhibited a slightly lower pH_i than wild-type islets (Table 1). Thus, in addition to depleting arginine, nNOS activity helps maintain a higher islet pH_i 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 *1*) requires the metabolism of glucose (2–4); *2*) is not dependent on insulin biosynthesis, elevation of cAMP, or ATP-sensitive K^+ channel function (2–4); and *3*) may involve cellular phosphoinositide metabolism (5,6). Previous studies in rat islets have demonstrated that TDP is independent of Ca^{2+} but critically dependent on pH_i (21).

The importance of pH_i for TDP is further evidenced by our recent finding that a forced decrease of pH_i enables glucose to induce TDP in mouse islets where this function is normally absent (19,20). 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.

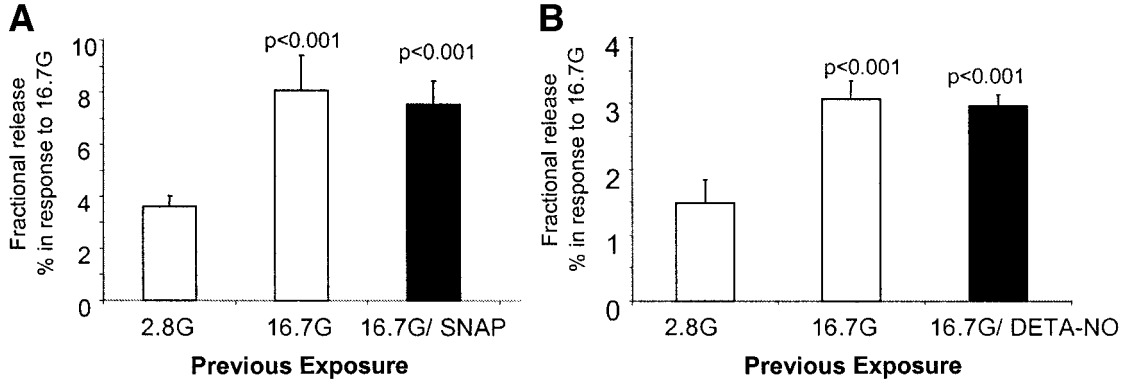

FIG. 2. Addition of exogenous NO does not inhibit TDP normally present in NOS-KO islets. Insulin secretion in response to 16.7 mmol/l glucose (G) in NOS-KO islets previously exposed to 16.7 mmol/l glucose in the presence or absence of the NO donors SNAP (0.1 mmol/l) or DETA-NO (0.5 mmol/l) with controls maintained in basal glucose. Glucose induces TDP in both the presence and absence of each NO donor (*t* test compares each condition with the corresponding control in basal glucose). No significant difference between high glucose with and without each NO donor ($n = 5$). The corresponding absolute amounts (ng/ml) for each condition from left to right are 4.4 ± 1.38, 13.0 ± 3.1, 9.1 ± 1.81, 4.5 ± 1.25, 7.76 ± 1.5, and 8.56 ± 0.99.

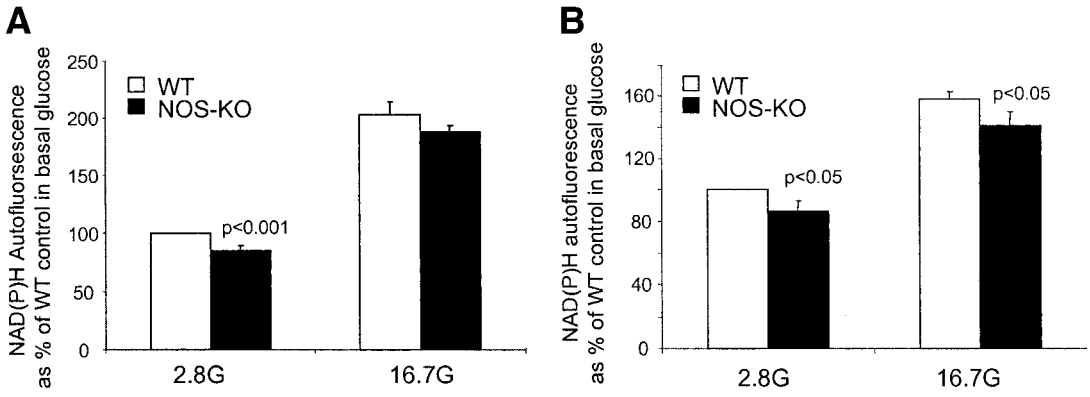

FIG. 3. Cellular NADPH autofluorescence in NOS-KO islets is not higher than that of wild-type (WT) islets. NADPH autofluorescence was measured in cultured islets using TPEM, in response to basal and high glucose (G). High glucose increases NADPH autofluorescence in both cytosol and mitochondria. NADPH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

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_i. To explore this possibility, we compared pH_i in wild-type and NOS-KO islets. Interestingly, NOS-KO islets consistently maintained a lower pH_i than wild-type islets under both basal and glucose-stimulated conditions (Table 1). The difference in pH_i is small, but significant, in comparison to the

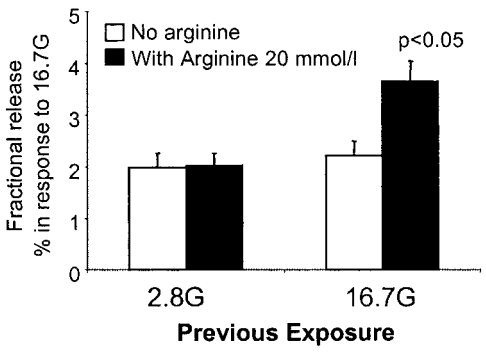

FIG. 4. Arginine enables glucose to induce TDP in wild-type islets. Insulin secretion in response to 16.7 mmol/l glucose (G) in wild-type islets previously exposed to glucose in the presence (■) or absence (□) of exogenous arginine (20 mmol/l) (n = 11). High glucose induces TDP in the presence of arginine. 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.

TABLE 1
Comparsion of pH_i 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 KRBH 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_i 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.

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