

Changes in the Dimeric State of Neuronal Nitric Oxide Synthase Affect the Kinetics of Secretagogue-Induced Insulin Response

Anne-Dominique Lajoix,^{1,2} Martine Pugnère,² Françoise Roquet,² Jean-Claude Mani,² Samuel Dietz,^{1,2} Nathalie Linck,² Fleur Faurie,² Gérard Ribes,² Pierre Petit,² and René Gross²

We previously showed that pancreatic β -cells express a neuronal isoform of nitric oxide synthase (nNOS) that controls insulin secretion by exerting two enzymatic activities: nitric oxide (NO) production and cytochrome c reductase activity. We now bring evidence that two inhibitors of nNOS, *N*- ω -nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole (7-NI), increase glucose-induced insulin secretion but affect β -cell function differently. In the presence of L-NAME, insulin response is monophasic, whereas 7-NI preserves the normal biphasic secretory pattern. In addition, the alterations of β -cell functional response induced by the inhibitors also differ by their sensitivity to a substitutive treatment with sodium nitroprusside, a chemical NO donor. These differences are probably related to the nature of the two inhibitors. Indeed, using low-temperature SDS-PAGE and real-time analysis of nNOS dimerization by surface plasmon resonance, we could show that 7-NI, which competes with arginine and tetrahydrobiopterin (BH₄), an essential cofactor for nNOS dimer formation, inhibits dimerization of the enzyme, whereas the substrate-based inhibitor L-NAME stabilizes the homodimeric state of nNOS. The latter effect could be reproduced by the two endogenous inhibitors of NOS, *N*- ω -methyl-L-arginine and asymmetric dimethylarginine, and resulted interestingly in a reduced ability of the protein inhibitor of nNOS (PIN) to dissociate nNOS dimers. We conclude that intracellular factors able to induce abnormalities in the nNOS monomer/dimer equilibrium could lead to pancreatic β -cell dysfunction. *Diabetes* 53: 1467–1474, 2004

Nitric oxide (NO) is a short-lived gas produced by a family of enzymes named NO synthases (NOSs). Three isoforms have been identified: the Ca²⁺-calmodulin-dependent constitutive NOS, including neuronal (nNOS) and endothelial NOS (eNOS), and a

Ca²⁺-calmodulin-independent inducible NOS (iNOS) (1). As homodimers (2), NOSs produce NO through the transfer of NADPH-derived electrons from the reductase domain to the heme of the adjacent oxygenase domain, so that the heme iron binds O₂ and catalyzes the mono-oxygenation of the substrate arginine. Accordingly, different classes of NOS inhibitors have been developed to assess the functional role of these enzymes and include substrate-based inhibitors such as arginine analogs (3–5) and imidazole derivatives (6), which compete with the substrate binding, flavoprotein (7), calmodulin inhibitors (8), and finally pterin antagonists such as indazole agents (9). These indazole agents compete with arginine and tetrahydrobiopterin (BH₄), an essential cofactor for NOS activity.

Concerning the pancreatic β -cell, conflicting data have been reported as to the cellular localization and the nature of the constitutive NOS isoform present in pancreatic islets (10–13). However, recent molecular, immunocytochemical, and electron microscopical studies from our laboratory demonstrate the expression of a neuronal isoform of NOS in insulin-secreting β -cells presenting a 99.8% homology with rat cerebellar NOS (14). From studies in isolated islets with the two nNOS inhibitors, *N*- ω -nitro-L-arginine methyl ester (L-NAME) and 7-nitroindazole (7-NI), it appears that pancreatic β -cell nNOS exerts a tonic inhibitory effect on insulin secretion (15–17). We also found such an effect in the isolated perfused rat pancreas, in which L-NAME not only amplifies glucose-induced insulin secretion but also completely suppresses the biphasic pattern of insulin response, an essential feature of normal β -cell function (17).

Brain NOS has been shown to exert two catalytic activities: first, the monooxygenation of arginine with NO production, which requires a homodimeric conformation of the enzyme, and, second, the nonoxidating reduction of cytochrome c (18), which, unlike NO synthesis, occurs regardless of the dimeric/monomeric state of nNOS. Concerning rat pancreatic nNOS, we were able to show that the enzyme can also produce NO and reduce cytochrome c and that an increase in the latter activity accounts for a great part of the alteration of insulin secretion after blockade with L-NAME (14). The mechanism through which nNOS blockade occurs with L-NAME (competitive with arginine at the substrate site level) and 7-NI (competitive with both arginine and BH₄, essential for nNOS dimerization) might differently affect the nNOS quaternary structure. This prompted us first to study and compare the

From ¹Inmodia SAS, Montpellier, France; and ²UMR 5160 CNRS, Montpellier, France.

Address correspondence and reprint requests to René Gross, CNRS UMR 5160, Institut de Biologie, 4 Boulevard Henri IV, 34960 Montpellier Cedex 2, France. E-mail: rene.gross@univ-montp1.fr.

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7-NI, 7-nitroindazole; ADMA, asymmetric dimethyl-arginine; BH₄, tetrahydrobiopterin; eNOS, endothelial NOS; iNOS, inducible NOS; L-NAME, *N*- ω -nitro-L-arginine methyl ester; L-NNA, *N*- ω -nitro-L-arginine; L-MMA, *N*- ω -methyl-L-arginine; NOS, nitric oxide synthase; nNOS, neuronal NOS; PIN, protein inhibitor of nNOS; SNP, sodium nitroprusside.

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effects of L-NAME and 7-NI on the kinetics and the magnitude of glucose-induced insulin secretion and then to evaluate their respective sensitivity to a substitutive treatment with an NO donor, sodium nitroprusside (SNP), and to an inhibitor of cytochrome c reductase activity, miconazole. Finally, we investigated if and how the differences we recorded relate to changes in the ability of nNOS to homodimerize in low-temperature SDS-PAGE and surface plasmon resonance studies in the presence of the two inhibitors.

RESEARCH DESIGN AND METHODS

Functional studies. Adult male Wistar rats weighing 330–370 g were used in this study. After anesthesia of animals with sodium pentobarbitone (60 mg/kg i.p.), pancreata were isolated from all neighboring tissues and transferred into a thermostated plastic chamber according to the procedure of Loubatières et al. (19). The organs were perfused through their own arterial system with Krebs-Ringer bicarbonate buffer (108 mmol/l NaCl, 1.19 mmol/l KH_2PO_4 , 4.74 mmol/l KCl, 2.54 mmol/l CaCl_2 , 1.19 mmol/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 18 mmol/l NaHCO_3) containing 2 g/l BSA and 5 mmol/l glucose. It was continuously bubbled with a mixture of 95% O_2 and 5% CO_2 , and the pH was maintained close to 7.35. Perfusions were carried out at a constant pressure of ~30–40 cm water, selected to provide a pancreatic flow rate of 2.5 ml/min. A 30-min equilibration period was allowed before the first sampling; two more control samples were collected 5 and 10 min later before any drug administration. Pancreatic effluents were measured in graduated cylinders and immediately frozen until insulin determination. The pharmacological drugs, L-NAME, L-arginine miconazole nitrate salt, 7-NI, and SNP dihydrate were purchased from Sigma-Aldrich (Steinheim, Germany).

Insulin was measured by the radioimmunochemical method of Herbert et al. (20) with rat insulin (Linco Research, St. Charles, MI) as a standard, allowing a sensitivity of 0.06 ng/ml. Insulin secretion was calculated by multiplying the hormone concentration (ng/ml) by the pancreatic flow rate. The values are given in the text and plotted in the figures as means \pm SE, but also as mean integrated data obtained by calculating the areas under the curve during the 20 min of high-glucose (11 mmol/l) administration. Both kinetic and integrated data were submitted to ANOVA followed by the Newman-Keuls multiple comparison test.

Cell culture and incubation experiments. The insulin-secreting cell line INS-1 (a gift from Professor C.B. Wollheim) was cultured in RPMI-1640 supplemented with 10% FCS, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2 mmol/l L-glutamine, 10 mmol/l HEPES, 1 mmol/l sodium pyruvate, and 50 mmol/l 2-mercaptoethanol, according to the method of Asfari et al. (21).

For incubation experiments, INS-1 cells were seeded in 6-cm dishes at the density of 2.5×10^6 and cultured for 5 days. The cells were first washed and preincubated for 1 h at 37°C in Krebs-Ringer bicarbonate buffer containing 2 g/l BSA in the absence of glucose. After removal of the medium, the cells were incubated for 1 h at 37°C in the same buffer in the presence of 5 mmol/l glucose and the different pharmacological drugs (see below). At the end of the incubation period, the medium was removed and the cells were solubilized in lysis buffer (see below).

Low-temperature SDS-PAGE and Western blot. Low-temperature SDS-PAGE has been used to study nNOS dimerization in vitro and in INS-1 cells (22). For in vitro analysis, low-temperature SDS-PAGE was performed on 1 μg recombinant rat nNOS (Alexis Biochemicals, Lausen, Switzerland), after incubation in 50 mmol/l triethanolamine buffer (pH 7) (22) for 1 h at 25°C in the absence or presence of BH_4 (100 $\mu\text{mol}/\text{l}$; Sigma-Aldrich), arginine (1 mmol/l), *N*- ω -nitro-L-arginine (L-NNA) (1 mmol/l; Sigma-Aldrich), 7-NI (100 $\mu\text{mol}/\text{l}$), *N*- ω -methyl-L-arginine (1 mmol/l; Sigma-Aldrich), and *N*- ω , *N*- ω -dimethylarginine (1 mmol/l; Sigma-Aldrich). For experiments with the protein inhibitor of nNOS (PIN), recombinant PIN (2, 5, and 10 molar excess vs. nNOS) was added to the pharmacological drugs for the last 45 min of a 1.5-h incubation. Incubations were terminated by the addition of cold 2 \times Laemmli buffer. Samples were then subjected to SDS-PAGE using a 4–12% gradient gel (Invitrogen, Carlsbad, CA) in a cold room at 4°C (22). After a 2-h migration, the gels were stained with the Silver Staining Kit of Amersham Biosciences (Little Chalfont, U.K.).

In the case of INS-1 cell extracts, low-temperature SDS-PAGE was followed by a Western blot. INS-1 cells were first incubated in 5 mmol/l glucose in the absence or presence of 5 mmol/l arginine, 5 mmol/l L-NAME, 5 mmol/l arginine + 5 mmol/l L-NAME, 100 $\mu\text{mol}/\text{l}$ 7-NI, and 100 $\mu\text{mol}/\text{l}$ 7-NI + 5 mmol/l arginine for 1 h. After incubation, the cells were homogenized in 20 mmol/l Tris lysis buffer, pH 7.4, containing 150 mmol/l NaCl, 1% Triton X-100, 0.1% SDS, and a cocktail of protease inhibitors (Roche Applied Science, Mannheim, Germany).

Then, 75 μg soluble proteins were separated on a 6% SDS-polyacrylamide gel at 10°C and transferred to a nitrocellulose membrane. Filters were first saturated with 5% dried skim milk and then incubated in the presence of a monoclonal anti-nNOS antibody (diluted 1:750; Transduction Laboratories, Lexington, KY). After washing, the membranes were finally incubated with a horseradish peroxidase-conjugated anti-mouse antibody (diluted 1:3,000; Sigma-Aldrich). Immunoreactivity was detected by an enhanced chemiluminescence reaction (Amersham Biosciences).

BIACORE analysis. nNOS dimerization was analyzed by surface plasmon resonance using a BIACORE 2000 apparatus (Biacore AB, Uppsala, Sweden). Recombinant rat nNOS (Alexis Biochemicals), at a concentration of 5 $\mu\text{g}/\text{ml}$ in 10 mmol/l acetate buffer (pH 5.5), was covalently immobilized on a flow cell of a CM5 sensor chip (Biacore AB) using the *N*-ethyl-*N*-(dimethylaminopropyl)carbodiimide protocol described by the manufacturer. The amount of bound nNOS was 1,500 pg/mm^2 . A second flow cell without immobilized protein was used as a control. The binding experiments were performed at 37°C in the eluent buffer HBS-EP (Biacore AB) at a flow rate of 30 $\mu\text{l}/\text{min}$. Next, 10 $\mu\text{g}/\text{ml}$ nNOS diluted in the eluent buffer was injected (over a period of 180 s) alone or in the presence of different combinations of BH_4 (10 $\mu\text{mol}/\text{l}$), L-NNA (0.1 mmol/l), or 7-NI (10 $\mu\text{mol}/\text{l}$). The nNOS-coated flow cell was first equilibrated with the eluent buffer containing the same combination of pharmacological drugs, and nNOS was added to this buffer immediately before injection to avoid dimerization in the tube. After the injection step, a 400-s dissociation step with eluent buffer was followed by a pulse of HCOOH 2M to regenerate the nNOS surface. The same experiments were performed with an irrelevant protein injected to measure the nonspecific binding. The immobilization level of nNOS and the flow rate were optimized to avoid mass transfer phenomena. Each experiment was repeated at least three times. The kinetic parameters of the binding reaction were determined using BIA evaluation 3.2 software (Biacore AB).

RESULTS

Comparative study of the alteration of insulin response to glucose by L-NAME and 7-NI. Raising the glucose concentration from 5 to 11 mmol/l provoked the classic biphasic insulin response with a mean integrated response of 178.6 ± 13.0 ng/ml \times 20 min (Fig. 1A). Administration of the competitive inhibitor of nNOS, L-NAME (5 mmol/l), 15 min before and during high glucose perfusion converted the biphasic pattern into a monophasic one with a significant increment of insulin output (596.5 ± 20.0 ng \times 20 min; $P < 0.001$) (Fig. 1A). The dose of L-NAME chosen has been previously shown not to produce a nonspecific depolarizing effect (23). The second inhibitor of nNOS, 7-NI, infused under the same conditions, dose-dependently (from 20 to 100 $\mu\text{mol}/\text{l}$; data not shown) potentiated insulin response to glucose. Indeed, at a concentration of 100 $\mu\text{mol}/\text{l}$, insulin secretion was markedly increased (895.1 ± 18.1 ng \times 20 min; $P < 0.001$) but remained clearly biphasic (Fig. 1A).

Effect of SNP and miconazole on alteration of the insulin response to glucose induced by L-NAME and 7-NI. A substitutive treatment with the NO donor SNP (30 $\mu\text{mol}/\text{l}$) was ineffective in the presence of 5 mmol/l L-NAME and high glucose (11 mmol/l) but significantly decreased the secretory effect of 100 $\mu\text{mol}/\text{l}$ 7-NI from 895.1 ± 98.1 to 389.7 ± 33.6 ng \times 20 min ($P < 0.001$) (Fig. 1B). At the higher concentration (300 $\mu\text{mol}/\text{l}$), SNP strongly increased insulin secretion in the presence of L-NAME (1274.0 ± 108.3 ng \times 20 min; $P < 0.001$) but drastically reduced the effect of 7-NI by 80% (180.8 ± 21.6 ng \times 20 min; $P < 0.001$). In contrast, the inhibitor of cytochrome c reductase activity, miconazole (10 $\mu\text{mol}/\text{l}$), significantly decreased both L-NAME- and 7-NI-induced increases in insulin outputs to 177.2 ± 12.8 (–70%) and 349.8 ± 29.7 (–61%) ng \times 20 min, respectively ($P < 0.001$) (Fig. 1B).

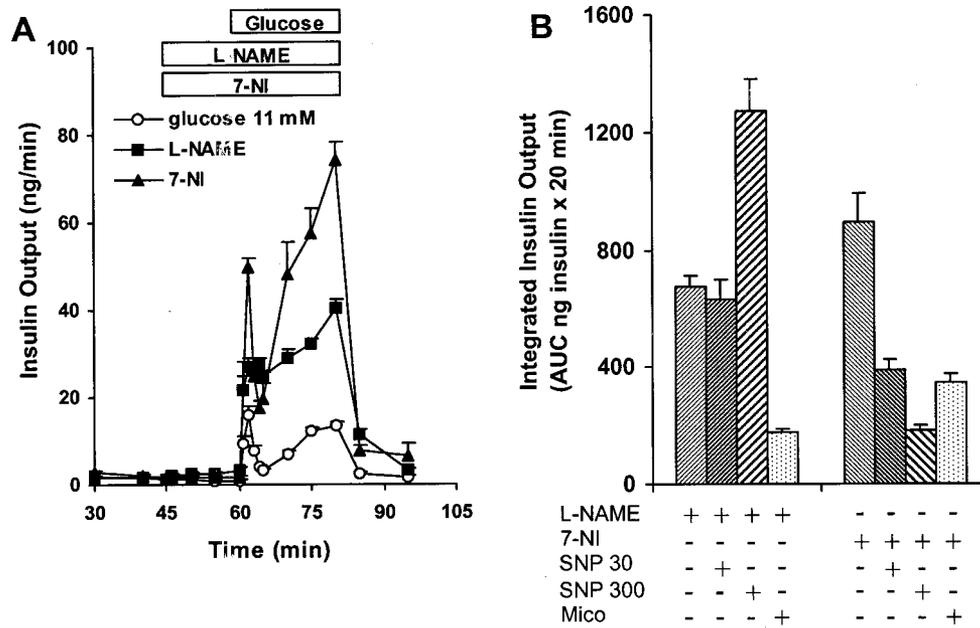


FIG. 1. Comparison of the alteration of glucose insulinotropic effect induced by L-NAME and 7-NI and the sensitivity to a substitutive treatment with SNP and miconazole in the isolated perfused rat pancreas. **A:** Effect of 5 mmol/l L-NAME ($P < 0.001$, $n = 5$, ■) and 100 µmol/l 7-NI ($P < 0.001$, $n = 7$, ▲) compared with the effect of glucose alone ($n = 6$, ○). Basal glucose background: 5 mmol/l. **B:** Effect of 30 µmol/l (NS, $n = 5$) and 300 µmol/l ($P < 0.001$, $n = 5$) SNP and 10 µmol/l miconazole (Mico, $P < 0.001$, $n = 6$) on L-NAME-induced alteration ($n = 7$) compared with the effect of 30 µmol/l ($P < 0.001$, $n = 6$) and 300 µmol/l ($P < 0.001$, $n = 6$) SNP and 10 µmol/l miconazole ($P < 0.001$, $n = 6$) on 7-NI-induced alteration ($n = 7$). AUC, area under the curve.

Effect of simultaneous administration of L-NAME and 7-NI on insulin response to glucose. Simultaneous infusion of 5 mmol/l L-NAME and 100 µmol/l 7-NI provoked a significant increase in insulin output ($P < 0.01$ vs. 7-NI alone; $P < 0.001$ vs. L-NAME alone) (Fig. 2A). The mean integrated insulin output for 20 min of high glucose administration reached $1,651.4 \pm 130.4$ ng \times 20 min and corresponds to the sum of the effects recorded in the presence of the inhibitors alone. In this combined treat-

ment, miconazole (10 µmol/l) and SNP (300 µmol/l) induced a 65 and 40% decrease, respectively ($P < 0.001$) (Fig. 2B). When infused together, they further decreased insulin output to 454.7 ± 59.3 ng \times 20 min (-72% ; $P < 0.001$).

Effect of a substitutive treatment with SNP and miconazole on the alteration of insulin response to glucose induced by 7-NI. Simultaneous administration of SNP (30 µmol/l) and miconazole (10 µmol/l) markedly

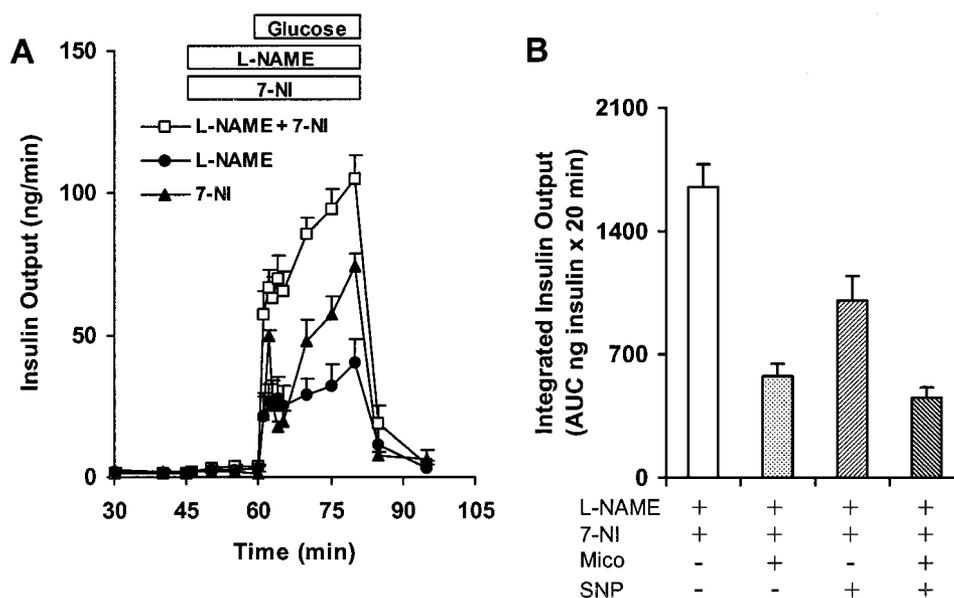


FIG. 2. Effect of a combined treatment with L-NAME and 7-NI on the insulin response to glucose in the isolated perfused rat pancreas. **A:** Effect of 5 mmol/l L-NAME + 100 µmol/l 7-NI in the presence of 11 mmol/l glucose ($n = 6$, □) compared with the effect of L-NAME ($P < 0.001$, $n = 5$, ●) and 7-NI alone ($P < 0.01$, $n = 6$, ▲). **B:** Effect of 10 µmol/l miconazole (Mico) ($P < 0.001$, $n = 6$), 300 µmol/l SNP ($P < 0.001$, $n = 5$), or a combination of miconazole + SNP ($P < 0.001$, $n = 4$) on insulin output induced by L-NAME + 7-NI in the presence of high glucose. AUC, area under the curve.

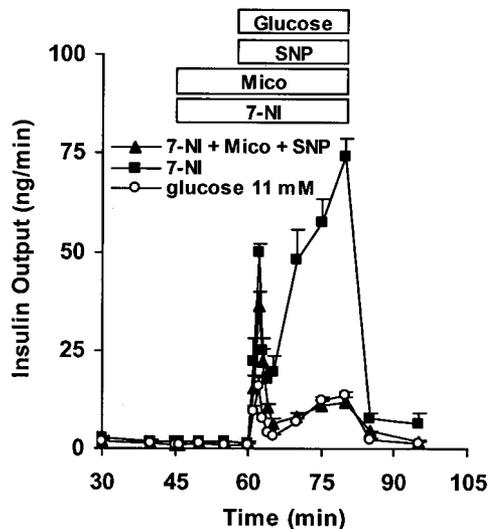


FIG. 3. Effect of a combined treatment with SNP and miconazole on the 7-NI-induced alteration of the insulin response to glucose in the isolated perfused rat pancreas. Effect of 30 $\mu\text{mol/l}$ SNP + 10 $\mu\text{mol/l}$ miconazole (Mico) on 7-NI-induced alteration ($P < 0.001$, $n = 6$, \blacktriangle) compared with the effect of 7-NI ($n = 7$, \blacksquare) and glucose alone ($n = 6$, \circ) is shown. Basal glucose background: 5 mmol/l.

reduced insulin output induced by 100 $\mu\text{mol/l}$ 7-NI (Fig. 3). The secretory response remained biphasic, reaching $229.4 \pm 22.3 \text{ ng} \times 20 \text{ min}$ (-74% ; $P < 0.001$ vs. 7-NI alone), a value close to that observed with glucose alone ($178.6 \pm 13.0 \text{ ng} \times 20 \text{ min}$).

Effect of 7-NI on arginine-induced insulin secretion. 7-NI (100 $\mu\text{mol/l}$), ineffective on insulin secretion when given alone on the 5 mmol/l glucose background, significantly increased the arginine (5 mmol/l) secretory effect from 151.9 ± 15.5 to $270.1 \pm 38 \text{ ng} \times 20 \text{ min}$ ($P < 0.01$) (Fig. 4). This increment was completely prevented by simultaneous administration of SNP (300 $\mu\text{mol/l}$).

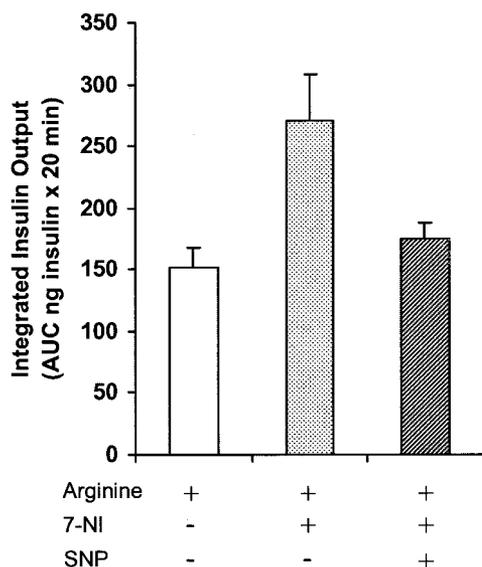


FIG. 4. Effect of 7-NI on arginine-induced insulin secretion in the isolated perfused rat pancreas. Effect of 100 $\mu\text{mol/l}$ 7-NI alone ($P < 0.01$, $n = 7$) or in the presence of 300 $\mu\text{mol/l}$ SNP ($n = 7$) on 5 mmol/l arginine-induced insulin secretion compared with arginine alone ($n = 7$) is shown. Basal glucose background: 5 mmol/l. AUC, area under the curve.

Effects of L-NAME and 7-NI on the dimeric state of nNOS in the INS-1 cells. Concerning nNOS, the heme moiety is the only indispensable factor for dimerization, but arginine and BH_4 are both necessary to stabilize the dimeric conformation (22,24). Because L-NAME and 7-NI act through different mechanisms to inhibit nNOS, we wondered if the different insulin secretory patterns in response to glucose could not result from the ability of the two inhibitors to differently affect the dimeric conformation of the enzyme.

Using low-temperature SDS-PAGE (22) and Western blotting, we investigated the monomer/dimer equilibrium of nNOS in the INS-1 cells after treatment with 5 mmol/l glucose in the presence or absence of arginine, L-NAME, and 7-NI during 60 min. Under basal conditions, we were able to detect faint amounts of SDS-resistant dimers, whereas in the presence of arginine, part of the monomers were converted to SDS-resistant dimers, with a 300-kDa molecular mass (Fig. 5). Incubation with L-NAME resulted in the appearance of two dimeric species, differing by their respective 300- and 270-kDa molecular masses. The amount of both dimer species was reinforced by simultaneous incubation with L-NAME and arginine. After incubation with 7-NI, we could not detect any SDS-resistant dimers, whereas some dimers did occur when arginine was also present, but they were less abundant than with arginine alone. Similar data could also be obtained after incubation of INS-1 cells during 20 min, a time corresponding roughly to the duration of the pretreatment period we applied before administrating secretagogues in the isolated perfused pancreas. Thus, in INS-1 cells, L-NAME, just like arginine, promotes nNOS dimerization, whereas 7-NI, on the contrary, inhibits the dimerization of the enzyme.

In vitro effects of L-NNA and 7-NI on the dimerization of nNOS. To further investigate the effects of the two inhibitors on nNOS dimerization, we used recombinant nNOS, incubated in the presence of 100 $\mu\text{mol/l}$ BH_4 , 1 mmol/l arginine, 1 mmol/l L-NNA, or 100 $\mu\text{mol/l}$ 7-NI. L-NNA was preferred to the prodrug L-NAME in these in vitro experiments because it is the final product of the hydrolytic bioactivation of L-NAME in the cells (25). After a 1-h incubation, BH_4 with arginine induced the formation of 300-kDa SDS-resistant dimers, whereas BH_4 with L-NNA led to the formation of two SDS-resistant dimeric species, one of 300-kDa and the other of 270-kDa molecular mass (Fig. 6A). Other bands are faint amounts of contaminants revealed by the silver staining. The smaller dimers were also faintly produced in the presence of BH_4 with arginine. Moreover, neither arginine nor L-NNA promoted dimer formation on their own in the absence of BH_4 (data not shown). In the presence of BH_4 and 7-NI, no SDS-resistant dimers could be detected. Furthermore, unlike L-NNA, the addition of 7-NI to BH_4 and arginine clearly inhibited the dimerization induced by the latter. These results clearly indicate that the combined action of BH_4 and L-NNA induces the formation of two types of dimers and that 7-NI inhibits BH_4 and arginine-induced dimer formation.

Real-time interaction analysis of nNOS dimerization by BIACORE. Influence of the different cofactors on nNOS dimerization was analyzed by surface plasmon resonance using the BIACORE technology. In the absence of added compound, there was no binding of nNOS on

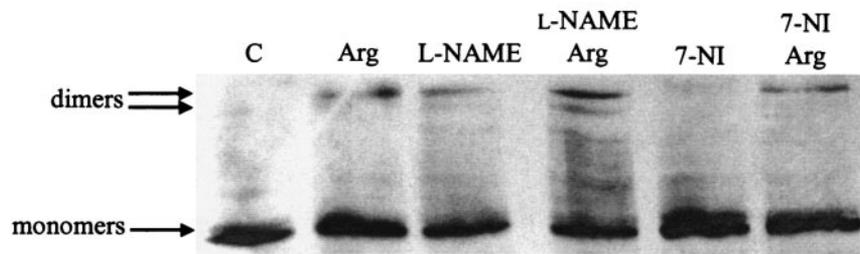


FIG. 5. Effect of L-NAME and 7-NI on the dimeric state of nNOS in INS-1 cells. INS-1 cells were incubated in the presence of 5 mmol/l glucose and different compounds as indicated. The dimer/monomer equilibrium was analyzed by low-temperature SDS-PAGE and subsequent Western blotting with an anti-nNOS antibody. Monomers and the two types of dimers are indicated by arrows. Arg, arginine; C, control.

immobilized nNOS (Fig. 7A). In contrast, the addition of BH_4 + L-NNA to the sample and eluent buffer induced nNOS binding to the immobilized nNOS (Fig. 7B), with an association rate constant (k_a) of $2.85 \pm 0.20 \times 10^5$ ($\text{mol/l})^{-1} \text{s}^{-1}$, a dissociation rate constant (k_d) of $2.76 \pm 0.06 \times 10^{-3} \text{s}^{-1}$, and an affinity constant measured at equilibrium (K_D) ($K_D = k_a/k_d$) of $9.68 \times 10^{-8} \text{mol/l}$ (Fig. 7). L-NNA without BH_4 was able to promote nNOS dimerization (Fig. 7C). Addition of 7-NI to BH_4 prevented the binding of nNOS to immobilized nNOS (Fig. 7D). Injections of an irrelevant protein to the immobilized nNOS in the same experimental conditions showed the absence of nonspecific binding (data not shown).

In vitro effects of endogenous inhibitors of nNOS.

Methyl-arginines are produced in vivo by enzymes known as arginine methyltransferases and are released into the cytosol after proteolysis of the methylated proteins (26). Among the methyl-arginines produced in mammals, only the asymmetric forms, such as *N*- ω -methyl-L-arginine (L-MMA) and asymmetric dimethyl-arginine (ADMA), are endogenous inhibitors of NOSs by competition with the substrate binding. We therefore questioned whether the physiological inhibitors L-MMA and ADMA could reproduce the same effects as L-NAME on nNOS dimerization. In the presence of 100 $\mu\text{mol/l}$ BH_4 , 1 mmol/l L-MMA or ADMA induced the formation of the same two dimeric species as L-NNA (Fig. 6B). These results clearly indicate that endogenous inhibitors of NOS promote nNOS dimerization, as does the pharmacological antagonist L-NNA.

Effects of arginine, L-NNA, L-MMA, and ADMA on PIN-induced dimer destabilization.

Using the yeast

two-hybrid screen, Jaffrey and Snyder (27) identified an 89-amino acid protein called PIN (protein inhibitor of nNOS) that interacts with the NH_2 -terminus of nNOS. PIN is a highly conserved protein that blocks the enzyme dimerization and subsequent NO production (27). In the pancreatic β -cells, we have shown the presence of PIN, whose overexpression provokes an increased insulin secretion in INS-1 cells, independently of nNOS catalytic activities (28). We then questioned whether the presence of L-NNA, L-MMA, and ADMA could affect the ability of PIN to dissociate nNOS dimers. Increasing concentrations of PIN almost completely suppressed BH_4 and arginine-induced dimers, whereas BH_4 - and L-NNA-induced dimers remained stable, even at the highest PIN concentration tested (Fig. 8A). Under the same conditions, PIN was also ineffective in destabilizing dimers produced in the presence of L-MMA or ADMA (Fig. 8B). These results clearly show that the stabilizing effect of L-NNA, L-MMA, or ADMA on nNOS dimers prevents PIN from inhibiting nNOS dimerization.

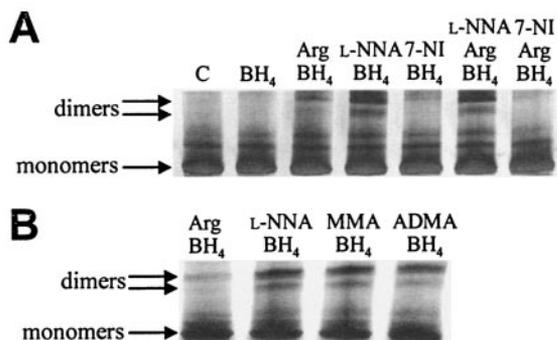


FIG. 6. In vitro effect of L-NNA, 7-NI (A), and endogenous inhibitors of nNOS, L-MMA, and ADMA (B) on the dimerization of nNOS. Recombinant nNOS was incubated in the presence of the indicated factors for 1 h. Dimers and monomers were separated by low-temperature SDS-PAGE, and the proteins were revealed by silver staining. Arrows indicate the monomers and the two types of dimers. Arg, arginine; C, control.

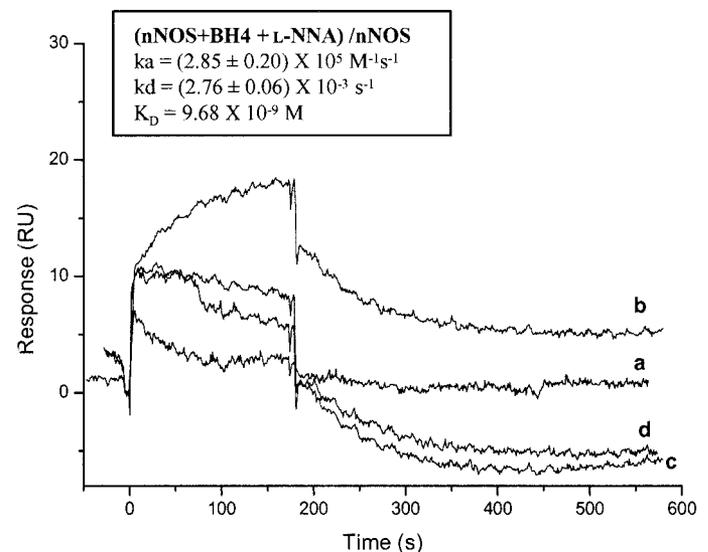


FIG. 7. Real-time analysis of nNOS dimerization by BIACORE. nNOS (10 $\mu\text{g/ml}$) was injected on recombinant nNOS immobilized on a CM5 sensor chip in different combinations of cofactors: binding of nNOS on nNOS, without cofactor (track a); in the presence of BH_4 (10 $\mu\text{mol/l}$) and L-NNA (0.1 mmol/l) (track b); in the presence of L-NNA (0.1 mmol/l) (track c); and in the presence of BH_4 (10 $\mu\text{mol/l}$) and 7-NI (10 $\mu\text{mol/l}$) (track d). The sensorgram (track b) illustrated the given kinetic parameters in the inset: k_a , association rate constant; k_d , dissociation rate constant; K_D , affinity constant measured at equilibrium ($K_D = k_a/k_d$). RU, resonance unit.

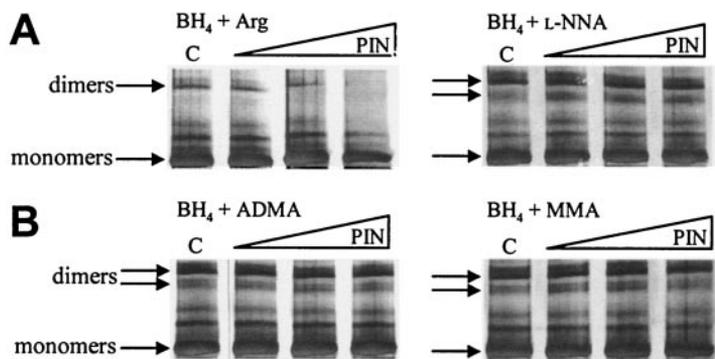


FIG. 8. In vitro effects of arginine, L-NNA (A), L-MMA, and ADMA (B) on PIN-induced dimer destabilization. Recombinant nNOS was first incubated in the presence of the indicated factors for 45 min and then with or without (C) increasing concentrations of PIN (2, 5, and 10 molar excess with respect to nNOS) for an additional 45 min. Dimers and monomers were separated by low-temperature SDS-PAGE, and the proteins were detected by silver staining. Arrows indicate the monomers and the dimers. Arg, arginine; C, control.

DISCUSSION

Our data demonstrate that the blockade of pancreatic nNOS results in strong amplification of glucose-induced insulin secretion, but that, depending on the nature and the molecular event challenged by the inhibitor, pancreatic β -cell secretory pattern is differently affected. In this respect, our molecular study clearly demonstrates that the two NOS antagonists, L-NAME and 7-NI, had different effects on the dimeric state of nNOS, both in vitro and in the β -cell line INS-1. L-NAME (or its final product L-NNA) was able to stabilize the nNOS dimers, whereas 7-NI destabilized them and induced monomerization of the enzyme.

In a recent study (14), we showed that insulin-secreting cells express a neuronal isoform of NOS and that the enzyme, just like the form present in brain (1,18), is able to exert two catalytic activities: production of NO and a nonoxidizing cytochrome c reductase activity. Indeed, we showed that the monophasic exaggerated insulin response to glucose in the presence of L-NAME resulted from both decreased NO production and increased nNOS-related cytochrome c reductase activity (14). We now report that 7-NI, another specific inhibitor of nNOS, is also able to increase glucose-induced insulin secretion, but, unlike L-NAME, it preserves the normal biphasic pattern of the β -cell response. Furthermore, if an increased cytochrome c reductase activity is, as for L-NAME, responsible for part of the amplifying effect of 7-NI, the latter is much more sensitive to the inhibitory effect of a substitutive treatment with SNP. Therefore, these data suggest that different mechanisms are involved in the alteration of insulin secretion induced by the two inhibitors. Such differences are probably related to the nature of the drugs. Indeed, L-NAME is known to compete with arginine binding, whereas 7-NI competes with arginine and BH_4 , an essential cofactor involved in the regulation of nNOS catalytic activity and quaternary structure. Concerning nNOS dimerization, the heme moiety is the sole factor absolutely required for the formation of dimers (24,29,30). These dimers are stable under native conditions, but BH_4 and arginine are necessary for their stabilization and for their resistance to SDS (22,30).

L-NNA is a substrate-based inhibitor, obtained by the insertion of a nitro group onto the guanidino terminus of arginine, that blocks NO production by competition with the substrate binding. Because of the strong structural homology between L-NNA and arginine, it could be expected that the binding of L-NNA to the substrate binding site mimics the stabilizing effect of arginine on nNOS

dimerization. Such a possibility is supported by a study showing that most arginine analogs promote iNOS dimerization to the same extent as arginine and sometimes even more efficiently (31). This was the case in our study, which showed that L-NNA is able to stabilize nNOS dimers in the presence of BH_4 . This effect can probably be explained by the fact that L-NNA binds to the arginine binding site by the same set of interactions as arginine (32–34). Indeed, arginine and its analogs are maintained above the heme moiety by interactions between the two nitrogen atoms of the guanidinium group and glutamic acid 371 (numbering of inducible NOS) (32). 7-NI is an indazole compound with a structure very different from L-NNA. This competitive inhibitor of NOS, more selective for nNOS, acts competitively with respect to arginine and BH_4 (9). Unlike L-NNA, 7-NI completely destabilized the nNOS dimer by preventing binding of BH_4 and arginine, both essential factors for dimer stabilization.

From these molecular studies, we conclude that the differences in the functional endocrine effects of the two nNOS inhibitors are related to their opposite effects on the dimeric state of the enzyme. Miconazole, the inhibitor of nNOS-induced reduction of cytochrome c, is effective in reducing the stimulating effect of both L-NAME and 7-NI, which is in accordance with the ability of nNOS to reduce cytochrome c in the dimeric as well as the monomeric conformation. As for the effect of the substitutive treatment with SNP, the clear inhibition observed with 7-NI fits well with a decreased endogenous NO production because of the inability of nNOS to synthesize NO as a monomer. Because NO has been shown to inhibit phosphofruktokinase (16), glyceraldehyde phosphate dehydrogenase (35), and cytochrome oxidase (36), suppression of an inhibitory tone on glycolytic and mitochondrial enzymes together with a rise in ATP production, resulting from an increased cytochrome c reductase activity, account for the whole increment in insulin secretion due to the blockade of nNOS with 7-NI. Consequently, because the effect of 7-NI results from increases in glycolytic and mitochondrial electron fluxes, the physiological biphasic pattern of the β -cell response is conserved. Such a possibility is further supported by the observation that the enhancement by 7-NI of the arginine insulinotropic effect is completely suppressed by the NO donor, which contrasts markedly with the stimulating effect of SNP on the strong potentiation of arginine-induced insulin secretion in the presence of L-NAME (37).

In contrast, stabilization of the dimeric state, in the presence of the nonmetabolizable arginine analog

L-NAME, induced a decreased NO production and an increased cytochrome c activity as well, as shown in our previous study (14). A striking difference with 7-NI is the minor role played by the inhibitory effect of SNP, whereas a clear stimulating effect occurs with L-NAME at a concentration (300 $\mu\text{mol/l}$) that completely inhibits the second phase of insulin response to glucose alone (17). This suggests that binding to nNOS of L-NNA issued from L-NAME during the pretreatment and treatment periods triggers molecular mechanisms less tightly coupled with metabolic fluxes than 7-NI and is responsible for the disappearance of the biphasic secretory pattern. Thus, our data bring evidence that the mechanisms involved in the modulation of insulin secretion by nNOS are probably more complex than merely restricted to NO production and cytochrome c reduction. The differences in the conformational changes induced by the two pharmacological inhibitors probably account, at least in part, for their functional secretory effects. Moreover, we recently showed that pancreatic β -cells express PIN, the overexpression of which provokes an increase in insulin secretion (28). PIN is known to act through inhibition of nNOS dimerization (27), and therefore our finding that L-NNA is able to prevent PIN-induced dissociation of nNOS dimers might be of physiopathological relevance and account for the abnormal pattern of insulin secretion recorded in the presence of L-NAME when compared with the effect of 7-NI. Furthermore, the second dimer species, in addition to that induced by arginine, might be related to an unknown noncatalytic activity of nNOS.

The control by nNOS of both the amplitude and the pattern of insulin response to secretagogues clearly depends on the quaternary structure of the enzyme. More interesting is our demonstration that the L-NNA molecular effects are shared by the two endogenous inhibitors of nNOS, L-MMA and ADMA, which are both able, as L-NAME, to increase insulin secretion (data not shown). These observations warrant further investigations in animal models of type 2 diabetes to determine if intracellular factors able to affect nNOS quaternary structure could be implicated in β -cell dysfunction. Interestingly, plasma concentrations of ADMA have been shown to be increased in patients with type 2 diabetes (38). Elevation of the concentration of ADMA is thought to be, at least in part, responsible for the depressed endothelium-dependent vasodilatation and could at the same time alter β -cell function.

In summary, the two inhibitors of nNOS, L-NAME and 7-NI, differently affect β -cell function in that they induce different secretory patterns. Changes in the two catalytic activities of nNOS (NO production and cytochrome c reductase activity) cannot on their own completely explain the different behavior of β -cells, especially the difference in their response to a substitutive treatment with NO in the presence of the two inhibitors. These differences are probably related to our demonstration that L-NAME and 7-NI induce opposite effects on the monomer/dimer equilibrium of nNOS: L-NAME stabilizes nNOS dimeric state, whereas 7-NI prevents dimerization of the enzyme. These differences may also be due to the inability of PIN to exert its physiological role in the control of insulin secretion. Finally, a major and original finding of our study is that the

L-NAME stabilizing effect was also observed for the physiological inhibitors of nNOS. We conclude that changes in the intracellular environment of nNOS, able to affect nNOS quaternary structure, might be implicated in certain pathological states characterized by β -cell dysfunction.

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