Protein Inhibitor of Neuronal Nitric Oxide Synthase (PIN) Is a New Regulator of Glucose-Induced Insulin Secretion

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We previously showed that pancreatic β-cells express neuronal nitric oxide synthase (nNOS) that controls insulin secretion through two catalytic activities: nitric oxide (NO) production and cytochrome c reductase activity. We now provide evidence that the endogenous protein inhibitor of nNOS (PIN) is expressed in rat pancreatic islets and INS-1 cells. Double-immunofluorescence studies showed a colocalization of PIN with both nNOS and myosin Vα in insulin-secreting β-cells. Electron microscopy studies confirmed that PIN is mainly associated with insulin secretory granules and colocalized with nNOS in the latter. In addition, PIN overexpression in INS-1 cells enhanced glucose-induced insulin secretion, which is only partly reversed by addition of an NO donor, sodium nitroprusside (SNP), and unaffected by the inhibitor of cytochrome c reductase activity, miconazole. In contrast, the pharmacological inhibitor of nNOS, Nω-nitro-arginine methyl ester, amplified glucose-induced insulin secretion, an effect insensitive to SNP but completely normalized by the addition of miconazole. Thus, PIN insulinotropic effect could be related to its colocalization with the actin-based molecular motor myosin Vα and as such be implicated in the physiological regulation of glucose-induced insulin secretion at the level of the exocytotic machinery. Diabetes 55:3279–3288, 2006
In previous studies, we showed that pancreatic β-cells express nNOS that displays three mutations when compared with cerebellar nNOS (29). Pancreatic β-cell nNOS is for a great part localized in insulin secretory granules but also in mitochondria and nucleus. The enzyme is implicated in the control of insulin secretion and, as previously shown for brain NOS, is able to exert two catalytic activities: NO production and a cytochrome c reductase activity (29,30). Furthermore, we could show that a normal balance between the two catalytic activities is essential for β-cell function, but the mechanisms that could regulate nNOS catalytic activities in pancreatic β-cells remain to be investigated. In an attempt to get insight into β-cell nNOS regulation, we hypothesized that PIN could be one of the factors implicated in nNOS regulation. This prompted us, first, to investigate if PIN is expressed in rat pancreatic β-cells, second, to study its subcellular localization, and finally to determine, by using the insulin-secreting cell line INS-1, whether PIN could play a role in the regulation of glucose-induced insulin secretion.

RESEARCH DESIGN AND METHODS

Cell culture and isolation of islets. The insulin-secreting cell line INS-1 (a generous gift from Professor C.B. Wollheim) was cultured as previously described by Asfari et al. (31). Islets were isolated from adult male Wistar rats (Iffa Credo, Lyon, France) using collagenase digestion and separation on a Ficoll density gradient (32). For electron microscopy, islets were then incubated for 1 h at 37°C in Krebs-Ringer bicarbonate buffer (10 mmol/l NaCl, 11.9 mmol/l KH2PO4, 4.74 mmol/l KCl, 2.54 mmol/l CaCl2, 1.19 mmol/l MgSO4·7H2O, and 18 mmol/l NaHCO3), pH 7.4, supplemented with 1 g/l BSA and 2.8 mmol/l glucose. For immunofluorescence studies, isolated cells from 6- to 10-week-old rats, not 5 weeks after isolation, were washed and preincubated for 1 h at room temperature. Sections were rinsed in deionized water, stained with 2% uranyl acetate for 20 min, and then observed with a transmission electron microscope (Hitachi H-7100; Hitachi, Dusseldorf, Germany). The specificity of the immune reaction was tested by incubating the sections with only the secondary antibody.

Transfection and incubation experiments. INS-1 cells were seeded on poly-l-lysine–coated 24-well plates at a density of 4 × 104 cells per well for 4 d. INS-1 cells were transfected per 8 × 106 cells per well for 48 h. After 3 days of culture, cells were transfected with a eukaryote expression vector (pCR3.1; Invitrogen) containing the cDNA of rat PIN using Lipofectamine Plus Reagent (Invitrogen), according to the manufacturer’s protocol. Control cells were obtained by transfection with the empty vector under the same conditions. Forty-eight hours after transfection, overexpression of PIN was estimated by Western blotting (31). At this time, the cells transfected, or not, were 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 another hour at 37°C in the same buffer in the presence of 5 mmol/l glucose with or without Nω-nitro-l-arginine methyl ester (L-NAME; 5 mmol/l), monomaze nitrate salt (10 μmol/l), and sodium nitrourapid hydride (3 mmol/l) (Sigma Aldrich). To investigate if PIN could regulate nNOS catalytic activities in pancreatic β-cells, second, to study its subcellular localization, and finally to determine, by using the insulin-secreting cell line INS-1, whether PIN could play a role in the regulation of glucose-induced insulin secretion.

RESULTS

PIN is expressed in rat pancreatic β-cells and INS-1 cells. To investigate whether PIN, previously identified in rat brain (16), is, as nNOS, also present in rat pancreatic β-cells (29), we used the RT-PCR approach with primers based on the sequence of rat brain PIN (accession no. U66461). As shown in Fig. 1A, a single band at the predicted size was obtained in islets of Langerhans and in INS-1 cells (representative of three experiments). Sequencing of this fragment revealed complete analogy with the sequence of rat brain PIN (data not shown). This result

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PIN is localized in insulin-secreting cells produce the PIN protein. INS-1 cells is lower than in testis and brain. These experiments). We identified a 10-kDa protein, displaying an anti-PIN antibody (Fig. 1)

Expression of PIN by RT-PCR. Total RNA from islets and INS-1 cells was isolated, and cDNA was amplified with primers based on the sequence of the PIN (representative of three experiments). β2-Microglobulin (β2-m) primers were used as positive controls. Molecular markers (MM) show bands corresponding to fragments of 2,000, 1,200, 800, 400, 200, and 100 bp. RT, negative control for PCR preparation performed without cDNA. A: Expression of PIN by Western blotting. Proteins from a testis, an INS-1 cell, an islet, and a brain extract were separated by 14% tricine polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with a monoclonal anti-PIN antibody. Immunoreactivity was detected by a chemiluminescence kit (representative of three experiments).

is consistent with the fact that PIN has been shown to be a highly conserved protein; a 90% sequence homology is observed among Chlamydomonas, Caenorhabditis elegans, Drosophila, and humans. Expression of the PIN protein was confirmed in islet and INS-1 cell extracts by Western blotting with a monoclonal anti-PIN antibody (Fig. 1B, representative of three experiments). We identified a 10-kDa protein, displaying a molecular mass identical to that observed in brain and testis. Nevertheless, the brain PIN band appeared slightly higher than in testis and islets, suggesting that different posttranslational modifications of the protein occur in these tissues. The level of PIN expression in the islets and INS-1 cells is lower than in testis and brain. These experiments provide evidence that pancreatic islets and INS-1 cells produce the PIN protein.

PIN is localized in insulin-secreting β-cells in association with nNOS and myosin Va. To confirm the presence of PIN in pancreatic β-cells, we performed double-immunofluorescence studies on isolated cells obtained from rat islets and on INS-1 cells using a monoclonal (Fig. 2B, C, H, and I) and a polyclonal (Fig. 2E, F, K, and L) anti-PIN antibody in combination with an anti-insulin antibody (Fig. 2A, C, D, F, G, I, J, and L). These experiments clearly show that PIN is expressed in islet β-cells (Fig. 2A–F) and in INS-1 cells (Fig. 2G–L). PIN is also present in glucagon-secreting α-cells and to a lesser extent in somatostatin-secreting δ-cells (data not shown). PIN staining was found to be cytoplasmic and displayed a punctuated aspect, identical for the two antibodies used. Furthermore, PIN was strongly colocalized with insulin, as shown by the appearance of a yellow color after merging the two fluorescences (Fig. 2C, F, I, and L). This strong colocalization suggests that PIN is associated with insulin secretory granules, as previously demonstrated for nNOS (29).

As PIN is able to interact with the NH2-terminal part of nNOS (19), we also studied the colocalization of the two proteins in islet β-cells (Fig. 3A–F) and in INS-1 cells (Fig. 3G–L). PIN staining was strongly associated with nNOS (Fig. 3B, C, E, F, H, I, K, and L) when using the monoclonal (Fig. 3A, C, G, and I) or the polyclonal (Fig. 3D, F, J, and L) antibodies, whereas nNOS was not exclusively colocalized with PIN. These results suggest that PIN is associated with nNOS at the level of the secretory granules.

As PIN is the light chain of dynein and myosin V (21,22), we then studied the colocalization of the inhibitor with these two motors. Our results confirm the expression of both motors in islet β-cells (Fig. 4A–C and G–I) and in INS-1 cells (Fig. 4D–F and J–L). Myosin Va staining is punctuated and partially colocalized with insulin (Fig. 4A–F), as previously shown by others (34,35). Interestingly, PIN displayed a strong colocalization with myosin Va (Fig. 4G–L). Concerning dynein, the labeling occurred mainly close to the membrane of the β-cell and to a lesser extent in the cytoplasm where a weak colocalization with PIN could be observed (data not shown).

To determine the subcellular localization of PIN, we performed electron microscopy on sections of pancreatic islets using the same set of anti-PIN antibodies (Fig. 5). A similar location was observed with the monoclonal (Fig. 5A and B) and the polyclonal (Fig. 5C–H) antibodies. In β-cells, PIN was mainly found to be associated with mature insulin secretory granules at the level of the dense core (large arrow head), the halo (thin arrow), and the membrane of the granules (large arrow) (Fig. 5A–C). Weak staining also appeared in less mature granules but only in association with the dense core (Fig. 5A, D, and E; empty arrow). A small part of PIN was also present in the cytoplasm (Fig. 5A; small arrow head), as well as in the mitochondria (data not shown). Interestingly, dimers of PIN could also be detected in the dense core (Fig. 5D; large arrow head), the halo (Fig. 5F; thin arrow), and the membrane (Fig. 5E; large arrow) of insulin secretory granules, suggesting that dimerization of PIN occurs in β-cells as previously shown in the brain (21). The occurrence of PIN dimers was confirmed by cross-linking experiments followed by Western blotting in INS-1 cells (data not shown). Double staining of PIN and nNOS confirmed colocalization of both proteins at the level of insulin granules (Fig. 5G and H).

PIN overexpression induced an enhanced glucose-induced insulin secretion in INS-1 cells. To assess the physiological function of PIN on insulin secretion, we performed overexpression experiments in INS-1 cells. After 48 h of transfection, we observed a clear increase in PIN mRNA by RT-PCR (Fig. 6A) and in INS-1 cells at 48 h. PIN staining was increased by 33% in cells overexpressing PIN compared with cells transfected with the control vector (P < 0.001, n = 6) (Fig. 6B).

To understand the mechanism by which PIN overexpression enhanced insulin secretion, we investigated if, and to what extent, the two catalytic activities of nNOS could interfere in PIN insulinotropic effect. A possible decrease in NO production was tested using sodium
nitroprusside (SNP), an exogenous NO donor, and an increased cytochrome c reductase activity was challenged with a specific inhibitor, miconazole (29). Data from PIN-overexpressing cells were also compared with those obtained in nontransfected cells in the presence of L-NAME, a nonmetabolizable analog of arginine, an inhibitor of nNOS activity. We first determined the dose of SNP able to decrease glucose-induced insulin secretion in INS-1 cells. The NO donor, at 3 and 30 nmol/l, decreased insulin secretion by 21% (P < 0.01 vs. glucose alone, n = 3) and 14% (P < 0.05 vs. glucose alone, n = 3), respectively, an inhibition no longer observed for higher SNP concentrations (data not shown). In PIN-overexpressing cells, SNP (3 nmol/l) reduced insulin secretion to the same extent as in control cells but failed to bring insulin secretion back to normal values (P < 0.01, n = 3) (Fig. 6B). Besides, the 65% increase in insulin release induced by L-NAME (P < 0.001 vs. glucose alone, n = 3), at a dose of 5 nmol/l, which was

FIG. 2. Localization of PIN in isolated β-cells from rat islets and in INS-1 cells by double immunofluorescence. Isolated β- (A–F) and INS-1 (G–L) cells were double labeled with an anti-insulin (A, D, G, and J) and a monoclonal (B, C, H, and I) or a polyclonal (E, F, K, and L) anti-PIN antibody, and fluorescence was analyzed by dual-channel confocal microscopy. Insulin and PIN stainings appear as green (A, D, G, and J) and red (B, E, H, and K), respectively, and coincidence of both fluorescences as yellow (C, F, I, and L). The scale bars are 10 μm.
previously shown not to produce nonspecific depolarizing effects (36), was not affected by SNP at increasing concentrations from 3 nmol/l (Fig. 6C, n = 3) to 300 μmol/l (data not shown). Concerning cytochrome c reductase activity, miconazole, at a dose of 10 μmol/l, which does not affect basal glucose response (29), only slightly and nonsignificantly reduced the effect of PIN overexpression (Fig. 6B, n = 6). This contrasts with the 95% reduction of the L-NAME secretory effect (P < 0.001, n = 3) (Fig. 6C), in accordance with previous data obtained in the isolated perfused rat pancreas (29). The secretory effect induced by blockade of nNOS with L-NAME is thus related to an increased cytochrome c reductase activity of the enzyme, which, in contrast, accounts for only a minor part of the positive insulinotropic effect of PIN, suggesting that the two nNOS inhibitors act through different mechanisms.

Therefore, we also studied the combined effects of PIN overexpression and L-NAME treatment in the presence of...
5 mmol/l glucose. In control cells, L-NAME again stimulated insulin secretion \((P < 0.001\) vs. control cells with glucose alone, \(n = 6\)), and the inhibitor produced, in PIN-overexpressing cells, a 48% increase \((P < 0.001\) vs. PIN-overexpressing cells with glucose alone) (Fig. 7A). Thus, L-NAME remains able to amplify insulin release in cells overexpressing PIN \((+20\%\) vs. control cells with L-NAME, \(P < 0.05\)), bringing additional evidence that PIN and L-NAME exert their positive effects on insulin secretion through different mechanisms. To ascertain whether the two inhibitors target nNOS, we measured the effects of PIN overexpression and L-NAME either alone or when...
FIG. 5. Subcellular localization of PIN in β-cells by electron microscopy performed on sections of isolated rat islets. A–C: Part of a β-cell rich in insulin secretory granules at different stages of maturation labeled with an anti-insulin antibody coupled to 5 nm gold particles and the monoclonal (A and B) and polyclonal (C) antibodies coupled to 10 nm (A) or 15 nm (B and C) gold particles. PIN staining is associated with mature insulin secretory granules at the level of the dense core (large arrow head), the halo (thin arrow), and the membrane of the granule (large arrow). PIN is also present in the cytoplasm (A; small arrow head) and in the dense core of less mature granules (A, D, and E; empty arrow). D–F: Dimers of PIN detected at the levels of the dense core (D; large arrow head), the halo (F; thin arrow), and the membrane (E; large arrow) of the granules. G and H: Double staining of PIN (15-nm particles) and nNOS (10-nm particles). Colocalization of both proteins occurs at the level of the dense core (G; large arrow head) and the membrane (H; large arrow) of the granules. Original magnification is ×60,000.
combined on nNOS catalytic activity in the presence of exogenously labeled arginine. PIN overexpression only slightly reduced (–11%; P = NS, n = 4) nNOS activity, whereas a 40% inhibition was observed with l-NAME in control cells (P < 0.01) versus 75% in PIN-transfected cells (P < 0.01 vs. control cells with glucose alone) (Fig. 7B).

**DISCUSSION**

Our data clearly show that PIN is expressed in rat pancreatic β-cells, as previously shown for nNOS (29), and positively modulates insulin secretion. Coincidence of nNOS and PIN also occurs in other tissues and has been reported in brain (18), kidney (20), and ventilatory muscles (19). nNOS is always coexpressed with its endogenous inhibitor, but PIN, which is moderately expressed in all rat and human tissues (16,17), does not necessarily occur with nNOS. The relative level of expression of the two proteins differs depending on both tissues and species. In brain, nNOS is mainly expressed in the cerebellum, whereas PIN is essentially found in the cortex, medulla, and hippocampus (18). Likewise, nNOS expression varies among species, which is not the case for PIN, and is found to be relatively constant (19). Therefore, it might be assumed that the magnitude of nNOS catalytic activity in a tissue is the result of the relative level of expression of both proteins.

In addition to coincidence of both proteins in pancreatic β-cells, immunofluorescence studies showed that PIN was strongly colocalized with nNOS, whereas the enzyme was not exclusively associated with the inhibitor. This discordance probably results from the fact that a part of nNOS is present in β-cell mitochondria, where PIN is only faintly expressed. In other tissues, PIN often colocalizes with nNOS in the intracellular compartments, as shown in kidney, where PIN is present in the apical membranes of the inner-medullary collecting duct, a site where nNOS is strongly expressed (20). Colocalization of PIN and nNOS in pancreatic β-cells makes it possible for them to interact at a site where a great part of nNOS is localized, the insulin secretory granules. This assumption is strongly supported by electron microscopy studies, which showed that PIN is mainly associated with insulin secretory granules and that PIN-nNOS association is detected at the levels of the vesicles membrane and the dense core. Interestingly, we also found that PIN colocalized with myosin Va, previously shown to be expressed in pancreatic β-cells (34,35). Thus, it is tempting to assume that PIN is associated on one hand with myosin Va and on the other hand with nNOS at the level of insulin secretory granules.

The physiological role played by PIN is not yet known. One function is the modulation of nNOS catalytic activity to prevent deleterious overproduction of NO. Indeed, it
has been reported that a cyclooxygenase-induced increase of PIN expression is, through an inhibition of the production of superoxide and NO by nNOS, able to prevent nerve growth factor–deprived PC12 cells from apoptosis (37). Concerning pancreatic β-cells, PIN overexpression resulted in a clear increase in insulin secretion, bearing evidence that this highly conserved protein plays a role in the stimulus-secretion coupling of the β-cell. The mechanism involved is, however, less tightly coupled to nNOS catalytic activity. Indeed, the NO donor SNP reduced insulin secretion in control cells but only poorly overcame the effect of PIN overexpression. Furthermore, in vitro, a strong increase in PIN mRNA did not significantly affect nNOS catalytic activity, which contrasts with the initial identification of PIN as a specific inhibitor of nNOS catalytic activity (16). This could not be confirmed by others (38), but it must be emphasized that, at present, all studies have been performed using recombinant proteins and cells transfected with exogenous nNOS. Therefore, our finding of a slight nonsignificant reduction of nNOS activity after overexpression of endogenous PIN in β-cells expressing nNOS warrants further investigations to assess if PIN does regulate nNOS activity. Taken together, our data suggest that the secretory effect of PIN may occur at a more distal level. Interestingly, PIN, as a subunit of dynein and myosin V, is involved in the trafficking of organelles and the association of intracellular proteins with the cytoskeleton. Thus, PIN has been shown to be essential for the retrograde particle movement of intrflagellar transport in Chlamydomonas reinhardtii (39). In addition, binding of PIN to different partners has been shown to be involved in cytosolic retention of the cytoplasmic inhibitor of nuclear factor-κB (24) and prevention of nuclear translocation of p53 induced by DNA damage (40). In pancreatic β-cells, PIN could thus be involved in the migration of insulin secretory granules as suggested by the PIN-nNOS colocalization at the level of the granules and the strong colocalization of PIN with myosin Va. Indeed, myosin Va has been shown to be implicated in the transport of dense-core secretory vesicles (34) and to control insulin granule recruitment during late-phase secretion (35). Dynemin, which locates essentially at the periphery of the β-cell, only partly colocalizes with PIN in the cytoplasmic part of the cells. This could be related to the finding that dynemin is involved in insulin granule recovery after rapid kiss-and-run exocytosis in β-cells (41) and in the subcellular distribution of mitochondria in HeLa cells (42).

Additional evidence for the role of PIN in intracellular trafficking events also finds support in data obtained using L-NAME and the ability of the latter to reinforce the effect of PIN overexpression. In previous studies, we showed that the nonmetabolizable analog of arginine stimulates glucose-induced insulin secretion in great part through an increase in cytochrome c reductase activity. We now confirm this in INS-1 cells, where miconazole, an inhibitor of nNOS-induced cytochrome c reductase activity, is able to suppress the L-NAME secretory effect. This is not the case in PIN-overexpressing cells, which further excludes a metabolic interplay in the PIN effect. However, that L-NAME reinforced PIN effects is likely the result of an increased reduction of cytochrome c, a well-known electron transporter for mitochondrial production of ATP that may act as fuel for PIN-associated ATP-driven molecular motors. Such an assumption is also supported by the inability of the natural analog of L-NAME, L-arginine, to strengthen the PIN effect (data not shown), as the positively charged amino acid is known to depolarize pancreatic β-cells via biophysical events not related to ATP production (43).

In summary, taken together, the data we obtained by immunofluorescence and electron microscopic studies, as well as in functional experiments, clearly point to an effect of PIN in the intracellular trafficking of insulin secretory granules. As a matter of fact, PIN and nNOS clearly interact in the insulin secretory granules, and such events also occur at the vesicle membrane. Since PIN colocalizes with myosin Va, it is tempting to assume that PIN and nNOS, as two specific interacting partners, interfere with other multiprotein complexes of the exocytic machinery.

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