Inhibitory Effect of IGF-I on Type 2 Nitric Oxide Synthase Expression in Ins-1 Cells and Protection Against Activation-Dependent Apoptosis

Involvement of Phosphatidylinositol 3-Kinase

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Challenge of Ins-1 cells, a rat β-pancreatic cell line, with lipopolysaccharide (LPS) and interferon-γ (IFN-γ) promoted the expression of type 2 nitric oxide synthase (NOS-2) in a cooperative way. Treatment of Ins-1 cells with IGF-I significantly inhibited the expression of NOS-2, especially at sub-saturating concentrations of LPS and IFN-γ. The inhibitory effect of IGF-I on NOS-2 expression was abrogated when cells were incubated with wortmannin or LY294002, two inhibitors of phosphatidylinositol 3-kinase. Transient expression of the p110 subunit of phosphatidylinositol 3-kinase impaired the LPS and IFN-γ-dependent NOS-2 promoter activity in cells transfected with a 1-kb fragment corresponding to the 5′-flanking region of the NOS-2 gene. However, expression of a dominant negative form of p85 abolished the inhibitory action of IGF-I on the NOS-2 promoter activity. Analysis of the decreased NOS-2 promoter activity in cells incubated with IGF-I showed a lower nuclear factor κB binding as determined by electrophoretic mobility shift assays. The synthesis of NO, produced after LPS and IFN-γ challenge, triggered an apoptotic response in these cells. IGF-I reduced apoptosis mainly through the decreased synthesis of NO. However, in activated cells treated with N-[3-(aminomethyl)benzyl]acetamide, a specific NOS-2 inhibitor, IGF-I completely abolished the NO-independent apoptotic response. This protection from apoptosis was dependent on phosphatidylinositol 3-kinase activity. These results suggest an important anti-inflammatory and anti-apoptotic role for IGF-I in β-pancreatic cells, with both actions depending on the activation of phosphatidylinositol 3-kinase. Diabetes 49:209–217, 2000
IκB-α and IκB-β, which keep the NF-κB complex inactive in the cytosol (19,20). Moreover, synthesis of high concentrations of NO has been shown to induce apoptosis in various cell types, including β-cells (21,22).

In view of the inhibitory effect exerted by IGF-I on the expression of NO-2 as well as on the NO- and cytokine-dependent apoptosis in isolated β-pancreatic cells (2,3), we investigated whether the signaling of IGF-I in these cells could be involved, via PI 3-kinase activation, in the abrogation of NO-2 expression under pro-inflammatory conditions. Results show that IGF-I impaired the expression of NO-2 in INS-1 cells treated with moderate doses of LPS and IFN-γ. This effect of IGF-I was dependent on PI 3-kinase activity. Moreover, the activation of PI 3-kinase elicited by IGF-I inhibited the apoptosis observed in INS-1 cells challenged with pro-inflammatory stimuli.

**RESEARCH DESIGN AND METHODS**

**Chemicals.** Reagents were from Sigma (St Louis, MO), Boehringer Mannheim (Mannheim, Germany), and Merck (Darmstadt, Germany) and were of the highest quality available. Antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), Pharmingen (San Diego, CA), and Upstate Biotechnology (Lake Placid, NY). Electrofocusing equipment and reagents were from Bio-Rad (Richmond, CA) and Amersham (Bucks, U.K.). Serum and media were from BioWhittaker (Walkersville, MD).

**Cell culture and characterization.** INS-1 cells were grown in RPMI 1640 medium as previously indicated (23), expanded to generate an original source of INS-1 cells treated with moderate doses of LPS and IFN-γ. The supernatants were stored at −80°C (soluble extracts), and the pellets were resuspended in 50 µl buffer A supplemented with 20mM glycerol and 0.4 mol/l KCl and then gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained by centrifugation at 13,000 × g for 15 min, and aliquots of the supernatant were stored at −80°C. Protein content was assayed using the Bio-Rad protein reagent. When preservation of the PI 3-kinase activity was required, the homogenization medium consisted of buffer B supplemented with 0.22 mol/l mannitol, 68 mmol/l sucrose, and 10 mol/l cytocalasin B. All steps of cell fractionation were carried out at 4°C (28).

**Characterization of proteins by Western blot.** Equal amounts of soluble nuclear lysate were size-separated in 10% SDS-PAGE. The gels were blotted onto a Hybond membrane (Amersham) and incubated with anti-NO-2, anti-IκB-α, anti-β-actin, anti–Bax, anti–Bcl-2, anti-cytochrome C, and anti–IκB complex antibodies (Santa Cruz Biotechnology). In experiments using anti-phospho(ser)154IκB-α antibody (New England Biolabs, Beverly, MA), the blot incubation solution contained 50 ng/ml of gluthathione-S-transferase (GST)–IκB-α (1–137) treated previously with alkaline phosphatase-agarose (29). The blots were incubated with distinct stimuli and its expression was not affected by IGF-I, LPS, LY 2 9 4 0 2 , and Merck (Darmstadt, Germany) and were of the highest quality available.

**Plasmids constructs and preparation.** The following plasmids were used: p2NOS CAT, containing a 1.0 kb fragment corresponding to the 5′ Flanking region of NO-2 fused to a promoterless CAT reporter gene previously described (19); rCD2p110, which encodes a constitutively active p110α catalytic subunit of PI 3-kinase, including the extracellular and transmembrane domains of the rat CD2 cell surface antigen; and rCD2p110k, a p110α kinase deficient mutant; pB2SN, which is unable to bind p110 and therefore inhibits the recruitment of p110 to the membrane (a generous gift from C.R.F., ICRF, London). The plasmids were previously described (24,25). An rCD2 vector was used as the control of specificity in response to stimuli, and its expression was not affected by IGF-I, LPS, LY 29402, and wortmannin. The expression of CD2 was determined by flow cytometry after labeling the cells with FITC-OX-42 (Pharmingen). Transfected cells expressing CD2 were sorted, kept immediately in culture, and stimulated with the indicated ligands. Plasmids were purified using EndoFree Qiagen columns (Hilden, Germany).

**Assay of PI 3-kinase activity.** INS-1 cells were washed twice with phosphate-buffered saline (PBS) and incubated with 1.5 ml of RPMI 1640 medium as previously indicated (23), expanded to generate an original source of INS-1 cells treated with moderate doses of LPS and IFN-γ. The supernatants were stored at −80°C (soluble extracts), and the pellets were resuspended in 50 µl buffer A supplemented with 20mM glycerol and 0.4 mol/l KCl and then gently shaken for 30 min at 4°C. Nuclear protein extracts were obtained by centrifugation at 13,000 × g for 15 min, and aliquots of the supernatant were stored at −80°C. Protein content was assayed using the Bio-Rad protein reagent. When preservation of the PI 3-kinase activity was required, the homogenization medium consisted of buffer B supplemented with 0.22 mol/l mannitol, 68 mmol/l sucrose, and 10 mol/l cytocalasin B. All steps of cell fractionation were carried out at 4°C (28).

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**Caspase assay.** The activity of DEVD-specific caspase (3 and 7) was determined in cell lysates using N-acetyl-DEVD-7-amino-4-methylcoumarin as fluorogenic substrate and following the instructions of the supplier (Pharmingen). The corresponding peptide aldehyde and Z-VAD.fmk were used to inhibit the caspase activity in vitro and in vivo, respectively, and to ensure the specificity of the reaction. The linearity of the caspase assay was determined over a 30-min reaction period (28).

**Statistical analysis.** The data shown are the means ± SE of three or four experiments. Statistical comparisons for significance between cells treated in the absence or presence of IGF-I were performed using analysis of variance followed by the two-tailed Student’s t test. *P < 0.05 was considered significant. In studies of Western blot analysis, linear correlations between increasing amounts of input protein and signal intensity were observed (correlation coefficients >0.8).

**RESULTS**

**Inhibition of NOS-2 expression by IGF-I in Ins-1 cells.** Incubation of Ins-1 cells with LPS failed to induce NO synthesis. However, combinations of LPS, IFN-γ, interleukin (IL)-1β, and tumor necrosis factor-α (TNF-α) resulted in a synergic effect in terms of the synthesis of NO, suggesting the expression of NOS-2 (Fig. 1A). A dose-dependent effect of both LPS and IFN-γ reflected the cooperation between these stimuli in the expression of NOS-2. Treatment of cells with 10 nmol/l IGF-I inhibited the synthesis of NO in response to low concentrations of LPS/IFN-γ, but this effect was notably less at higher concentrations or with combinations of IFN-γ, IL-1β, and TNF-α (Fig. 1A and B). The inhibitory effect of IGF-I on NO synthesis in cells stimulated with 250 ng/ml LPS and 10 U/ml IFN-γ exhibited the half-maximal effect (I_{50}) at 1 nmol/l and was due to a decrease in the levels of NOS-2 protein (Fig. 1C). The presence of IGF-I during the initial 4-h period after stimulation with LPS/IFN-γ was required for the observation of the inhibition of NO synthesis, suggesting that IGF-I acts mainly through the inhibition of NOS-2 expression (Fig. 1D).

Signaling through the IGF-I receptor involves the phosphorylation of several tyrosine residues of IRS-1 and IRS-2 that activate, among others, PI 3-kinase activity (11,32,33). To determine the relevance of this lipid kinase in the expression of NOS-2 in response to an LPS/IFN-γ challenge, Ins-1 cells were treated with LY294002 and wortmannin, two inhibitors of PI 3-kinase, and rapamycin, an inhibitor of p70 S6 kinase, an enzyme downstream of the PI 3-kinase pathway (4,34). As Fig. 2A shows, these inhibitors failed to modify (wortmannin even enhanced) the synthesis of NO elicited by a low concentration of LPS/IFN-γ. However, treatment of cells with LY294002 or wortmannin, but not rapamycin, abolished completely the inhibitory effect of IGF-I on NO synthesis. The activation of PI 3-kinase in response to IGF-I was confirmed by measuring the lipid kinase activity of anti-PY20 IP from these cells (Fig. 2B). Interestingly, LPS dose-dependently enhanced the PI 3-kinase activity measured in anti-phosphotyrosine IP, but this effect was at least ninefold lower than that elicited by IGF-I under identical activation conditions. This lipid kinase activity was inhibited when cells were treated with LY294002 and the drug maintained during the assay. These results indicate that the inhibitory effect of IGF-I on NOS-2
expression at subsaturating concentrations of LPS/IFN-γ was due to the activation of PI 3-kinase.

NOS-2 activity is mainly regulated at the transcription level, and activation of NF-κB is an essential requirement for the expression of this gene (19,24). Treatment of Ins-1 cells with IGF-I inhibited IKK activation in response to an LPS/IFN-γ challenge, an effect that was reversed, at least in part, when cells were treated with LY294002. The decrease in the levels of IκB-α and IκB-β measured in these cells reflected the activation state of IKK (Fig. 3A). Analysis by EMSA of the NF-κB activity of nuclear protein extracts showed that IGF-I decreased the binding in cells activated with LPS/IFN-γ, a situation antagonized after treatment with LY294002 (Fig. 3B). Figure 3D shows the time-dependent activation of NF-κB in cells treated with LPS/IFN-γ.

The IGF-I–dependent inhibition of NOS-2 expression was analyzed at the mRNA and protein levels. Treatment of Ins-1 cells with MG 132 (an inhibitor of the 26S proteasome that blocks NF-κB activation) suppressed the expression of NOS-2 in response to LPS/IFN-γ, indicating the necessity of NF-κB activity for the process (Fig. 4). An important inhibition of NOS-2 RNA (sampling at 8 h; 73% inhibition) and protein (sampling at 24 h; 62% inhibition) levels was observed after incubation with IGF-I. Treatment with LY294002 sup-
pressed the inhibitory action of IGF-I. In addition to these experiments, the effect of PI 3-kinase on the expression of NOS-2 in these cells was evaluated using transient expression of various subunits of PI 3-kinase together with a vector encoding the 1 kb 5'-flanking sequence of the NOS-2 promoter (p2NOS.CAT) linked to a CAT reporter gene. As Fig. 5A shows, the reporter activity induced after stimulation with low concentrations of LPS/IFN-γ was abolished by IGF-I, but this effect was less notable at higher LPS/IFN-γ concentrations. When cells expressed an active form of p110, the response to the LPS/IFN-γ challenge was reduced, regardless of the treatment with IGF-I (Fig. 5B). However, in cells expressing a kinase-deficient p110, the response to IGF-I was reestablished. Expression of a dominant negative form of p85 significantly attenuated the ability of IGF-I to inhibit the expression of the CAT reporter gene (Fig. 5D).

**FIG. 5.** Effect of transient expression of PI 3-kinase on the activity of NOS-2 promoter. Cells (2 × 10⁶) were transfected by lipofection with 5 µg of plasmids encoding several forms of PI 3-kinase and 3 µg of p2NOS.CAT. After treatment for 18 h with the indicated stimuli, CAT activity was measured and expressed as a percentage with respect to the CAT activity from cells transfected with a kSV2.CAT vector. Results show the mean ± SE of three experiments. *P < 0.01, **P < 0.001 with respect to the same condition in the absence of IGF-I.
IGF-I inhibition of LPS and IFN-γ-dependent apoptosis.

Incubation of Ins-1 cells with LPS/IFN-γ induced the appearance of apoptotic bodies after staining with propidium iodide (not shown). Analysis of markers of apoptosis in these cells showed a decrease of Bcl-2 after 24 h of incubation with LPS/IFN-γ, a process that was inhibited in the presence of IGF-I (Fig. 6A and B). The levels of Bax, an apoptogenic member of the Bcl-2 family, increased in cells activated for 24 h with LPS and IFN-γ. This effect was abrogated when cells were treated with IGF-I and restored when the PI 3-kinase inhibitor LY294002 was present, reflecting the necessity of a functional PI 3-kinase for protection against the rise in Bax levels. Determination of the presence of cytochrome C in the cytosol well reflected the protective effect exerted by IGF-I against LPS/IFN-γ-induced apoptosis at the time that suggested a contribution of mitochondrial signaling to the triggering of apoptosis in these cells (35–37). When DEVD-specific caspase activity (caspases 3 and 7) was measured (Fig. 6C), the NO-dependent increase in activity induced by incubation with S-nitrosoglutathione (GSNO) (0.5 mmol/l) was not affected by treatment with IGF-I. However, a 4.5-fold decrease of caspase activity was observed when cells activated with LPS/IFN-γ were treated with IGF-I; this effect was abolished after inhibition of PI 3-kinase.

To better analyze the opposite effects of IGF-I and LPS/IFN-γ stimulation in the regulation of apoptosis, the release of nucleosomal moieties from the nucleus to the cytosol was determined as a quantitative marker of the extent of DNA fragmentation. As Fig. 7A shows, the protection exerted by IGF-I against apoptosis was effective at low concentrations of LPS, but decreased at higher doses. To assess the contribution of NO-dependent and -independent pathways to LPS/IFN-γ induction of apoptosis, cells were treated with 50 µmol/l of N-[3-(aminomethyl)benzyl]acetamide (1400W), a NOS-2 inhibitor (apparent Iₜₐₜₐₜ = 5 µmol/l, not shown). The 1400W completely abolished NO synthesis (Fig. 7B and C). Under these conditions, apoptosis was reduced 64%, reflecting the existence of a significant NO-independent apoptosis (36%). Interestingly, this apoptosis still decreased after treatment with IGF-I, which indicates that this growth factor, in addition to its action lowering NO synthesis, also exerts an efficient protection from apoptosis by interacting through additional apoptotic pathways activated by LPS. However, the anti-apoptotic effects of IGF-I disappeared when PI 3-kinase was inhibited. These results suggest a role for PI 3-kinase in the regulation of apoptosis in these cells. To analyze this point more specifically, cells were transfected with CD2-tagged PI 3-kinase constructs and, after sorting by flow cytometry the cells expressing CD2, the apoptotic response was determined by measuring oligonucleosomal release. As Fig. 8 shows, resistance to LPS/IFN-γ-dependent apoptosis was observed in cells that expressed the active p110 kinase, but not in those encoding a kinase-deficient form. Inhibition of PI 3-kinase with LY294004 suppressed the protective effect dependent on p110 activity.
DISCUSSION

We have studied in INS-1 cells the effect of IGF-I on the response to the pro-inflammatory molecules LPS and IFN-γ. The use of this cell line allowed the study of effects on β-cells in the absence of contribution of stimuli released by other cells, such as islet-infiltrated macrophages and T-cells (38,39). INS-1 cells required the coordinate action of two or more stimuli (i.e., TNF-α + IFN-γ + IL-1β or LPS + IFN-γ) to promote the expression of NOS-2. In this regard, the use of IFN-γ as co-activator provided a model reminiscent of viral infection of islet β-cells, where this cytokine stimulates the synthesis of NO and impairs insulin secretion, leading to islet degeneration by apoptosis (40,41). Moreover, the action of IFN-γ in these cells is poorly documented, although it has been recognized as a causal agent of immune islet degeneration, even in the absence of NO synthesis (41). In contrast to IFN-γ, the dysfunction dependent on IL-1β release by infiltrating macrophages is better documented in the pathogenesis of type 1 diabetes (39,42).

The NO synthesized after expression of the high-output NOS-2 has been shown to be an important cause of immune-induced β-cell destruction in type 1 diabetes as deduced by biochemical, genetic, and pharmacological data (39,43,44). Inhibition of NO synthesis by substrate analogs of NOS-2 protects or attenuates β-cell dysfunction and destruction (44). Moreover, in mice with a NOS-2 disrupted gene, pro-inflammatory stimuli such as IL-1β failed to impair islet function in vitro and showed a reduced incidence of hyperglycemia after challenge with multiple low doses of streptozotocin in vivo (45).

The role of IGF-I as inhibitor of NOS-2 expression in response to pro-inflammatory stimuli exhibits a certain cell specificity: in renal mesangial cells, this growth factor enhances NO synthesis (46), whereas in rat islets it has the opposite effect (3). This protective action of IGF-I on islet function seems to be physiologically relevant, as deduced from the observation that in nonobese mice, IGF-I is present in the islets of prediabetic animals, but not in islets with immune infiltration, suggesting an inhibitory role for IGF-I against cytotoxic actions of pro-inflammatory stimuli (2). In the experimental model used, the inhibitory action of IGF-I on NOS-2 expression was observed in the early steps of LPS and IFN-γ signaling, since after 6 h of activation, addition of IGF-I had no effect on NO synthesis and favored cell viability. According to our data, the mechanism by which IGF-I impaired NOS-2 expression was compatible with an inhibition of the IKK activity, and therefore of the IκB phosphorylation and degradation (30,31). This inhibition of IκB targeting and degradation was confirmed by three independent criteria: 1) the impairment of specific ser32 phosphorylation of IκBα; 2) the attenuation of the decrease of the IκBα and IκBβ levels; and 3) the important reduction of NF-κB activity as deduced by EMSA.
These data suggest the requirement of NF-κB activation for the expression of NOS-2 in these cells. Indeed, abrogation of NF-κB activity by pharmacological agents or by heat shock treatment of isolated islets impaired the expression of NOS-2 in response to IL-1β (47,48). It is worth mentioning that the protective effects of IGF-I were observed at moderate stimulation of Ins-1 cells with LPS and IFN-γ, but the effects were lost under higher activation conditions, such as those that trigger a high rate of NO synthesis (for example, after the concerted action of IL-1β, IFN-γ, and TNF-α).

The observation that the effect of IGF-I on NOS-2 expression was abrogated when cells were treated with the PI 3-kinase inhibitors LY294002 and the structurally unrelated molecule wortmannin (not shown) was reminiscent of the inhibitory action of this kinase in macrophages and glial cells after challenge with LPS (15,16). In addition to the use of PI 3-kinase inhibitors, transfection of cells with plasmids expressing several forms of p85 and p110 subunits reflected the necessity of this pathway to mediate the effects of IGF-I. Moreover, rapamycin, an inhibitor downstream of the PI 3-kinase pathway (6), did not affect the synthesis of NO, suggesting that the observed effects were dependent on PI 3-kinase activity directly. In Ins-1 cells, the sequence of events following PI 3-kinase inhibition was compatible with a prominent role of attenuated NF-κB activation, likely affecting a step involved in the activation of IKK. Interestingly, when inflammatory activation was accomplished at saturation of LPS and IFN-γ stimulation, the important inhibitory action of IGF-I on NOS-2 expression was significantly attenuated. This might be due to the cooperative action of LPS and IFN-γ in the activation of the NOS-2 promoter, decreasing the requirements of NF-κB activity for the expression of this gene (17,49). In addition to this, the activation of PI 3-kinase by IGF-I is well documented (6,50). However, the existence of different isoenzymes of the p110 (α, β, and δ in mammals) and the p85 (α, β, and p55γ) subunits, with specific patterns of cell expression and regulation, suggests the occurrence of distinct signaling pathways mediated by defined p85-p110 complexes. The physiological relevance of this remains to be established (13,51).

The other aspect investigated in this work was the effect of IGF-I on the control of apoptosis in these cells. Activation of Ins-1 cells with LPS and IFN-γ induced an extensive apoptosis that after 24 h of culture involved ~25–30% of the cells as deduced by microscopic observation of apoptotic features. Indeed, treatment of Ins-1 cells with NO donors, such as GSNO, triggered apoptosis when the release of NO was in the range of the concentrations prevailing after expression of NOS-2 (0.5 mmol/l of donor), indicating that NO induces apoptosis in these cells. Moreover, it has been described that NO by itself upregulates the expression of Fas by β-cells, favoring the induction of apoptosis after interaction with islet-infiltrating T-cells that express Fas ligand (52) and reinforcing the apoptotic capacity of this molecule for β-cells. In this regard, the apoptosis induced after activation with LPS and IFN-γ was mainly due to the release of NO, because the specific NOS-2 inhibitor L-NAME suppressed 70–75% of the apoptosis. Interestingly, the remaining NO-independent apoptosis was inhibited after treatment of cells with IGF-I. However, IGF-I was unable to influence the apoptosis triggered by GSNO. This protection against apoptosis elicited by IGF-I might be due to the inhibition of the expression of Bax and to the moderate accumulation of Bcl-2 observed in cells treated with pro-inflammatory stimuli. In this pathway, an important effect was also observed on the PI 3-kinase–dependent signaling. Indeed, the anti-apoptotic action of IGF-I and PI 3-kinase are well documented (2,4,53,54). The protection from apoptosis exerted by activation of PI 3-kinase in these cells was confirmed by additional experiments of transient expression of PI 3-kinase molecules. Taking advantage of the use of PI 3-kinase and CD2 chimeric proteins, an important protection, LY294002-inhibitable, was characterized when cells expressed a p110 catalytically active subunit, but not when cells were transfected with the kinase-deficient form of p110.

In summary, the results reported in this work show an important role for IGF-I in the maintenance of cell viability of β-cells. This effect appears to be mainly dependent on the activation of PI 3-kinase, although the precise contribution of the distinct isoforms of the PI 3-kinase present in Ins-1 cells deserves further work. Also, the results suggest that IGF-I is very efficient in the protection of β-cell function against moderate inflammatory signaling, but not under acute processes, at least regarding the expression of NOS-2. This view of IGF-I as regulator of inflammatory processes in β-cells might be related to the observation of a wide variability in circulating IGF-I levels in adult human populations; a fact that, although related to the initiation of severe neoplastic processes, could prevent β-cell injury in the course of local inflammatory processes and perhaps contribute as an additional factor in the susceptibility to developing type 1 diabetes in humans (55). Further work on the pharmacological management of downstream IGF-I signaling, in particular after the establishment of the relevant isoenzymes of PI 3-kinase activated by IGF-I in β-cells, could benefit the development of new strategies for the therapeutic control of diabetes.

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