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

Antonio Castrillo, Oscar G. Bodelón, and Lisardo Boscá

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 subsaturating 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]acetamidine, a specific NOS-2 inhibitor, IGF-I completely abolished the NO-independent apoptosis. 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

From the Instituto de Bioquímica (Centro Mixto CSIC-UCM), Facultad de Farmacia, Universidad Complutense, Madrid, Spain.

Address correspondence and reprint requests to Lisardo Boscá, Instituto de Bioquímica, Facultad de Farmacia, 28040 Madrid, Spain. E-mail: boscal@eucmax.sim.ucm.es.

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1400W, *N*-[3-(aminomethyl)benzyl]acetamidine; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; GSNO, S-nitrosoglutathione; GST, glutathione-S-transferase; $I_{0.5}$, half-maximal effect; IFN-γ, interferon-γ; IKK, IκB kinase; IL, interleukin; IP, immunoprecipitation; IRS, insulin receptor substrate; LPS, lipopolysaccharide; NOS, nitric oxide synthase; PBS, phosphate-buffered saline; PI, phosphatidylinositol; PMSF, phenylmethylsulfonyl fluoride; TLC, thin-layer chromatography; TNF-α, tumor necrosis factor-α.

IGF-I has been implicated in the regulation of physiological and pathological functions of various cell types, including pancreatic β-cells (1–5). Signaling by IGF-I involves the activation of the IGF-I receptor, a $\alpha_2\beta_2$ heterotetrameric molecule, through its ligand-induced tyrosine kinase activity, leading to the phosphorylation of the cytoplasmic domain of the β-subunits (6,7). Receptor activation promotes the rapid phosphorylation on multiple tyrosine residues of insulin receptor substrate (IRS)-1 and IRS-2, which allows the binding, through the phosphorylated sequences, of *src* homology domain 2 (SH2) domains of various proteins, initiating further IGF-I–dependent signaling events (8,9). Among the proteins interacting with IRS-1 and IRS-2 are *1*) Grb-2, which activates the Ras/Raf/ERK signaling pathway; *2*) Shc, which in addition to the interaction with IRS-1 can be activated directly by phosphorylation through the IGF-I receptor, depending on the cell type; and *3*) the p85 subunit of the phosphatidylinositol (PI) 3-kinase (8–10). PI 3-kinase is a family of isoenzymes with dual lipid and serine/threonine kinase activities that acts as a key regulator in a variety of cellular responses, including glucose transport, mitogenesis and differentiation, and membrane dynamics, and as an anti-apoptotic mediator (4,11–14). The kinase activity is located in the p110 subunit, whereas p85 acts as the regulatory part of the complex. Interaction between phosphorylated IRS-1 and p85 allows the activation of p110 and the localization of this subunit in the proximity of its substrates near the membrane, among them p85 and IRS-1 (12,14). The signaling downstream PI 3-kinase is a subject of current interest because a large number of targets appear to be modulated through this pathway (13).

Recently, it has been shown that inhibition of PI 3-kinase leads to an overexpression of nitric oxide synthase (NOS)-2, the enzyme responsible for the high-output NO synthesis in macrophages, microglia, and other cells stimulated with lipopolysaccharide (LPS) or pro-inflammatory cytokines (15,16). Expression of NOS-2 in rodent cells has been studied exhaustively. The process is regulated through synergistic cooperation between the transcription factors NF-κB, Stat-1, and IRF-1, at least in response to LPS and IFN-γ challenge (17,18). Some data indicate that NF-κB activation is a critical event in the expression of NOS-2, and this process depends on the degradation of the corresponding inhibitory proteins

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 NOS-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 NOS-2 expression under pro-inflammatory conditions. Results show that IGF-I impaired the expression of NOS-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). Electrophoresis 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 cells, and used up to 3–5 passages. Cells were characterized morphologically and biochemically as described by Asfari et al. (23). Subconfluent cells ($0.8–1 \times 10^5$/cm^2). were maintained for 2 days in culture, and the medium was replaced by phenol-red free RPMI 1640 containing 0.5 mmol/l arginine and 5% fetal calf serum (FCS). When cells were treated with distinct stimuli, PI 3-kinase inhibitors (wortmannin and LY292004) were added before IGF-I, and this preceded cell activation with LPS and IFN-γ.

Plasmid constructs and preparation. The following plasmids were used: p2NOS.CAT, containing a 1.0 kb fragment corresponding to the 5′-flanking region of NOS-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 rCD2p110kd, a p110 kinase deficient mutant; p85dn, which is unable to bind p110 and therefore inhibits the recruitment of p110 to the membrane (a generous gift from Dr. D.A. Cantrell; 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, LY294002, and wortmannin. The expression of CD2 was determined by flow cytometry after labeling the cells with FITC-OX-34 (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).

Transfection of Ins-1 cells and assay of CAT activity. Ins-1 cells were washed twice with phosphate-buffered saline (PBS) and incubated with 1.5 ml of RPMI medium without FCS in 6-cm diameter dishes. Cells were transfected for 8 h by lipofection with DOTAP following the instructions of the supplier (Boehringer Mannheim). After transfection, the culture medium was replaced, and cells were maintained for 24 h before stimulation with RPMI 1640 medium containing 5% FCS. Equal amounts of DNA were used in the transfection experiments, using a basic p.CAT plasmid (Promega, Madison, WI) to normalize the DNA content. A kSV_2·CAT plasmid was used as an internal control for the efficiency of the transfection. After 18 h of treatment with the indicated stimuli, the cells were homogenized and CAT activity was determined by thin-layer chromatography (TLC) following a previously described protocol (18). The amount of acetylated chloramphenicol was quantified in a FUJI BAS1000 radioactivity detection system.

Characterization of NOS-2 expression by Northern blot. Total RNA ($2–4 \times 10^6$ cells) was extracted using the guanidinium thiocyanate method and size-separated by electrophoresis in a 0.9% agarose gel containing 2% formaldehyde. The levels of NOS-2 mRNA were determined using an [α-^{32}P]dCTP labeled *Eco*RI-*Hind*II fragment from the NOS-2 cDNA (18,26). The intensity of the bands detected by Northern blot was measured by laser densitometry (Molecular Dynamics, Sunnyvale, CA). Hybridization with an 18S ribosomal probe was used to normalize the lane charge.

Determination of NO synthesis. NO was measured as the accumulation of nitrite in the incubation medium after reduction of nitrate to nitrite with nitrate reductase. Nitrite was determined spectrophotometrically with Griess reagent (24,27).

Assay of PI 3-kinase activity. The cell layers were washed twice with ice-cold buffer A (10 mmol/l HEPES, pH 7.9; 1 mmol/l EDTA, 1 mmol/l EGTA, 10 mmol/l KCl, 1 mmol/l dithiothreitol [DTT], 0.5 mmol/l phenylmethylsulfonyl fluoride [PMSF], 2 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml tosyl-lysyl-chloromethane, 5 mmol/l NaF, 1 mmol/l $NaVO_4$, 10 mmol/l Na_2MoO_4) containing 120 mmol/l NaCl. Lysis of the cells was performed at 4°C with 1 ml of buffer A supplemented with 0.5% Nonidet P-40 and under continuous shaking. After centrifugation in an Eppendorf centrifuge, the supernatant was treated with anti-PY20 antibody following the instructions of the supplier (Upstate Biotechnology). The activity of PI 3-kinase present in the resuspended immunoprecipitation (IP) was determined using PI (20 μg) and [γ-^{32}P]ATP (24). After TLC, the amount of phosphorylated lipids was evaluated using a FUJI BAS1000 detector.

Preparation of soluble and nuclear extracts. Ins-1 cells ($2–3 \times 10^6$) were washed twice with PBS, scraped off the dishes, transferred to a 1.5-ml tube, and centrifuged. The cell pellets were homogenized with 100 μl buffer A. After 10 min at 4°C, Nonidet P-40 was added to reach 0.5% concentration. The tubes were gently vortexed for 15 s, and nuclei were collected by centrifugation at 8,000*g* for 15 min. The supernatants were stored at –80°C (soluble extracts), and the pellets were resuspended in 50 μl buffer A supplemented with 20% 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 mitochondrial integrity was required, the homogenization medium consisted of buffer A supplemented with 0.22 mol/l mannitol, 68 mmol/l sucrose, and 10 μmol/l cytochalasin B. All steps of cell fractionation were carried out at 4°C (28).

Characterization of proteins by Western blot. Equal amounts of soluble protein extracts were size-separated in 10% SDS-PAGE. The gels were blotted onto a Hybond P membrane (Amersham) and incubated with anti–NOS-2, anti–IκB-α, anti–IκB-β, anti-Bax, anti–Bcl-2, anti–cytochrome C, and anti–IκB kinase 2 (IKK2) antibodies (Santa Cruz Biotechnology). In experiments using anti-phospho(ser^{32})IκB-α antibody (New England Biolabs, Beverly, MA), the blot incubation solution contained 50 ng/ml of glutathione-S-transferase (GST)–IκB-α(1–137) treated previously with alkaline phosphatase-agarose (29). The blots were submitted to sequential reprobing with antibodies after treatment with 100 mmol/l β-mercaptoethanol and 2% SDS in Tris-buffered saline and heating at 60°C for 30 min. The blots were revealed by enhanced chemiluminescence following the manufacturer's instructions (Amersham).

Measurement of IKK activity. Ins-1 cells (3×10^6) were homogenized in 1.2 ml buffer A and centrifuged for 10 min in a microcentrifuge. The supernatant (1 ml) was precleared, and IKK2 was immunoprecipitated with 1 μg of anti-IKK2 antibody and protein A-agarose (30,31). After extensive washing of the IP with buffer A, the pellet was resuspended in kinase buffer (20 mmol/l HEPES, pH 7.4, 0.1 mmol/l EDTA, 100 mmol/l NaCl, 1 mmol/l DTT, 0.5 mmol/l PMSF, 2 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml tosyl-L-lysine chloromethyl ketone, 5 mmol/l NaF, 1 mmol/l $NaVO_4$, 10 mmol/l Na_2MoO_4, and 10 nmol/l okadaic acid). The kinase activity was assayed in 100 μl buffer A containing 100 ng IP, 100 ng GST–IκB-α (1–317), and 1 mmol/l MgATP. Aliquots of reaction were stopped at various times in 50 μl of Laemmli sample buffer and analyzed by Western blot using a specific anti-phospho(ser^{32})IκB-α antibody and reprobing the membrane with anti-IκBα antibody (Santa Cruz Biotechnology). The linearity of the kinase reaction was confirmed over a period of 30 min.

Electrophoretic mobility shift assays. The oligonucleotide sequence 5′TGCT AGG-GGGATTTTCCCTCTCTCTGT3′ corresponding to the consensus NF-κB binding site (nucleotides –978 to –952) of the murine NOS-2 promoter was used (17,24). Oligonucleotides were annealed with their complementary sequence by incubation for 5 min at 85°C in 10 mmol/l Tris-HCl, pH 8.0, 50 mmol/l NaCl, 10 mmol/l $MgCl_2$, and 1 mmol/l DTT, and an aliquot (50 ng) was end-labeled with Klenow enzyme in the presence of 50 μCi of [α-^{32}P]dCTP and the other unlabeled dNTPs. There was 5×10^4 dpm of the DNA probe used for each binding assay of nuclear extracts as follows: 3 μg protein was incubated for 15 min at 4°C with the DNA and 2 μg poly(dI:dC), 5% glycerol, 1 mmol/l EDTA, 100 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l DTT, and 10 mmol/l Tris-HCl, pH 7.8, in a final volume of 20 μl. The DNA protein complexes were separated on native 6% polyacrylamide gels in 0.5% Tris-borate-EDTA buffer (24). Supershift assays were carried out after incubation of the nuclear extracts with the antibodies against c-Rel proteins (0.5 μg) for 1 h at 4°C, followed by electrophoretic mobility shift assay (EMSA) (not shown).

Measurement of apoptosis. Apoptosis was determined by the release of oligonucleosomes from the nucleus to the cytosol following an established protocol using a cell death kit (Boehringer) in which the histone-associated DNA fragments released to the cytosol were detected using a sandwich enzyme immunoassay with anti-histone and anti–DNA peroxidase antibodies. The relative degree of apoptosis was quantitatively determined by measuring the peroxidase activity at 405 nm and calculating the ratio between the enzyme activity of a sample incubated for a given period of time and the corresponding value from untreated cells. In addition to this, the changes in cellular morphology and the appearance of apoptotic bodies were determined by confocal microscopy (not shown).

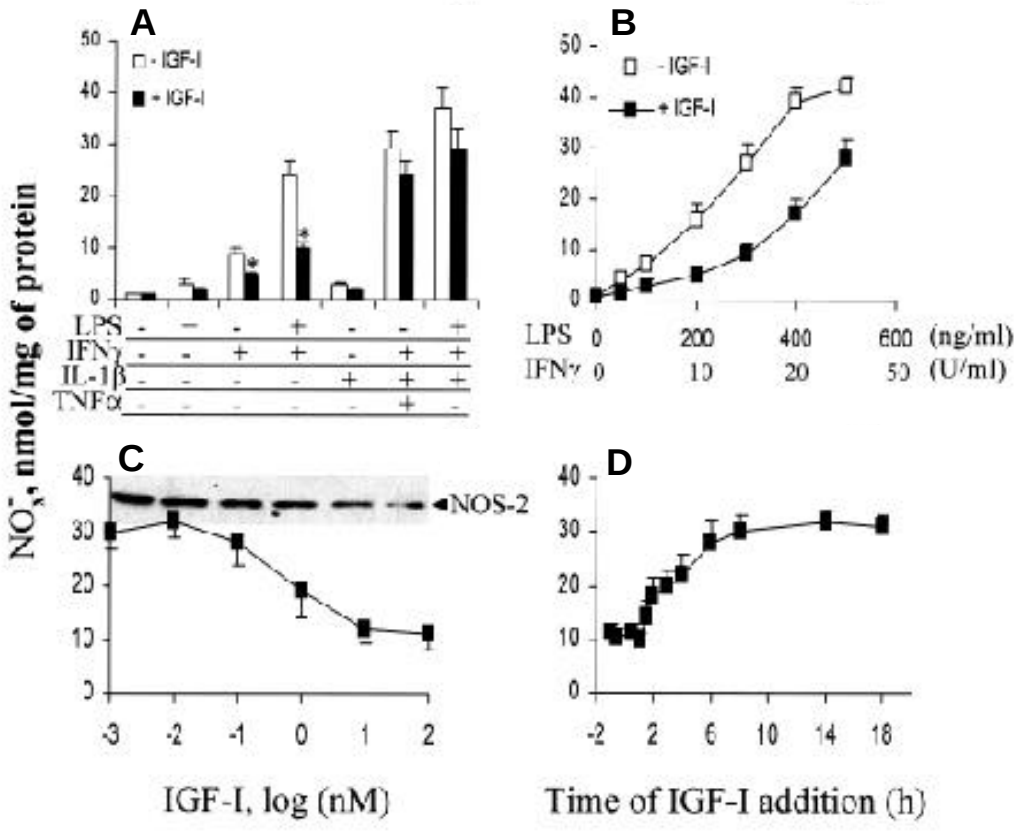

FIG. 1. Inhibition by IGF-I of NO synthesis in Ins-1 cells treated with LPS and IFN-γ. Cells were incubated for 24 h with 10 nmol/l IGF-I, 300 ng/ml LPS, 10 U/ml IFN-γ, 10 ng/ml IL-1β, 5 ng/ml TNF-α, or combinations of these, except as otherwise stated. The synthesis of NO was determined by the accumulation of nitrate plus nitrite in the incubation medium (*A–D*). At the end of the incubation period (300 ng/ml LPS, 10 U/ml IFN-γ, and varying concentrations of IGF-I), soluble extracts were prepared and the amount of NOS-2 was determined by Western blot (*C*). The effect on NO synthesis of IGF-I added at various times with respect to LPS/IFN-γ challenge (300 ng/ml and 10 U/ml, respectively) was determined (*D*). Results show the mean ± SE of three experiments. **P* < 0.05 with respect to the corresponding condition in the absence of IGF-I.

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. 1*A*). 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. 1*A* 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 ($I_{0.5}$) at 1 nmol/l and was due to a decrease in the levels of NOS-2 protein (Fig. 1*C*). 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. 1*D*).

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. 2*A* 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. 2*B*). 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

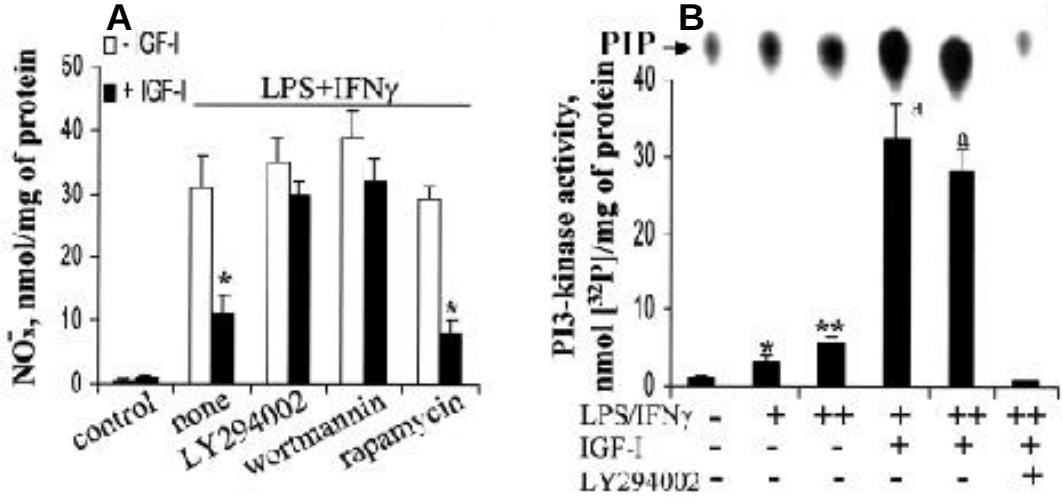

FIG. 2. PI 3-kinase inhibitors abolished the effect of IGF-I on NO synthesis. Ins-1 cells were incubated for 24 h with IGF-I (10 nmol/l), 300 ng/ml LPS, 10 U/ml IFN-γ, 20 µmol/l LY294002, 250 nmol/l wortmannin, and 10 µmol/l rapamycin, and the synthesis of NO was measured (*A*). In the same experiment, cells incubated for 10 min with the indicated stimuli (+) or with 1 µg/ml LPS and 50 U/ml IFN-γ (++) were homogenized and treated with anti-PY20 antibody. The lipid kinase present in the IP was assayed. When cells were treated with LY294002, this was added to the kinase assay (*B*). Results show the mean ± SE of three experiments. $*P < 0.01$, $**P < 0.001$ with respect to the condition in the absence of IGF-I (*A*) or to the control condition (*B*). $^{a}P < 0.001$ with respect to the corresponding condition in the absence of IGF-I (*B*)

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. 3*A*). 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. 3*B*). Figure 3*D* 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-

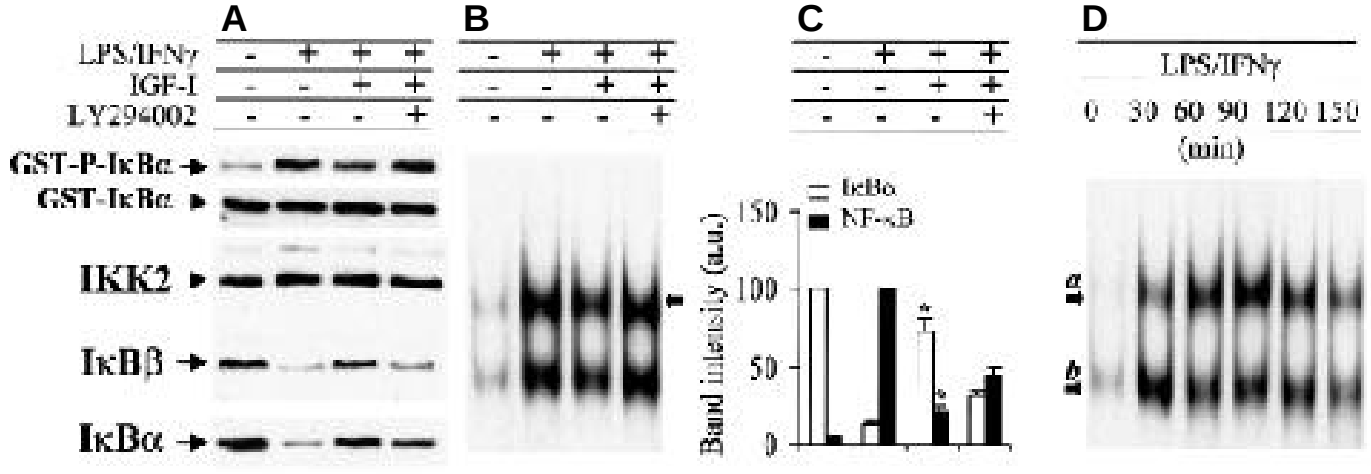

FIG. 3. IGF-I inhibits IκB degradation and NF-κB activity. Ins-1 cells were treated for 15 min with the indicated stimuli (300 ng/ml LPS, 10 U/ml IFN-γ, 10 nmol/l IGF-I, 20 µmol/l LY294002), and after homogenization and IP with anti-IKK2 antibody, IKK activity was determined in vitro using GST–IκB-α as substrate. The amount of GST-phospho(ser^{32})IκB-α, GST–IκB-α, and IKK present in each assay was determined by Western blot (*A*, *upper panel*). The endogenous levels of IκB-α and IκB-β were determined by Western blot in soluble extracts after 1 h of treatment (*A*). The binding of nuclear proteins to the distal κB motif of the murine NOS-2 promoter was determined by EMSA using extracts of cells stimulated for 1 h (*B*). The intensity of the bands corresponding to the levels of IκB-α and the upper complex of the EMSA are shown (mean ± SE, $n = 3$; $*P < 0.01$ with respect to the corresponding condition in the absence of IGF-I) (*C*). The time course of NF-κB activation is shown. *Band a*, p50.p65; *band b*, p50.p50 complexes determined by supershift assays (*D*).

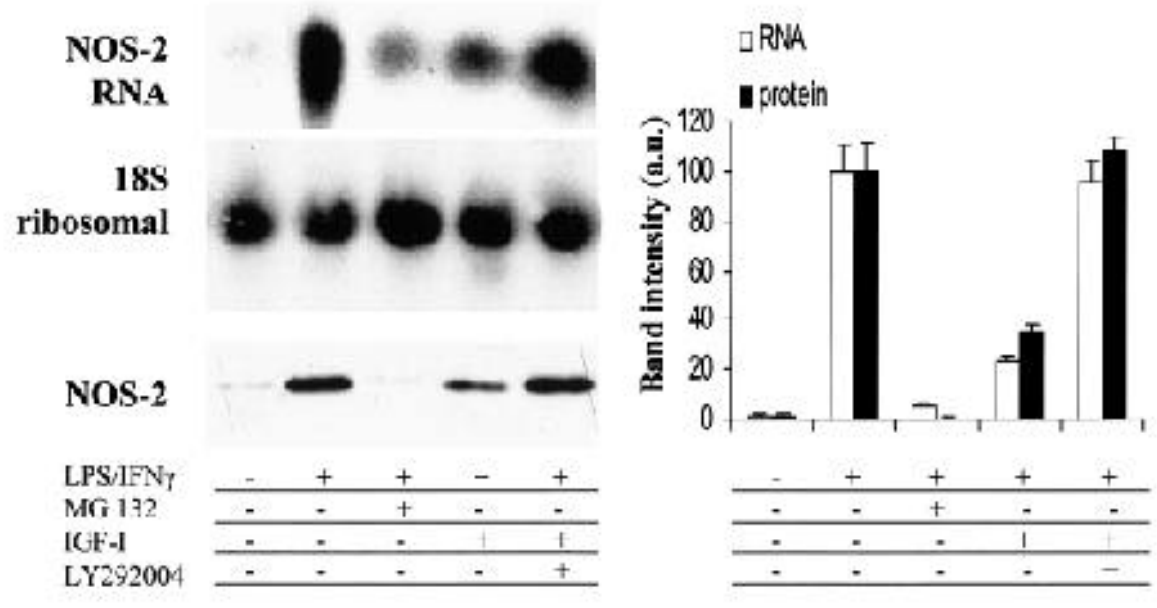

FIG. 4. Effect of IGF-I on NOS-2 mRNA and protein levels. Ins-1 cells were stimulated as described in Fig. 3. Cell extracts were prepared at 8 and 24 h to determine the mRNA and protein levels of NOS-2, respectively. MG 132 was used at 20 µmol/l. Results show a representative experiment out of three (*left*) and the mean ± SE of the corresponding band intensities. mRNA levels were calculated after normalization for the content of 18S ribosomal RNA.

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. 5*A* 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. 5*B*). 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. 5*D*).

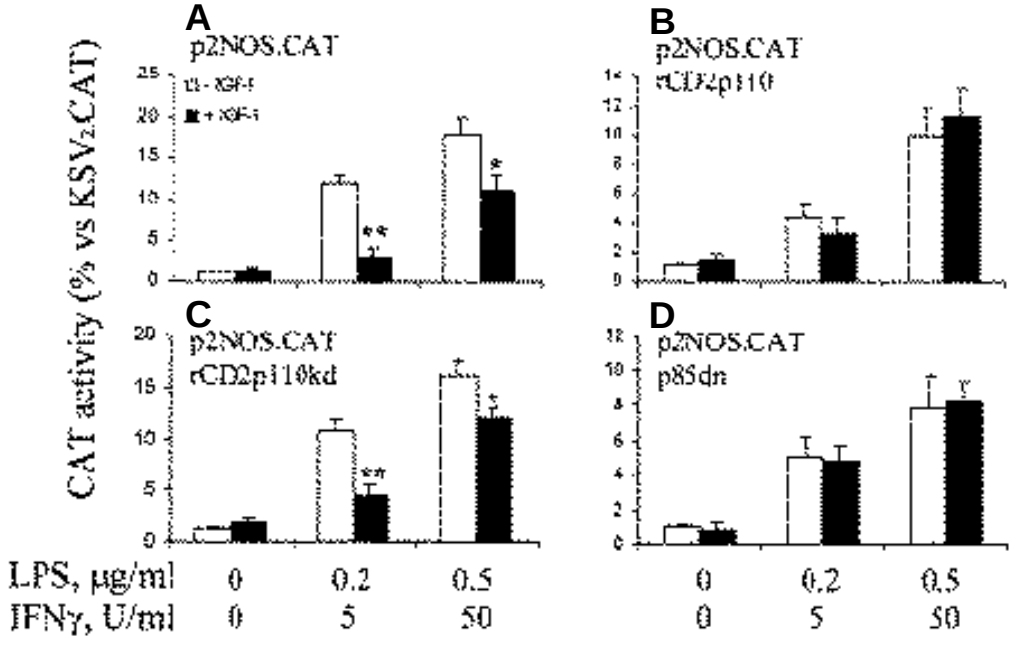

FIG. 5. Effect of transient expression of PI 3-kinase on the activity of NOS-2 promoter. Cells (2×10^6) were transfected by lipofection with 5 µg of plasmids encoding several forms of PI 3-kinase and 3 µg of p2NOS.CAT. After treatment for 18 h with the indicated stimuli, CAT activity was measured and 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.

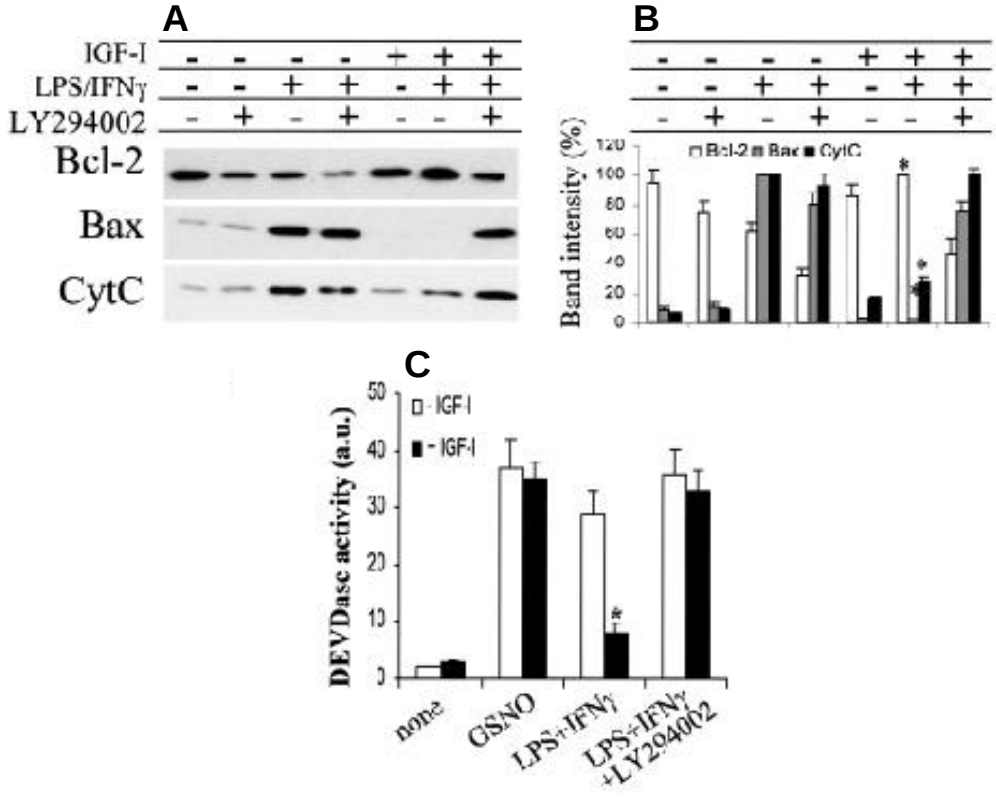

FIG. 6. Effect of IGF-I and PI 3-kinase inhibitors on apoptosis markers and DEVD-caspase activity in Ins-1 cells. Cells were treated for 24 h with the indicated ligands at the concentrations described in Fig. 3. After homogenization of the cells, the levels of Bcl-2 and Bax were determined by Western blot in the soluble extracts. The release of cytochrome C from the mitochondria to the cytoplasm was determined in homogenates preserving the mitochondrial integrity (*A*). The densitometry of the bands is shown as percentage of the maximal intensity for each protein (*B*). DEVDase activity (caspases 3 and 7) was measured spectrofluorometrically using a specific substrate and the corresponding aldehyde as specific inhibitor (*C*). Results show the mean ± SE of three experiments. **P* < 0.001 with respect to the 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. 6*A* 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. 6*C*), 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. 7*A* 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]acetamidine (1400W), a NOS-2 inhibitor (apparent $I_{0.5}$ = 5 µmol/l, not shown). The 1400W completely abolished NO synthesis (Fig. 7*B* 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 LY292004 suppressed the protective effect dependent on p110 activity.

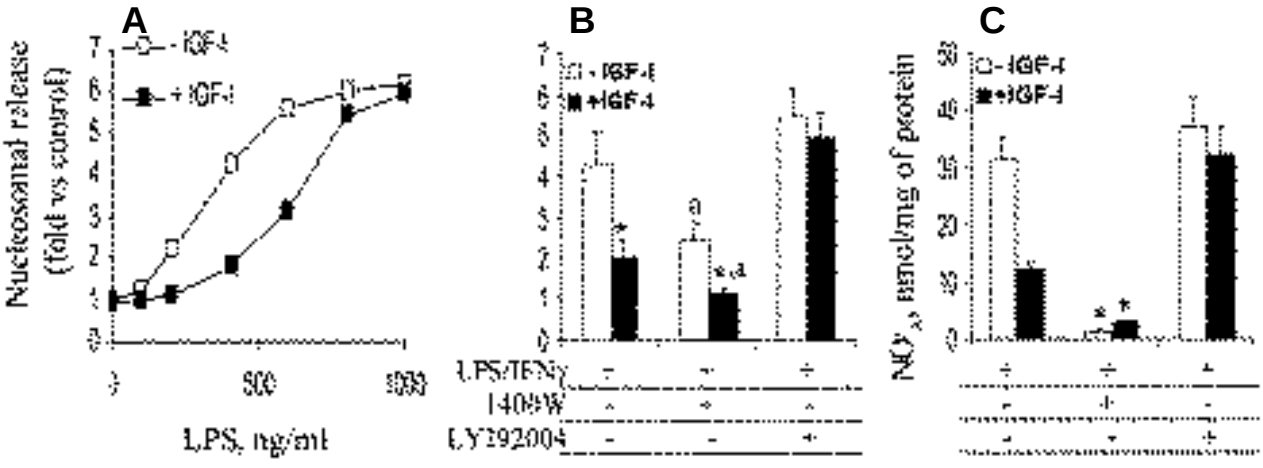

FIG. 7. Measurement of apoptosis in Ins-1 cells by the accumulation of oligonucleosomes in the cytoplasm. Cells were treated for 24 h with 10 U/ml of IFN-γ and the indicated concentrations of LPS. The amount of nucleosomal DNA present in cytosolic extracts was determined with an enzyme-linked immunosorbent assay cell-death kit (*A*). The effect on apoptosis (*B*) and on NO synthesis (*C*) of the NOS-2 and PI 3-kinase inhibitors 1400W (50 µmol/l) and LY292004 (20 µmol/l), respectively, was measured. Results show the mean ± SE of three experiments. **P* < 0.01, [a]*P* < 0.01 with respect to the condition in the absence of IGF-I and in the absence of 1400W, respectively (*B*). **P* < 0.001 with respect to the condition in the absence of 1400W (*C*).

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 ser[32] phosphorylation of IκB-α; *2*) the attenuation of the decrease of the IκB-α and IκB-β levels; and *3*) the important reduction of NF-κB activity as deduced by EMSA.

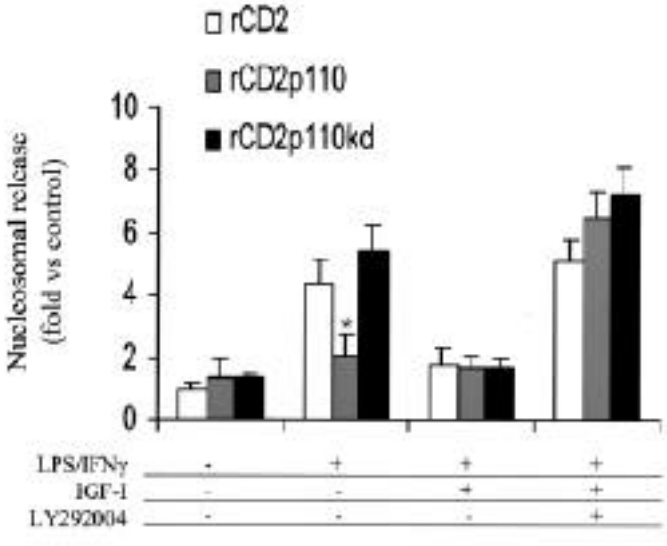

FIG. 8. Effect of PI 3-kinase expression on apoptosis. Cells (2–3 × 10^6) were transfected with the indicated plasmids for 18 h. $CD2^+$ cells were sorted and kept in culture. After 2 h to favor adhesion to the dishes, the cells were stimulated, and the release of oligonucleosomes from the nucleus to the cytosol was determined. Results show the mean ± SE of three experiments assayed per duplicate. **P* < 0.01 with respect to cells transfected with rCD2 and rCD2p110kd.

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 apoptogenic 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 1400W 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 several neoplasic 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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