

Evidence for Involvement of the Proteasome Complex (26S) and NF κ B in IL-1 β -Induced Nitric Oxide and Prostaglandin Production by Rat Islets and RINm5F Cells

Guim Kwon, John A. Corbett, Scott Hauser, Jeanette R. Hill, John Turk, and Michael L. McDaniel

Interleukin-1 β (IL-1 β) has been implicated as an effector molecule of β -cell destruction in autoimmune diabetes. IL-1 β inhibits insulin secretion from pancreatic β -cells by stimulating the expression of inducible nitric oxide synthase (iNOS) that generates the free radical nitric oxide. IL-1 β also induces the coexpression of the inducible isoform of cyclooxygenase (COX-2) that results in the overproduction of proinflammatory prostaglandins. The current studies were designed to characterize the involvement of protease(s) in the signaling pathway of IL-1 β -induced iNOS and COX-2 expression by rat islets and transformed rat pancreatic β -cells. Because of the limitations of cell numbers of purified primary β -cells obtained from rat islets, biochemical and molecular studies were performed using the rat insulinoma β -cell line RINm5F. A serine protease inhibitor, *N* α -*P*-tosyl-L-lysine chloromethyl ketone (TLCK), and a proteasome complex (26S) inhibitor, MG 132, inhibited IL-1 β -induced nitrite formation, an oxidation product of nitric oxide produced by iNOS, in a concentration-dependent manner, with complete inhibition observed at 100 μ mol/l and 10 μ mol/l, respectively. Both TLCK and MG 132 also inhibited iNOS gene expression at the level of mRNA and protein. In an analogous manner, TLCK (100 μ mol/l) and MG 132 (10 μ mol/l) inhibited IL-1 β -induced COX-2 enzyme activity (PGE₂ formation) and COX-2 gene expression at the level of mRNA and protein. In human islets, the proteasome inhibitor MG 132 also inhibited the formation of the products of iNOS and COX-2 enzyme activity, nitrite, and PGE₂, respectively. These findings suggest that the inhibitory action of TLCK and MG 132 on iNOS and COX-2 expression precedes transcription. The transcription factor NF κ B is essential for activation of a number of cytokine-inducible enzymes and was evaluated as a possible site of protease action necessary for IL-1 β -induced coex-

pression of iNOS and COX-2. TLCK and MG 132 inhibited both IL-1 β -induced activation of NF κ B and degradation of I κ B α by islets and RINm5F cells. These results implicate protease activation as an early signaling event in IL-1 β -induced inhibition of β -cell function. This study also suggests that IL-1 β -induced iNOS and COX-2 coexpression by pancreatic β -cells share a common signaling pathway in utilizing the proteasome complex (26S) and the transcription factor NF κ B, and it identifies sites of intervention to prevent the overproduction of their inflammatory products. *Diabetes* 47:583-591, 1998

Insulinitis, a local inflammatory reaction in and around pancreatic islets, is a key feature of type 1 diabetes in both humans and animals (1-3). Proinflammatory agents such as cytokines, free radicals, and eicosanoids are proposed to play key roles in culminating insulinitis. The cytokine interleukin-1 β (IL-1 β) has been shown to cause dysfunction and destruction of rat pancreatic β -cells (4-6). IL-1 β stimulates the expression of inducible nitric oxide synthase (iNOS) by β -cells, resulting in the overproduction of the free radical nitric oxide (NO) that mediates inhibition of insulin secretion and β -cell cytotoxicity (7-11). IL-1 β also induces the expression of the inducible isoform of cyclooxygenase (COX-2) by rat islets within the same time frame as iNOS expression (12). Furthermore, NO stimulates the enzymatic activity of cyclooxygenase (both constitutive and inducible isoforms), augmenting the production of proinflammatory prostaglandins, in particular prostaglandin E₂ (PGE₂) (12,13). In light of the possible role of the proinflammatory mediators NO and PGE₂ in the pathogenesis of immunologically mediated diabetes, delineation of signaling pathways involved in IL-1 β -induced NO and PGE₂ production by islets is of particular interest.

The coexpression of iNOS and COX-2 by islets appears to share a common signaling pathway based on several lines of evidence: 1) the time course of IL-1 β -induced NO and PGE₂ production by islets is similar (12), 2) both iNOS and COX-2 expression involve activation of a tyrosine kinase(s) (14), 3) the IL-1 β receptor antagonist protein (IRAP or IL-1 β ra) blocks IL-1 β -induced formation of both proinflammatory mediators, NO and PGE₂ (15,16), and 4) the promoter regions of iNOS and COX-2 genes share consensus sequences for the binding of transcription factors NF κ B and activator protein-1 (17,18).

The transcription nuclear factor κ B (NF κ B) has recently

From the Department of Pathology (G.K., J.R.H., J.T., M.L.M.), Washington University School of Medicine; and Molecular and Cellular Biology (S.H.), Searle Research and Development, St. Louis, Missouri.

Address correspondence and reprint requests to Dr. Michael L. McDaniel, Department of Pathology, Box 8118, Washington University School of Medicine, 660 South Euclid Ave., St. Louis, MO 63110-8118.

Received for publication 19 September 1997 and accepted in revised form 15 December 1997.

COX-1, cyclooxygenase-1; COX-2, cyclooxygenase-2; IFN- γ , γ -interferon; IL-1 β , interleukin-1 β ; I κ B α , inhibitor of NF κ B; iNOS, inducible nitric oxide synthase; MEM, minimal essential medium; NF κ B, nuclear factor κ B; NMMA, *N*⁶-monomethyl-L-arginine; NO, nitric oxide; PBS, phosphate-buffered saline; PGE₂, prostaglandin E₂; TLCK, *N* α -*P*-tosyl-L-lysine chloromethyl ketone; TPCK, *N* α -tosyl-L-phenylalanine chloromethyl ketone; TNF- α , tumor necrosis factor- α .

been proposed to regulate a number of cytokine-inducible enzymes. NF κ B is present in the cytoplasm of cells as an inactive form complexed with I κ B, an inhibitory factor of NF κ B. Cytokines induce dissociation of this complex, presumably by phosphorylation of I κ B and/or activation of proteolytic degradation of I κ B that then allows NF κ B to translocate from the cytoplasm to the nucleus, where it interacts with DNA recognition sites necessary to mediate gene transcription. Recent studies have focused on the possible function of the proteasome complex to mediate proteolytic processing of the inactive NF κ B complex. The 26S (1,500 kDa) proteasome complex is highly conserved and is present in the cytoplasm and nucleus of all eukaryotic cells (19). Its function is to remove abnormal and short-lived regulatory proteins, including I κ B α (19). The enzymatic activity of the proteasome complex requires multiple steps, including covalent conjugation of target proteins with multiple ubiquitin (Ub) molecules through ATP-dependent mechanisms (20,21). MG 132 (carbobenzoxyl-leucinyll-leucinyll-leucinyll-H, also called Z-LLL) is a potent inhibitor of the proteasome complex (26S). *N* α -*P*-tosyl-L-lysine chloromethyl ketone (TLCK), a serine protease inhibitor, has been shown to protect β -cells from deleterious effects of IL-1 β (22). The site of action of TLCK appears to be at the proteasome complex (26S), although TLCK may have other sites of action.

In the present study, we provide evidence that IL-1 β -induced iNOS and COX-2 coexpression by pancreatic islets and the β -cell line RINm5F share a common signaling pathway in utilizing the proteasome complex (26S) and NF κ B. A protease inhibitor, TLCK, and a specific inhibitor of the proteasome complex (26S), MG 132, inhibit IL-1 β -induced iNOS and COX-2 expression at the level of enzymatic activity, mRNA, and protein. Both inhibitors, TLCK and MG 132, also block NF κ B activation and I κ B degradation. An understanding of the signaling pathway of IL-1 β -induced NO and PGE₂ production by β -cells will provide strategies of intervention to further evaluate the role of nitric oxide and PGE₂ in mediating β -cell dysfunction and destruction associated with autoimmune diabetes.

RESEARCH DESIGN AND METHODS

Male Sprague-Dawley rats (160–180 g) were purchased from Sasco (O'Fallon, MO). Collagenase type P was obtained from Boehringer Mannheim (Indianapolis, IN). Tissue culture medium (CMRL-1066), minimal essential medium (MEM), penicillin, streptomycin, Hanks' balanced salt solution, and L-glutamine were obtained from GIBCO Laboratories (Grand Island, NY). Fetal bovine serum was obtained from Hyclone (Logan, UT). RINm5F cells were obtained from the Washington University Tissue Culture Support Center. Human islets were a kind gift from Dr. David Scharp (Islet Isolation Core Facility, Washington University School of Medicine). *N*⁶-monomethyl-L-arginine (NMMA) acetate was obtained from Calbiochem (San Diego, CA), and IL-1 β (2×10^8 U/mg) was obtained from Cistron Biotechnology (Pine Brook, NJ). Tumor necrosis factor- α (TNF- α ; 1×10^8 U/mg) and γ -interferon (IFN- γ ; 2×10^7 U/mg) were obtained from Boehringer Mannheim. The cDNA probe for cyclophilin was a gift from Dr. Jeffrey Milbrandt (Washington University). [α -³²P]dCTP was obtained from Amersham (Arlington Heights, IL). Oligonucleotide labeling kits were from Pharmacia (Piscataway, NJ). ³⁵S-*trans*-labeled methionine (1117 Ci/mmol) was from ICN (Costa Mesa, CA), and rabbit iNOS antiserum was a gift from Dr. Thomas Misko (Monsanto Corporation). NF κ B consensus oligonucleotides (5'-GATCCGAGGGGACTTTCCTCCGCT-GGGGACTTTCAGG-3') and T4 polynucleotide kinase were obtained from Oncogene Science (Uniondale, NY). Rabbit antisera for NF κ B p50 and p65, I κ B α , and its control peptide were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The proteasome inhibitor MG 132 was a gift from Proscript (Cambridge, MA). In vitro transcription and ribonuclease protection assay kits and cyclophilin RNA probes were obtained from Ambion (Austin, TX). Rabbit COX-2 antisera and PGE₂ radioimmunoassay kits were from Cayman Chemical (Ann Arbor, MI).

Isolation of rat pancreatic islets. Islets were isolated from 12–15 male Sprague-Dawley rats by collagenase digestion as described previously (23). Iso-

lated islets were cultured overnight in complete CMRL-1066 medium containing 2 mmol/l L-glutamine, 10% (vol/vol) heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

Nitrite determination. Rat islets (200) were treated with 5 U/ml IL-1 β and various concentrations of TLCK or MG 132 and incubated for 24 h in 500 μ l of complete CMRL-1066. Human islets were incubated for 48 h at 37°C in complete CMRL-1066 before the initiation of experiments. Following this culture period, human islets were washed three times in complete CMRL-1066, counted (200 per condition), and placed in 24-well plates. Human islets were treated with cytokine mixtures (75 U/ml IL-1 β , 3.5 nmol/l TNF- α , and 750 U/ml IFN- γ) and various concentrations of MG 132 as indicated in the figure legends and incubated for 24 h. The culture supernatant was removed, and 50- μ l aliquots were then mixed with 50 μ l of Griess reagent (24). Nitrite production was determined at an absorbance of 540 nm using a Titertek Multiskan MCC/340 plate reader.

RNA isolation and Northern blot analysis. Following incubation of RINm5F cells ($\sim 70 \times 10^5$ in 10 ml of complete CMRL-1066) for 6 h with 10 U/ml IL-1 β and/or various concentrations of either TLCK or MG 132, cells were washed three times with phosphate-buffered saline (PBS, pH 7.4). Total RNA isolation and Northern blot analyses were performed as described previously (25).

Determination of iNOS and COX-2 protein expression. iNOS was immunoprecipitated from metabolically labeled islet cells as described previously (12). Briefly, islets (200 in 500 μ l complete CMRL-1066) were washed three times with 500 μ l methionine-deficient MEM (9 parts MEM without methionine:1 part MEM containing methionine) and incubated at 37°C for 5 h. Islets were then treated with 5 U/ml IL-1 β \pm 100 μ mol/l TLCK and 300 μ Ci of ³⁵S-methionine Trans-Label (ICN) and further incubated for 19 h. Islets were then isolated by centrifugation and washed. Then iNOS was immunoprecipitated using antiserum prepared against the COOH-terminal 27 amino acid of mouse macrophage iNOS (a kind gift from Dr. Thomas Misko).

In other experiments, iNOS and COX-2 expression by islets was determined by Western blot analysis. Islets (200 in 500 μ l of complete CMRL) were treated with IL-1 β \pm TLCK or MG 132 for 24 h as indicated in the figure legends. Islets were washed three times with PBS (pH 7.4) and solubilized in 20 μ l of SDS sample mix (0.25 mol/l Tris-HCl, 20% β -mercaptoethanol, and 4% SDS, 5 mmol/l EDTA, and 1 mmol/l EGTA) and 10 μ l dH₂O and boiled for 4 min, followed by the addition of 2 μ l of loading dye (0.05% bromophenol blue in 80% glycerol). SDS gel electrophoresis was carried out under standard conditions (26). Protein was transferred from the SDS gel to nitrocellulose membranes (Hoeffer, San Francisco, CA) under semidry transfer conditions (Millipore) at 120 milliamp for 40 min. Blots were blocked overnight at room temperature in 5% nonfat dry milk in TBST (20 mmol/l Tris, 500 mmol/l NaCl, pH 7.5, 0.1% Tween 20). iNOS blots were incubated for 2 h at room temperature with rabbit anti-mouse macrophage iNOS (Alexis Biochemicals, San Diego, CA) at a dilution of 1:1,000, washed four times in TBST, and then incubated for 90 min at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit antisera (Jackson ImmunoResearch, West Grove, PA) at a dilution of 1:7,000. The blots were then washed four times in TBST. For COX-2 blots, the same procedure was followed except that rabbit anti-mouse COX-2 (Cayman Chemical, Ann Arbor, MI) was used as a primary antibody. Both COX-2 and iNOS were detected by enhanced chemiluminescence using Hyperfilm and enhanced chemiluminescence reagents (Amersham).

PGE₂ determination. PGE₂ was measured by enzyme immunoassay as described previously (12) using reagents obtained from Cayman Chemical.

Ribonuclease protection assays. Islets were treated with 5 U/ml IL-1 β \pm various reagents for the indicated time periods as described in the figure legends. Total RNA was prepared by the method of Kwon et al. (25). COX-1, COX-2, and cyclophilin mRNA levels were determined by ribonuclease protection assay. Rat COX-1 and COX-2 cDNA fragments were subcloned into the *Nco* I and *Hind*III *Bam*HI sites of pGEM 5Z and pGEM 7Z, respectively (27). The resultant plasmids were linearized with *Sph* I and *Hind*III, respectively, and then used as templates for in vitro synthesis of ³²P-labeled antisense COX-1 and COX-2 RNA probes with [α -³²P]UTP (800 Ci/mmol from DuPont-NEN) and SP-6 RNA polymerase. The sizes of the transcribed RNA probes for COX-1 and COX-2 were 280 and 190 bases, respectively. Ribonuclease protection assays were carried out with ~ 5 μ g of total RNA and 1×10^5 cpm of the RNA probe/assay using a kit from Ambion (RPAIL) as described in the instructions from the supplier. The protected RNAs were separated by electrophoresis in 7.5 mol/l urea/8% acrylamide sequencing gels. Gels were dried and exposed to film at room temperature. The relative intensities of each protected fragment were determined by Phosphor Imager analysis (Molecular Dynamics, Sunny Vale, CA). COX values are expressed relative to a cyclophilin probe (Ambion).

Nuclear extract preparation and electrophoretic mobility shift analysis. RINm5F cells ($5\text{--}6 \times 10^7$) were pretreated for 30 min with 100 μ mol/l TLCK or 10 μ mol/l MG 132. Cells were then treated with 10 U/ml IL-1 β and incubated for an additional 30 min. Cells were washed three times with PBS (pH 7.4), and nuclear extracts were prepared by the method of Flanagan et al. (28). Double-stranded

synthetic oligonucleotide probes for NF κ B were end-labeled using [γ - 32 P]ATP and T4 polynucleotide kinase. Nuclear extracts (10 μ g) were incubated with oligonucleotide probes (60,000 cpm) for 35 min at room temperature, and protein-DNA complexes were resolved by polyacrylamide gel electrophoresis (29). Supershift assays were performed by adding 2 μ g of antibodies for NF κ B p65 or p50 subunits to the reaction mixture along with nuclear extracts and oligonucleotide probes.

I κ B α degradation. Islets (200) and RINm5F cells (5×10^5) were metabolically labeled as described above. Islets and RINm5F cells were then treated with IL-1 β for the indicated time periods as shown in the figure legends. Islets and cells were isolated by centrifugation and washed, and then I κ B α was immunoprecipitated using antiserum prepared against the COOH-terminal amino acids (297–317) of human I κ B α /MAD-3 (Santa Cruz Biotechnology, Santa Cruz, CA) by the method of Corbett et al. (12).

Statistical analysis. Statistical comparisons were made between groups using a one-way analysis of variance with significant differences ($P < 0.05$) evaluated using a Scheffe's F-test post-hoc analysis (Statview V 4.0, Abacus Concepts).

RESULTS

Effects of TLCK and MG 132 on iNOS expression. Protease activation has been previously implicated as an early signaling event associated with IL-1 β -induced inhibition of pancreatic β -cell function (22). To determine whether protease activity is required for IL-1 β -induced iNOS expression, we examined the effects of a serine protease inhibitor, TLCK, and a proteasome complex (26S) inhibitor, MG 132, on IL-1 β -induced nitrite formation by rat islets. IL-1 β induces approximately seven- to ninefold increases in nitrite (an oxidation product of nitric oxide) by rat islets (Fig. 1A and B, bar 2). Both TLCK and MG 132 inhibited IL-1 β -induced nitrite formation by rat islets in a concentration-dependent manner, with complete inhibition observed at 100 μ mol/l and 10 μ mol/l, respectively (Fig. 1A and B). A competitive inhibitor of nitric oxide synthase, NMMA (0.5 mmol/l), also completely inhibited IL-1 β -induced nitrite formation. TLCK (100 μ mol/l) and MG 132 (10 μ mol/l) in the absence of IL-1 β had no effect on nitrite levels (data not shown). TLCK (100 μ mol/l) and MG 132 (10 μ mol/l) also did not alter Trypan blue dye exclusion or [35 S]methionine uptake as a measure of protein synthesis by rat islets and RINm5F cells (data not shown), suggesting that these inhibitory effects produced by TLCK and MG 132 were not due to nonspecific cytotoxicity. In addition, MG 132 (up to 10 μ mol/l) alone had no effect on a basal or glucose-stimulated insulin secretion. Our initial findings show that MG 132 (5–10 μ mol/l) partially reverses the inhibitory effects of IL-1 β on glucose-stimulated insulin secretion by rat islets (data not shown).

To determine the site of action of TLCK and MG 132, the effects of these reagents on iNOS gene transcription were studied by Northern blot analysis. RINm5F cells were incubated with 10 U/ml IL-1 β in the presence and absence of various concentrations of TLCK or MG 132 for 6 h. Previous time course studies indicated that iNOS mRNA expression by RINm5F cells peaked after 6 h of treatment with IL-1 β (30). Both TLCK and MG 132 inhibited IL-1 β -induced iNOS mRNA expression in a similar dose-dependent manner consistent with their ability to block nitrite formation (Fig. 2A and B). Complete inhibition of iNOS mRNA expression was observed at 100 μ mol/l TLCK (Fig. 2A, lane 5) or in the range of 1–5 μ mol/l MG 132 (Fig. 2B, lanes 4 and 5). Consistent with their inhibitory effects on nitrite formation and iNOS mRNA expression, TLCK (100 μ mol/l) and MG 132 (~1–5 μ mol/l) also completely inhibited iNOS expression at the level of protein, as shown in Figure 3A and B by rat islets. Taken together, these results suggest that the sites of action of

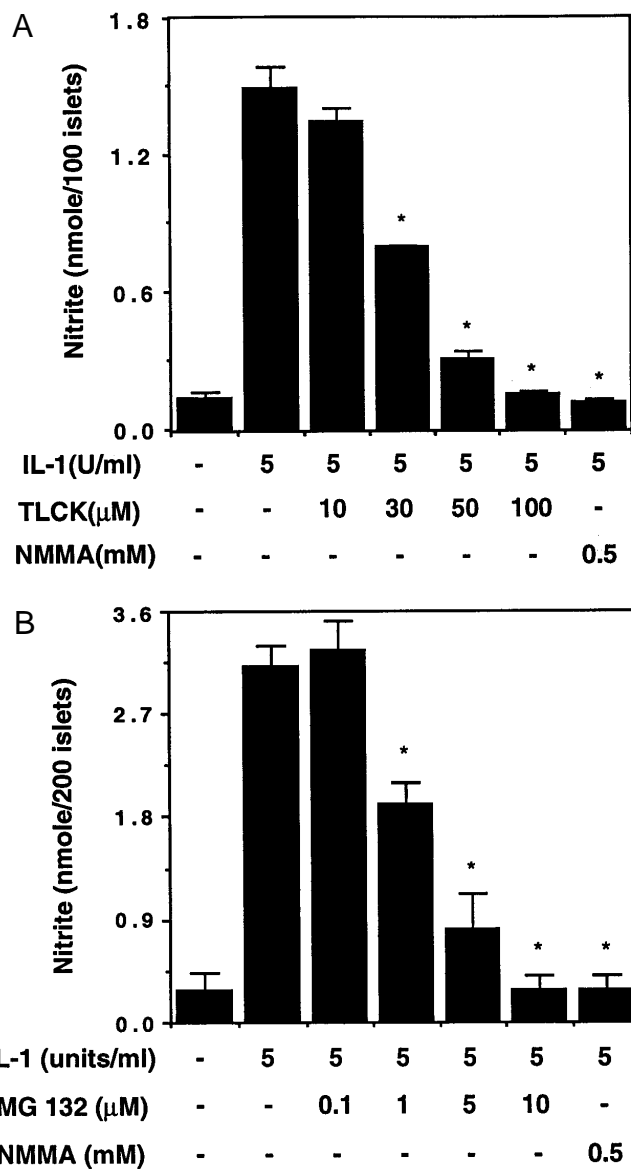


FIG. 1. Effects of TLCK and MG 132 on IL-1 β -induced nitrite production by rat islets. Rat islets (200) were treated with 5 U/ml IL-1 β and the indicated concentrations of TLCK (A) or MG 132 (B) for 24 h in 500 μ l of complete CMRL-106. As a control, 0.5 mmol/l NMMA, a competitive inhibitor of NO synthase, was incubated with IL-1 β . Nitrite production in the culture media was determined by the Griess reagent as described in METHODS. Results are the average of three individual experiments containing three replicates in each experiment. *Statistically significant inhibition of nitrite formation ($P < 0.001$), as compared with the IL-1 β activated group (determined by analysis of variance).

TLCK and MG 132 are prior to iNOS gene transcription.

Effects of TLCK and MG 132 on COX-2 expression. Next, we examined the effects of TLCK and MG 132 on IL-1 β -induced COX-2 expression by rat islets. TLCK (100 μ mol/l) and MG 132 (10 μ mol/l) significantly blocked PGE $_2$ production, the major enzyme product of COX-2, with inhibition of 80 and 100%, respectively (Table 1). We initially studied IL-1 β -mediated COX-1 and COX-2 gene transcription by rat islets using RNase protection assays. Based on

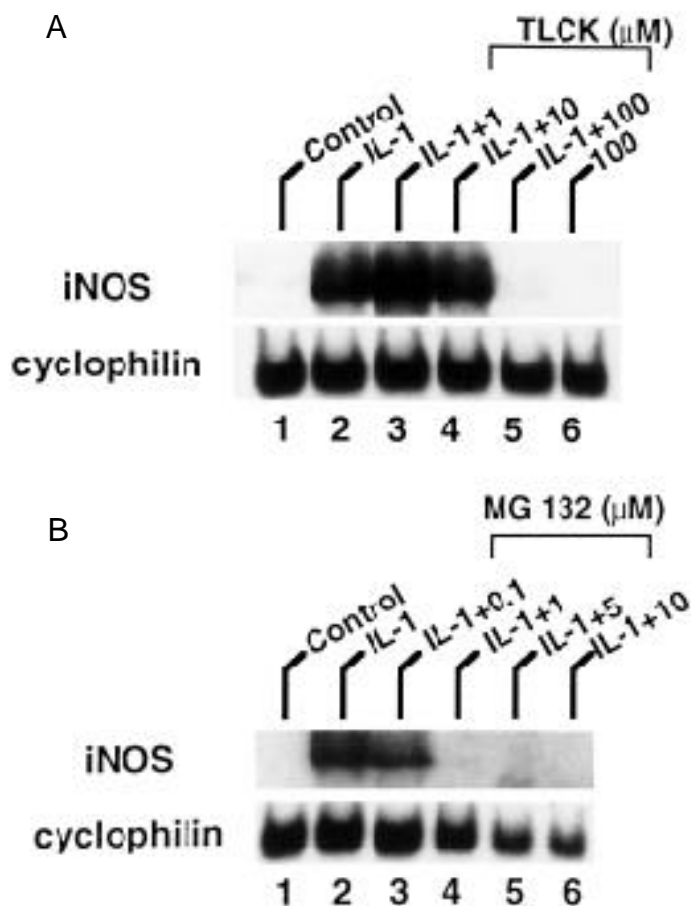


FIG. 2. Effects of TLCK and MG 132 on IL-1 β -induced iNOS mRNA expression by RINm5F cells. RINm5F cells were exposed to IL-1 β (10 U/ml) and/or various concentrations of TLCK (**A**) or MG 132 (**B**) for 6 h as indicated. Total cellular RNA (50 μ g) was analyzed by Northern blot analysis using iNOS and cyclophilin DNA probes. Results are representative of three individual experiments.

our previous observations that IL-1 β induces NO and PGE₂ production in a similar time-dependent manner (12) and that iNOS mRNA peaks ~6 h after IL-1 β treatment (30), we incubated islets with 5 U/ml IL-1 β for 6 h. IL-1 β stimulated a significant increase in COX-2 mRNA level (Fig. 4, lane 2, upper panel), which was completely blocked by actinomycin D (1 μ mol/l) (Fig. 4, lane 3, upper panel). Treatment of islets with 10 μ mol/l cycloheximide significantly enhanced IL-1 β -induced COX-2 mRNA expression (Fig. 4, lane 4, upper panel), probably because of increased levels of NF κ B activation resulting from the lack of newly synthesized I κ B α . COX-1 mRNA expression by islets was constant regardless of IL-1 β treatment (Fig. 4, lower panel), except under conditions of actinomycin D treatment (Fig. 4, lane 3, lower panel).

Both TLCK (100 μ mol/l) and MG 132 (10 μ mol/l) significantly inhibited IL-1 β -induced COX-2 mRNA expression (Fig. 5A, lanes 3 and 4, upper panel), but they did not significantly affect COX-1 mRNA levels. Figure 5B shows quantitation of COX-1 and COX-2 mRNA levels normalized with cyclophilin. Both TLCK (100 μ mol/l) and MG 132 (10 μ mol/l) also completely inhibited COX-2 protein expression, deter-

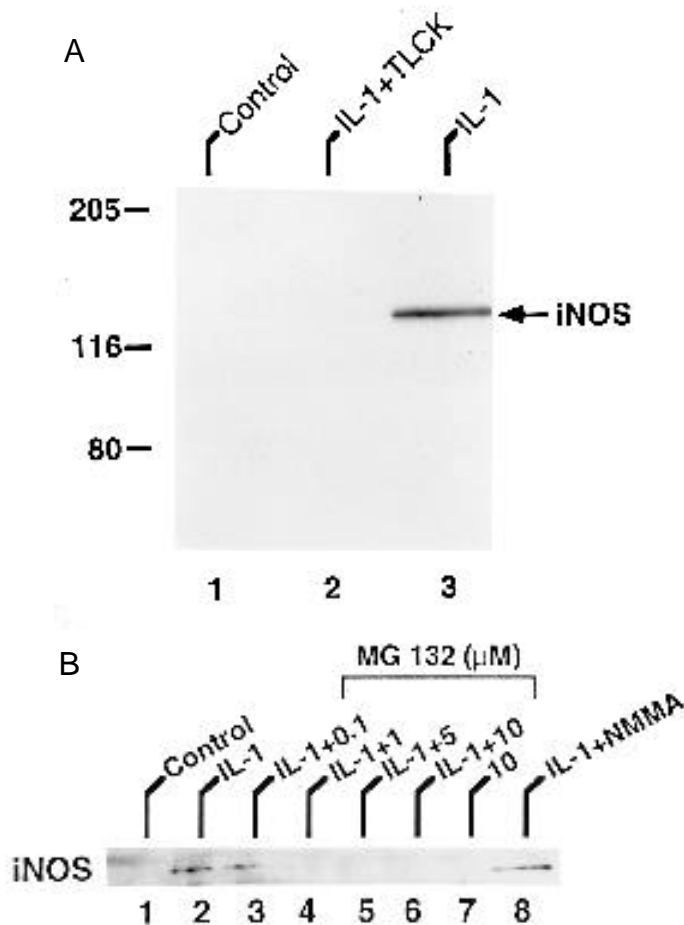


FIG. 3. Effects of TLCK and MG 132 on IL-1 β -induced expression of iNOS by rat islets. iNOS was immunoprecipitated from [³⁵S]methionine metabolically labeled islets treated with 5 U/ml IL-1 β in the presence or absence of 100 μ mol/l TLCK (**A**) or various concentrations of MG 132 (**B**). Immunoprecipitated samples were separated on 8% polyacrylamide gels, and proteins were visualized by fluorography. Results are representative of three individual experiments.

mined by Western blot analysis using rabbit antisera for human COX-2 (Fig. 6).

Effects of MG 132 on human islets. We also examined the effects of the specific proteasome complex inhibitor, MG 132, on cytokine-induced nitrite and PGE₂ production by human islets. Human islets were treated with a combination of cytokines (IL-1 β , TNF- α , and IFN- γ) and various concentrations of MG 132 or NMMA (0.5 mmol/l) as shown in Fig. 7. MG 132 inhibited cytokine-induced nitrite production (■) by human islets in a similar concentration-dependent manner, with complete inhibition at ~1 μ mol/l MG 132 as observed previously with rat islets. MG 132 also blocked PGE₂ production with complete inhibition at ~1–5 μ mol/l MG 132 (▣). Treatment of human islets with NMMA (0.5 mmol/l) in the presence of cytokines significantly inhibited PGE₂ production, which is consistent with previous findings that NO activates COX-2 enzymatic activity (12,13).

Effects of TLCK and MG 132 on NF κ B activation. We recently reported that activation of the transcription factor NF κ B mediates IL-1 β -induced NO generation by rat islets and RINm5F cells (25). To determine if TLCK and MG 132 inhibit

TABLE 1

Effects of TLCK or MG 132 on IL-1-induced PGE₂ production by rat islets

Treatment	PGE ₂ (ng · 200 islets ⁻¹ · 24 h ⁻¹)
Control	15.0 ± 3.6
IL-1 (5 U/ml)	115.7 ± 12.4
IL-1 + TLCK (100 μmol/l)	36 ± 4.6*
TLCK alone	22.4 ± 5.0
IL-1 + MG 132 (10 μmol/l)	14.7 ± 2.2*
MG 132 alone	13.5 ± 3.6

Data are means ± SE. Groups of islets (220) were cultured at 37°C for 24 h in 500 μl of CMRL-1066 or in CMRL-1066 containing 5 U/ml IL-1 ± TLCK (100 μmol/l) or MG 132 (10 μmol/l). The culture medium was removed, and PGE₂ was determined by enzyme immunoassay as stated in METHODS. Results are the average of four individual experiments. *Statistically significant inhibition of PGE₂ levels ($P < 0.001$), as compared with the IL-1-activated group, were determined by analysis of variance.

iNOS and COX-2 gene transcription by blocking NFκB activation, the effect of TLCK and MG 132 on IL-1β-induced NFκB translocation by RINm5F cells was studied (Fig. 8A and B). Our previous time course studies demonstrated that IL-1β-induced NFκB activation is detected as early as 15 min, peaks between 30 and 60 min, and is sustained for up to 3 h after IL-1β treatment (25). Therefore, following the pretreatment with TLCK (100 μmol/l) or MG 132 (10 μmol/l) for 30 min, RINm5F cells were treated with IL-1β for 30 min. IL-1β induced translocation of NFκB to the nucleus (Fig. 8A and B, lane 2), and both TLCK and MG 132 completely blocked this translocation (Fig. 8A and B, lane 3). TLCK or MG 132 alone did not affect translocation of NFκB (Fig. 8A and B, lane 4). Specificity of the nucleoprotein complex was demonstrated by supershift assays using antibodies against NFκB subunits p50 and p65 (Fig. 8C). Antibodies against NFκB subunits shifted NFκB DNA-protein complexes to more slowly migrating species (Fig. 8C, lanes 2 and 3). The supershift assays also suggest that the components of the complex consist of heterodimers of p50 and p65.

Effects of TLCK and MG 132 on IκBα degradation. Phosphorylation of IκBα and its subsequent degradation has been shown to activate NFκB in other cell systems (31,32). To test whether TLCK and MG 132 inhibit NFκB activation by blocking IκBα degradation, we initially studied IL-1β-induced IκBα degradation by immunoprecipitating IκBα from metabolically labeled islets or RINm5F cells using rabbit IκBα antiserum prepared against the COOH-terminal 21 amino acids of human IκBα/MAD-3. IκBα degradation was detected at 15 and 30 min after IL-1β treatment (Fig. 9, lanes 2 and 3). The addition of the peptide used to raise IκBα antiserum completely blocked immunoprecipitation of IκBα from rat islets (Fig. 9, right panel). Pretreatment of cells with MG 132 (10 μmol/l) for 30 min completely inhibited IL-1β-induced IκBα degradation, whereas TLCK (100 μmol/l) inhibited only partially (Fig. 10A, lanes 3 and 5, respectively). Figure 10B shows quantitation of IκBα degradation by laser densitometry from this study. MG 132 or TLCK alone did not affect IκBα levels (Fig. 10A and B, lanes 4 and 6, respectively).

DISCUSSION

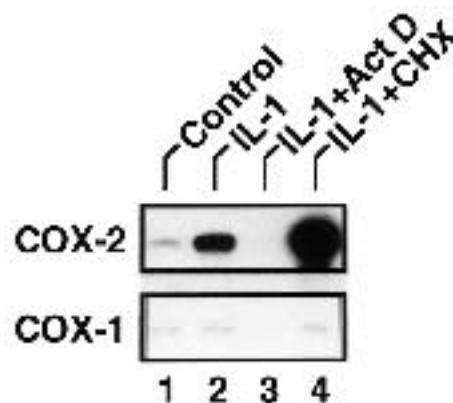


FIG. 4. Effects of actinomycin D and cycloheximide on IL-1β-induced COX-1 and COX-2 mRNA expression by rat islets. Islets were treated with 5 U/ml IL-1β ± 1 μmol/l actinomycin D or 10 μmol/l cycloheximide for 6 h at 37°C. Islets were washed three times with PBS (pH 7.4), and total RNA was isolated. Ribonuclease protection assays were carried out with ~5 μg of total RNA and 1 × 10⁵ cpm of the RNA probe/assay as described in METHODS. Results are representative of three individual experiments.

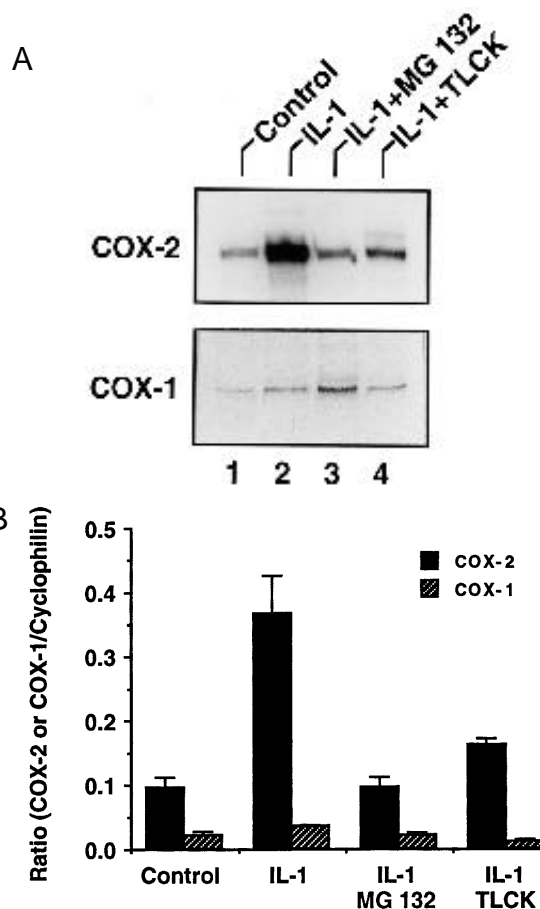


FIG. 5. Effects of MG 132 and TLCK on IL-1β-induced COX-1 and COX-2 mRNA expression by rat islets. **A:** Rat islets were treated with 5 U/ml IL-1β ± MG 132 (10 μmol/l) or TLCK (100 μmol/l) for 6 h at 37°C. Islets were washed three times with PBS (pH 7.4), and total RNA was isolated. COX-1, COX-2, and cyclophilin mRNA levels were determined by ribonuclease protection assays as described in METHODS. Results are representative of two individual experiments. **B:** The relative intensities of each protected fragment were determined by Phosphor Imager analysis. COX values are expressed relative to cyclophilin. Results are the average of two individual experiments.

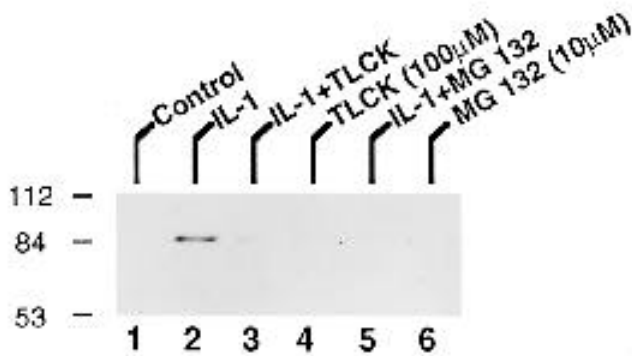


FIG. 6. Effects of TLCK and MG 132 on IL-1 β -induced COX-2 expression by rat islets. Rat islets (200 in 500 μ l of CMRL-1066) were treated with 5 U/ml IL-1 β \pm TLCK (100 μ mol/l) or MG 132 (10 μ mol/l) for 24 h at 37°C. Islets were washed, solubilized in SDS sample buffer, and processed for Western blot analysis as described in METHODS. Results are representative of four individual experiments.

This study demonstrates that the serine protease inhibitor TLCK and a specific inhibitor of the proteasome complex (26S), MG 132, inhibit IL-1 β -induced iNOS and COX-2 coexpression at the level of enzymatic activity, mRNA, and protein by rat islets and the β -cell line RINm5F. Both inhibitors also block IL-1 β -induced NF κ B activation and I κ B degradation. Activation of the proteasome complex and NF κ B seems to be a common sequence of events in the signaling pathways of IL-1 β -induced iNOS and COX-2 coexpression by rat islets. MG 132 also blocks cytokine-induced nitrite and PGE₂ formation by human islets, suggesting that activation of the proteasome complex (26S) and NF κ B is also involved in iNOS and COX-2 coexpression by human islets.

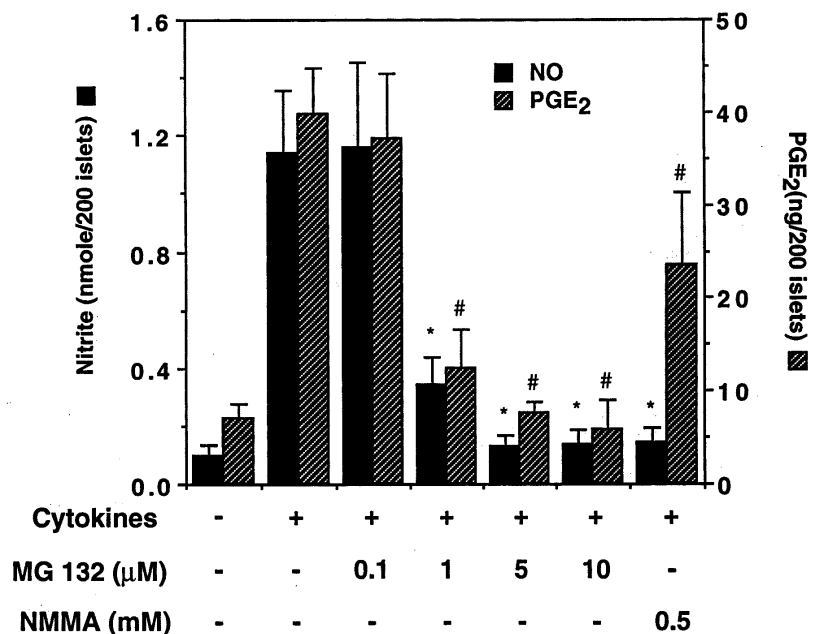
The site of action of MG 132 appears to be at the proteasome complex (26S), as demonstrated by its ability to block I κ B α degradation (Fig. 10A and B). The site of action of TLCK seems slightly different from MG 132 because TLCK

prevents I κ B α degradation only partially compared with MG 132. TLCK has been reported to have multiple sites of action in other systems that include modification of NF κ B and inhibition of its DNA binding activity (33). Serine protease inhibitors, including TLCK and *N*- α -tosyl-L-phenylalanine chloromethyl ketone (TPCK), were also shown to block phosphorylation of I κ B α and p105 (33–35), suggesting that these protease inhibitors may possibly prevent NF κ B activation through their inhibition of I κ B α phosphorylation rather than through its degradation.

Protease involvement in IL-1 β -mediated dysfunction of pancreatic islets has been reported by Welsh et al. (22). TLCK was shown to counteract the deleterious effects of IL-1 β on pancreatic islet functions, including inhibition of glucose oxidation, insulin biosynthesis, insulin content, and insulin release (22). In subsequent reports, Saldeen and Welsh (36) demonstrated that TLCK blocks IL-1 β -induced activation of NF κ B by RINm5F cells based on electrophoretic mobility shift assays. Our present study shows that TLCK inhibits I κ B α degradation, and this is correlated with NF κ B activation, also based on electrophoretic mobility shift assays. TLCK appears to protect β -cell function by blocking the formation of NO and cyclooxygenase metabolites. TLCK and its analog, TPCK, were also shown to block cytotoxic functions of immune cells, including superoxide anion production, cytokine release, cell-mediated cytolysis, and NO-related macrophage functions, by blocking NF κ B activation (37).

Human islets, unlike rat islets, require a combination of cytokines (IL-1 β , TNF- α , and IFN- γ) to stimulate NO and prostaglandin production (14,38). Cytokine-induced expression of iNOS mRNA by human islets is delayed compared with that by rat islets (12 vs. 3 h; 14,30). The cellular signaling mechanisms by which cytokines induce the expression of iNOS and COX-2 by human islets are not completely understood. Corbett et al. (14) have recently reported that cytokine-induced iNOS and COX-2 expression by human islets involves activation of tyrosine kinase(s), based on studies using genistein and herbimycin A, inhibitors of tyrosine kinase with dif-

FIG. 7. Effects of MG 132 on cytokine-induced nitrite and PGE₂ production by human islets. Human islets (200 in 500 μ l complete CMRL-1066) were treated with mixtures of cytokines (75 U/ml IL-1 β , 3.5 nmol/l TNF- α , and 750 U/ml IFN- γ) \pm various concentrations of MG 132 (0.1–10 μ mol/l) for 24 h. At the end of the incubation period, supernatants were collected and the media content of nitrite and PGE₂ was determined. Results are the average of four individual experiments. Statistically significant inhibition of cytokine-induced nitrite (*) and PGE₂ (#) levels ($P < 0.001$) by MG 132, as compared with the group treated with cytokines alone, was determined by analysis of variance.



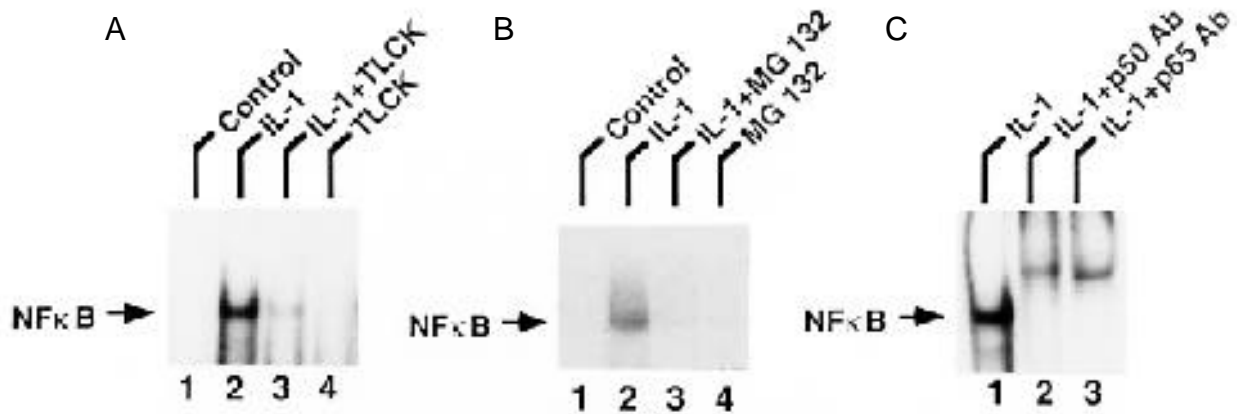


FIG. 8. Effects of TLCK and MG 132 on IL-1 β -induced translocation of NF κ B from cytosol to the nucleus in RINm5F cells. *A* and *B*: RINm5F cells ($5\text{--}6 \times 10^7$) were pretreated with 100 $\mu\text{mol/l}$ TLCK or 10 $\mu\text{mol/l}$ MG 132 for 30 min, followed by the addition of 10 U/ml IL-1 β and an additional 30-min incubation. Nuclear extracts were prepared, and electrophoretic mobility shift assays were performed as described in METHODS. The arrows denote a specific IL-1 β inducible NF κ B complex. *C*: Supershift of NF κ B complexes with antisera specific to NF κ B p50 and p65 subunits. Specific antiserum (2 μg) was added to binding reactions, and electrophoresis was performed as described in METHODS. Results are representative of three individual experiments.

ferent modes of action. In the present study, we show that the signaling pathways of cytokine-induced production of NO and PGE₂ appear to involve activation of the proteasome complex and the transcription factor NF κ B (Fig. 10). In addition, Flodstrom et al. (39) have also reported that NF κ B mediates cytokine-induced iNOS expression by human islets of Langerhans. Although there are differences in requirements for the initiating signals in human islets (where a combination of three cytokines is required) and rat islets (where IL-1 β alone is effective), there appear to be several common steps in the signal transduction pathways of NO and PGE₂ production by pancreatic islets from these two species.

Autoimmune diabetes is characterized by selective destruction of insulin-secreting β -cells from the islets of Langerhans. In vitro studies suggest that cytokine-induced production of proinflammatory agents, such as NO and prostaglandins, may participate in dysfunction and destruction of β -cells associated with autoimmune diabetes (12,38,40). This study suggests that the signaling pathways of cytokine-induced iNOS and COX-2 coexpression are tightly associated. Not only do the signaling pathways of iNOS and COX-2 expression share some intermediate steps, but NO also stimulates COX-2 activity (12,13). The possible roles of NO in β -cell dysfunction and destruction associated with autoimmune diabetes have been extensively studied, but the effects of cyclooxygenase

metabolites in β -cell function are not clear. In HIT cells, PGE₂ has been shown to inhibit glucose-induced insulin secretion, and inhibitors of PGE₂ synthesis augment this event (40,41). Specific mechanisms of the inhibitory action of PGE₂ on β -cell function are unknown. Cyclooxygenase metabolites, in vivo, are speculated to be involved in vasodilatation of local vessels at the site of injury or by chemotactic processes of immune cells. Further studies are required to understand the roles of the free radical NO and proinflammatory prostaglandins in autoimmune diabetes.

In summary, a schematic model for IL-1 β -induced iNOS and COX-2 coexpression by pancreatic β -cells is proposed in Fig. 11. In this scheme, interaction of IL-1 β with its specific type 1 receptor (IL-1 β R) located on the β -cell surface stimulates both postreceptor tyrosine kinase activity and the proteasome complex (26S), resulting in I κ B α phosphorylation and its subsequent degradation. This modification of I κ B α then allows dissociation of NF κ B from I κ B α and its translocation from the cytosol to the nucleus. NF κ B then binds to its consensus binding sites located in the upstream region of iNOS and COX-2 genes and activates transcription, translation, and enzymatic activity. The identification of a requirement for proteasome activity and activation of NF κ B in the signaling pathway of IL-1 β -induced NO and PGE₂ production by β -cells provides a strategy of intervention to further eval-

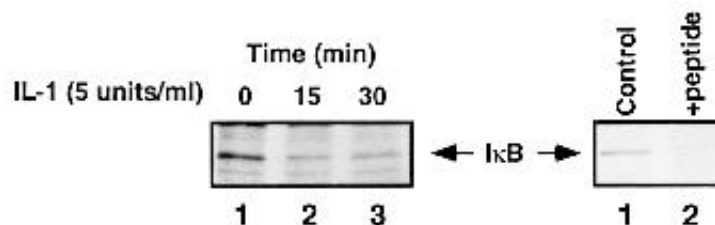


FIG. 9. IL-1 β -induced I κ B α degradation by rat islets. I κ B α was immunoprecipitated from metabolically labeled rat islets. Rat islets (200) were incubated in 500 μl methionine-deficient MEM (9 parts MEM without methionine:1 part MEM containing methionine) for 5 h at 37°C. After 5-h incubation, 300 μCi of [³⁵S]methionine Trans-Label was added and further incubated for 19 h. Islets were then treated with 5 U/ml IL-1 β for the indicated time periods. Islets were isolated by centrifugation and washed, and I κ B α was immunoprecipitated as described in METHODS. As a control, a peptide (10 μg) used to raise I κ B α antiserum was included to compete endogenous I κ B α . Results are representative of three individual experiments.

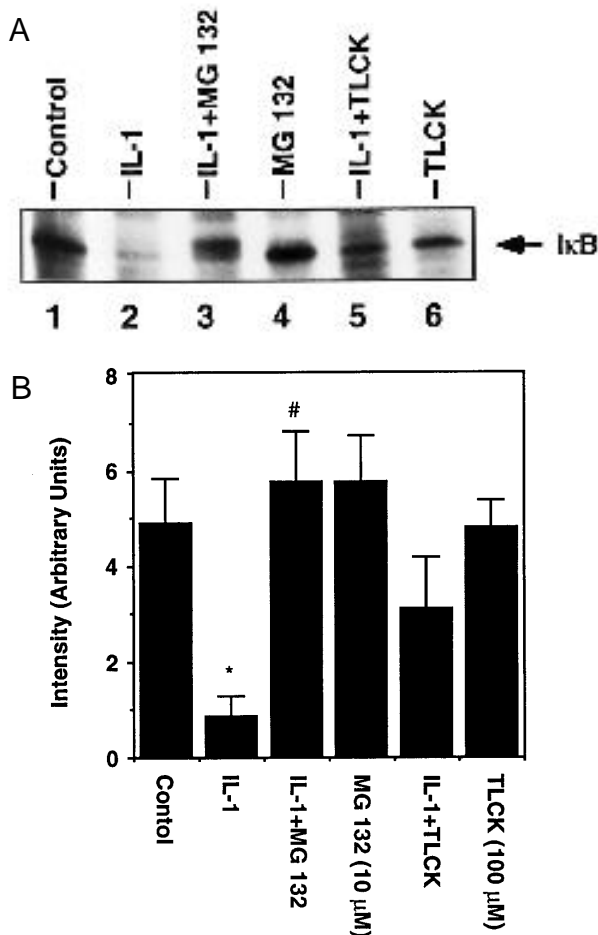


FIG. 10. Effects of MG 132 and TLCK on IL-1 β -induced I κ B α degradation by RINm5F cells. **A:** Metabolically labeled RINm5F cells (5×10^5) were pretreated with MG 132 (10 μ M) or TLCK (100 μ M) for 30 min, followed by the addition of 10 U/ml IL-1 β and an additional incubation for 30 min. I κ B α was then immunoprecipitated as described in Fig. 9. **B:** Quantitation of I κ B α by laser densitometry. Statistically significant IL-1 β -induced I κ B α degradation ($P < 0.009$; *) and its protection by MG 132 ($P < 0.005$; #) were determined by analysis of variance. Results are the average of four individual experiments.

uate the role of NO and PGE₂ in autoimmune diabetes.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants DK-06181 (M.L.M.), DK-34338 (J.T.), and T32-DK007296, Career Development Award from the Juvenile Diabetes Foundation International (J.A.C.), and a Lucille P. Markey Pathway postdoctoral fellowship (G.K.).

We would like to thank Connie Marshall, Joan Fink, and Mary Mueller for expert technical assistance.

REFERENCES

- Gepts W: Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* 14:619-633, 1965
- Nakhoda AF, Like AA, Chappel CI, Wei CN, Marliss EB: The spontaneously diabetic Wistar rat (the BB rat): studies prior to and during development of the overt syndrome. *Diabetologia* 14:199-207, 1978
- Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K, Tochino Y: Breeding of a non-obese, diabetic strain of mice. *Exp Anim* 29:1-13, 1980
- Mandrup-Poulsen T, Bendtzen K, Nielsen JH, Bendixen G, Nerup J: Cytokines cause functional and structural damage to isolated islets of Langerhans. *Allergy* 40:424-429, 1985

IL-1-induced iNOS and COX-2 Expression by Rat Pancreatic β -cells

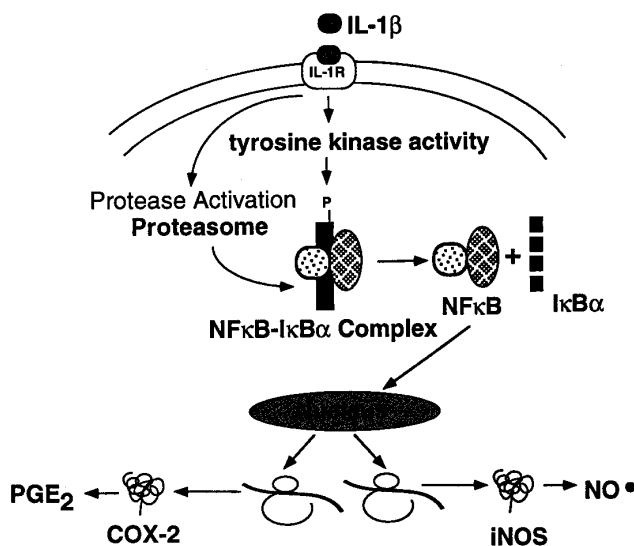


FIG. 11. A proposed schematic of IL-1 β -induced iNOS and COX-2 expression by rat pancreatic β -cells.

- Mandrup-Poulsen T, Bendtzen K, Nerup J, Egeberg J, Nielsen JH: Mechanisms of pancreatic islet cell destruction. *Allergy* 41:250-259, 1986
- Corbett JA, Wang JL, Misko TP, Zhao W, Hickey WF, McDaniel ML: Nitric oxide mediates IL-1 β -induced islet dysfunction and destruction: prevention by dexamethasone. *Autoimmunity* 15:145-153, 1993
- Southern C, Schulster D, Green IC: Inhibition of insulin secretion by interleukin-1 β and tumor necrosis factor- α via an L-arginine-dependent nitric oxide generation mechanism. *FEBS Lett* 276:42-44, 1990
- Corbett JA, Lancaster JR, Sweetland MA, McDaniel ML: Interleukin-1 β -induced formation of EPR-detectable iron-nitrosyl complexed in islets of Langerhans. *J Biol Chem* 266:21351-21354, 1991
- Welsh N, Eizirik DL, Bendtzen K, Sandler S: Interleukin-1 β -induced nitric oxide production in isolated rat pancreatic islets requires gene transcription and may lead to inhibition of the Krebs cycle enzyme aconitase. *Endocrinology* 126:3167-3173, 1991
- Corbett JA, McDaniel ML: Does nitric oxide mediate autoimmune destruction of β -cells? Possible therapeutic interventions in IDDM. *Diabetes* 41:897-903, 1992
- Laychock SG, Modica ME, Cavanaugh CT: L-arginine stimulates cyclic guanosine 3', 5'-monophosphate formation in rat islets of Langerhans and RINm5F insulinoma cells: evidence for L-arginine:nitric oxide synthase. *Endocrinology* 129:3043-3052, 1991
- Corbett JA, Kwon G, Turk J, McDaniel ML: IL-1 β induces the co-expression of both nitric oxide synthase and cyclooxygenase by islets of Langerhans: activation of cyclooxygenase by nitric oxide. *Biochemistry* 32:13767-13770, 1993
- Salvemini D, Misko TP, Masferrer JL, Seibert K, Currie MG, Needleman P: Nitric oxide activates cyclooxygenase enzymes. *Proc Natl Acad Sci USA* 90:7240-7244, 1993
- Corbett JA, Kwon G, Marino MH, Rodi CP, Sullivan PM, Turk J, McDaniel ML: Tyrosine kinase inhibitors prevent cytokine-induced expression of iNOS and COX-2 by human islets of Langerhans. *Am J Physiol* 270:C1581-C1587, 1996
- Corbett JA, McDaniel ML: Intraislet release of interleukin-1 inhibits β -cell function by inducing β cell expression of inducible nitric oxide synthase. *J Exp Med* 181:559-568, 1995
- Porreca E, Reale M, Febbo CD, Gioacchino MD, Babacane RC, Castellani ML, Baccante G, Conti P, Cuccurullo F: Down-regulation of cyclooxygenase-2 (COX-2) by interleukin-1 receptor antagonist in human monocytes. *Immunology* 89:424-429, 1996
- Xie Q-W, Whisnant R, Nathan C: Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon γ and bacterial lipopolysaccharide. *J Exp Med* 177:1779-1784, 1993
- Herschman HR, Gilbert RS, Xie W, Luner S, Reddy ST: The regulation and role of TIS 10 prostaglandin synthase-2. *Adv Prostaglandin Thromboxane Leukot Res* 23:23-28, 1995

19. Palombella VJ, Rando OJ, Goldberg AL, Maniatis T: The ubiquitin-proteasome pathway is required for processing the NF κ B1 precursor protein and the activation of NF κ B. *Cell* 78:773-785, 1994
20. Hershko A, Ciechanover A: The ubiquitin system for protein degradation. *Annu Rev Biochem* 61:761-807, 1992
21. Jentsch S: The ubiquitin-conjugation system. *Annu Rev Genet* 26:179-207, 1992
22. Welsh N, Bendzen K, Sandler S: Influence of protease on inhibitory and stimulatory effects of interleukin 1 β on β -cell function. *Diabetes* 40:290-294, 1991
23. McDaniel ML, Colca JR, Kotagal N, Lacy PE: A subcellular fractionation approach for studying insulin release mechanisms and calcium metabolism in islets of Langerhans. *Methods Enzymol* 98:182-200, 1983
24. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR: Analysis of nitrate, nitrite, and [¹⁵N]nitrate in biological fluids. *Anal Biochem* 126:131-138, 1982
25. Kwon G, Corbett JA, Rodi CP, Sullivan P, McDaniel ML: IL-1 β -induced nitric oxide synthase expression by rat pancreatic β -cells: evidence for the involvement of NF κ B in the signaling mechanism. *Endocrinology* 136:4790-4795, 1995
26. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685, 1970
27. Seibert K, Zhang Y, Leahy K, Hauser S, Masferrer J, Perkins W, Lee L, Isakson P: Pharmacological and biochemical demonstration of the role of cyclooxygenase 2 in inflammation and pain. *Proc Natl Acad Sci USA* 91:12013-12017, 1994
28. Flanagan WM, Corthesy B, Bram RJ, Crabtree GR: Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin. *Nature* 352:803-807, 1991
29. Sen R, Baltimore D: Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* 46:705-716, 1986
30. Kwon G, Corbett JA, McDaniel ML: Interleukin-1-induced Fos and Jun do not regulate inducible nitric oxide synthase in rat islets of Langerhans and RINm5F cells. *Endocrinology* 137:825-830, 1996
31. Scherer DC, Brockman JA, Chen Z, Maniatis T, Ballard DW: Signal-induced degradation of I κ B α requires site-specific ubiquitination. *Proc Natl Acad Sci USA* 92:11259-11263, 1995
32. Traenckner EB-M, Pahl HL, Henkel T, Schmidt KN, Wilk S, Baeuerle PA: Phosphorylation of human I κ B- α on serines 32 and 36 controls I κ B- α proteolysis and NF κ B activation in response to diverse stimuli. *EMBO J* 14:2876-2883, 1995
33. Finco TS, Beg AA, Baldwin AS Jr: Inducible phosphorylation of I κ B α is not sufficient for its dissociation from NF κ B and is inhibited by protease inhibitors. *Proc Natl Acad Sci USA* 91:11884-11888, 1994
34. Mellits KH, Hay RT, Goodbourn S: Proteolytic degradation of MAD3 (I κ B α) and enhanced processing of the NF κ B precursor p105 are obligatory steps in the activation of NF κ B. *Nucleic Acids Res* 21:5059-5066, 1993
35. Sun S-C, Elwood J, Beraud C, Greene WC: Human T cell leukemia virus type I tax activation of NF κ B. Rel involves phosphorylation and degradation of I κ B and RelA (p65)-mediated induction of the c-rel gene. *Mol Cell Biol* 14:7377-7384, 1994
36. Saldeen J, Welsh N: Interleukin-1 β induced activation of NF κ B in insulin producing RINm5F cells is prevented by the protease inhibitor N α -P-tosyl-L-lysine chloromethylketone. *Biochem Biophys Res Comm* 203:149-155, 1994
37. Kim H, Lee HS, Chang KT, Ko TH, Baek KJ, Kwon NS: Chloromethyl ketones block induction of nitric oxide synthase in murine macrophages by preventing activation of nuclear factor- κ B. *J Immunol* 154:4741-4748, 1995
38. Corbett JA, Sweetland MA, Wang J-L, Lancaster JR, McDaniel ML: Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. *Proc Natl Acad Sci USA* 90:1731-1735, 1993
39. Flodstrom M, Welsh N, Eizirik, DL: Cytokines activate the nuclear factor κ B (NF κ B) and induce nitric oxide production in human pancreatic islets. *FEBS Lett* 385:4-6, 1996
40. Robertson RP, Tsai P, Little SA, Zhang HJ, Walseth TF: Receptor-mediated adenylate cyclase-coupled mechanism for PGE₂ inhibition of insulin secretion in HIT cells. *Diabetes* 36:1047-1053, 1987
41. Seaquist ER, Walseth TF, Nelson DM, Robertson RP: Pertussis toxin-sensitive G protein mediation of PGE₂ inhibition of cAMP metabolism and phasic glucose-induced insulin secretion in HIT cells. *Diabetes* 38:1439-1445, 1989