IL-1β Induces the Coexpression of Both Nitric Oxide Synthase and Cyclooxygenase by Islets of Langerhans: Activation of Cyclooxygenase by Nitric Oxide†

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ABSTRACT: Autoimmune diabetes is characterized by an early infiltration of lymphocytes into and around islets, which is followed by selective destruction of the insulin-secreting β-cell. Cytokines released during this inflammatory reaction have been implicated as effector molecules which mediate β-cell destruction. In vitro treatment of rat islets with the cytokine IL-1β results in an inhibition of glucose-stimulated insulin secretion that is mediated by the overproduction of nitric oxide. IL-1β also stimulates the production of the cyclooxygenase (COX) product prostaglandin E_2 (PGE_2). In this study we have examined the effects of IL-1β on both inducible nitric oxide synthase (iNOS) and inducible cyclooxygenase (iCOX) expression, and the direct effects of nitric oxide on the activity of COX. Treatment of rat islets with 5 units/mL IL-1β induces a similar time-dependent production of both nitrite and PGE_2. IL-1β-induced nitrite and PGE_2 production is attenuated by the NOS inhibitor N^G-monomethyl-L-arginine (NMMA), but NMMA has no inhibitory effect on the expression of either iCOX or iNOS as determined by immunoprecipitation. Actinomycin D prevents IL-1β-induced iCOX and iNOS expression and the production of both nitrite and PGE_2 by islets, suggesting that mRNA transcription is required for IL-1β-induced expression of both iNOS and iCOX. The effects of exogenous arachidonic acid on both constitutive COX (cCOX) and iCOX activity were also investigated. Treatment of islets for 10 min with 50 μM arachidonic acid stimulated a ~10-fold increase in the production of PGE_2 by islets cultured with IL-1β before the arachidonic acid stimulation. NMMA significantly attenuated (70% inhibition) PGE_2 production induced by arachidonic acid, providing additional evidence for a direct effect of nitric oxide on the activity of iCOX. Arachidonic acid induced accumulation of PGE_2 by both untreated islets and islets treated with IL-1β and actinomycin D was also inhibited by NMMA (60% inhibition), suggesting that nitric oxide may also stimulate the activity of cCOX. These results show that IL-1β induces the coexpression of both iCOX and iNOS by islets and that nitric oxide stimulates the activity of both cCOX and iCOX, resulting in the overproduction of the inflammatory mediator PGE_2.

Nitric oxide has been implicated as an effector molecule that mediates both cytokine-induced β-cell dysfunction (Corbett & McDaniel, 1992; Rabinovitch, 1992) and macrophage-mediated β-cell destruction associated with autoimmune diabetes (Rabinovitch, 1992; Kroncke et al., 1991; Kolb & Kolb-Bachofen, 1992). Treatment of rat islets with IL-1β induces a concentration- and time-dependent inhibition of glucose-stimulated insulin secretion that is prevented by inhibitors of nitric oxide synthase (NOS),[1] such as NMMA (Welsh et al., 1991; Corbett et al., 1991), aminoguanidine (Corbett et al., 1992b), and nitro-L-arginine methyl ester (Southern et al., 1990). NMMA and aminoguanidine also prevent IL-1β-induced inhibition of insulin secretion by purified populations of primary rat β-cells (Corbett et al., 1992c) and cytokine-induced inhibition of insulin secretion by human islets (Corbett et al., 1993a). Nitric oxide is believed to inhibit insulin secretion by targeting intracellular iron–sulfur-containing mitochondrial enzymes, specifically islet cell aconitase (Welsh et al., 1991; Corbett et al., 1992c; Welsh & Sandler, 1992) and possibly the electron-transport chain at complexes I and II (Corbett et al., 1992a). Interactions of nitric oxide with non-heme iron–sulfur centers also have been demonstrated by electron spin resonance detection of iron–nitrosyl complexes in both rat islets treated with IL-1β (Corbett et al., 1991; Corbett et al., 1992a,b,c) and human islets treated with IL-1β, TNF-α, and IFN-γ (Corbett et al., 1993a).

Nitric oxide also interacts with heme-containing enzymes. Binding of nitric oxide to the heme moiety of soluble guanylate cyclase results in a potent activation of this enzyme and the production of cGMP (Moncada et al., 1990). Cyclooxygenase (COX) is a heme-containing enzyme catalyzing the first reaction in the biosynthetic pathway responsible for the production of inflammatory prostaglandins, prostacyclin, and thromboxane (Needleman et al., 1986). The interaction of nitric oxide with the heme moiety of COX was first demonstrated in 1972 (Yonetani et al.), and this interaction has been used as an electron paramagnetic resonance spectroscopic probe to determine the electromagnetic properties and heme coordination of COX (Karthein et al., 1987). Only recently

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[1] Abbreviations: NOS, nitric oxide synthase; iNOS, inducible nitric oxide synthase; cNOS, constitutive nitric oxide synthase; COX, cyclooxygenase; iCOX, inducible cyclooxygenase; cCOX, constitutive cyclooxygenase; PGE_2, prostaglandin E_2; NMMA, N^G-monomethyl-L-arginine; AA, arachidonic acid.

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has the effect of nitric oxide on the production of COX enzymatic products been investigated. Paradoxically, nitric oxide appears to both stimulate prostaglandin E_2 (PGE_2) production by murine macrophage cell line RAW 264.7, fetal fibroblasts (Salvemini et al., 1993), and norepinephrine-stimulated hypothalamic fragments (Rettori et al., 1992) and inhibit the production of COX products by resident liver macrophages (Stadler et al., 1993).

Autoimmune diabetes is characterized by a localized inflammatory reaction within the islet resulting in the selective destruction of insulin-secreting β-cells. We have previously shown that IL-1β stimulates the production of PGE_2 by isolated rat islets (Hughes et al., 1989), but the importance of this prostaglandin release remains unknown. In this study we have examined the potential interplay between nitric oxide and COX and present evidence demonstrating that IL-1β induces the expression of both iCOX and iNOS and that nitric oxide activates iCOX, resulting in the production of PGE_2 by islets.

EXPERIMENTAL PROCEDURES

Materials. Male Sprague–Dawley rats (150–200 g) were purchased from Sasco Inc. (O'Fallon, MO). Collagenase type P was obtained from Boehringer Mannheim (Indianapolis, IN). Minimal essential medium (MEM), CMRL-1066 tissue culture medium, heat-inactivated fetal bovine serum, L-glutamine, penicillin, and streptomycin were from Gibco Laboratories (Grand Island, NY). IL-1β was obtained from Cistron Biotechnology (Pine Brook, NJ). N^G-Monomethyl-L-arginine acetate was obtained from Calbiochem (San Diego, CA). Actinomycin D was from Sigma Chemical Co. (St. Louis, MO). ^{35}S-*trans*-labeled methionine (1117 Ci/mmol) was from ICN (Costa Mesa, CA). PGE_2 radioimmunoassay kits were from Cayman Chemical Co. (Ann Arbor, MI). Rabbit iCOX and iNOS rabbit antisera were gifts from Drs. Karen Seibert and Thomas Misko (Monsanto Corporate Research).

Islet Isolation. Islets were isolated from male Sprague–Dawley rats by collagenase digestion as described previously (McDaniel et al., 1983). In brief, on the day prior to each experiment, pancreases were inflated with Hank's buffered salts solution, and the tissue was isolated, minced, and digested with 7.5 mg of collagenase/pancreas for 7 min at 39 °C. Islets were separated on a Ficoll density gradient and then selected with a stereomicroscope to exclude any contaminating tissues. Islets were then cultured overnight under an atmosphere of 95% air and 5% CO_2 at 37 °C in complete CMRL-1066 medium (CMRL-1066 containing 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin).

Immunoprecipitation of Inducible NOS and COX. iNOS and iCOX were immunoprecipitated using methods described previously (Springer, 1991). In brief, 100 islets were washed three times in methionine-deficient MEM (MEM –Met; 9 parts MEM without methionine:1 part MEM containing methionine) and incubated for 5 h with 5 units/mL IL-1β, 0.5 mM NMMA, or 1 μM actinomycin D in 400 μL of MEM –Met. Three hundred microcuries of [^{35}S]methionine *trans*-label was added, and the islets were incubated for 13 additional hours at 37 °C. The islets were isolated by centrifugation, washed three times in 0.1 M PBS, and then lysed for 1 h at 4 °C in 1 mL of TSA (0.01 M Tris-HCl, pH 8.0, 0.14 M NaCl, and 0.025% NaN_3) containing 1.0% Triton X-100, 1.0% bovine serum albumin (BSA), 1 mg/mL aprotinin, 1 mM phenylmethanesulfonyl fluoride, 1 mg/mL leupeptin, and 1 mM iodoacetamide. Cellular debris was removed by centrifugation (30 min, 10 000*g*, 4 °C), and the supernatant was precleared for 1 h with 20 μL of cyanogen bromide activated protein A–Sepharose (protein A–Sepharose swelled in TSA; Sigma, St. Louis, MO) diluted 1:1 with dilution buffer (1.0% Triton X-100 and 1.0% BSA in TSA). The supernatant was cleared by centrifugation (1 min, 4 °C, 200*g*) and then divided into two equal 0.5-mL portions. Rabbit anti-iNOS (1:750 dilution) or rabbit anti-iCOX (1:750 dilution) antiserum and 40 μL of protein A–Sepharose were added to paired samples, and the lysates were incubated for 2 h at 4 °C. The protein A–Sepharose antibody complex was isolated by centrifugation and washed twice with dilution buffer (1 mL/wash), once with 1 mL of TSA, and once with 1 mL of 0.05 M Tris-HCl, pH 6.8. The protein A–Sepharose antibody complex was treated with 30 μL of SDS sample mix (0.25 M Tris-HCl, 20% β-mercaptoethanol, and 4% SDS) and boiled for 4 min to dissociate the protein A–Sepharose antibody complex. The supernatant was obtained by centrifugation, and the immunoprecipitates were analyzed on 10% SDS polyacrylamide gels (Laemmli, 1970) and visualized by fluorography.

PGE_2 and Nitrite Determinations. PGE_2, extracted by the method of Powell (1982), was measured by enzyme immunoassay as described previously (Pradelles et al., 1985). Nitrite release by islets was determined by mixing 50-μL portions of culture medium with 50 μL of Griess reagent (Green et al., 1982), and the absorbance at 540 nm was measured using a Titertek Multiskan MCC/340 plate reader.

Statistics. Statistical comparisons were made between groups using a one-way analysis of variance. Significant differences ($p < 0.05$) were evaluated with a post hoc analysis using Scheffe's *F*-test, as indicated (*).

RESULTS AND DISCUSSION

Time-Dependent Effects of IL-1β and NMMA on PGE_2 and Nitrite Accumulation by Rat Islets. Hughes et al. (1989) have previously demonstrated that treatment of rat islets with IL-1β results in a concentration- and time-dependent accumulation of the COX product PGE_2, and we have previously shown that IL-1β induces a similar time-dependent production of nitric oxide by rat islets (Corbett et al., 1992a). Because of the time-dependent nature of these two IL-1β-induced islet responses and the potential role of both nitric oxide and PGE_2 in β-cell destruction, we examined the possible interactions between nitric oxide and the production of PGE_2 by islets. Figure 1 shows that IL-1β-induced nitrite formation (panel a) occurs in a temporal manner similar to IL-1β-induced PGE_2 production by rat islets (panel b). The production of PGE_2 is attenuated, and the formation of nitrite is completely prevented, by treatment of islets with 0.5 mM NMMA in addition to IL-1β, suggesting that nitric oxide may activate the enzymatic activity of islet COX.

Effects of Actinomycin D and NMMA on iCOX and iNOS Expression. The activation of iCOX by nitric oxide is further demonstrated in Figure 2. Treatment of rat islets for 18 h with the transcriptional inhibitor actinomycin D (1 μM) prevents both IL-1β-induced nitrite formation and PGE_2 production (Figure 2). NMMA also prevents IL-1β-induced nitrite formation and inhibits 65% of IL-1β-induced PGE_2 production by islets. NMMA does not inhibit PGE_2 production by blocking IL-1β-induced expression of iCOX by islets. As shown in Figure 3, treatment of rat islets with IL-1β induces the expression of iCOX and iNOS (lane 2 for both proteins) as determined by immunoprecipitation of iCOX and iNOS from metabolically labeled islets. iNOS and iCOX are not expressed in control islets (lanes 1) or islets treated with actinomycin D (1 μM) in addition to IL-1β (lanes 3).

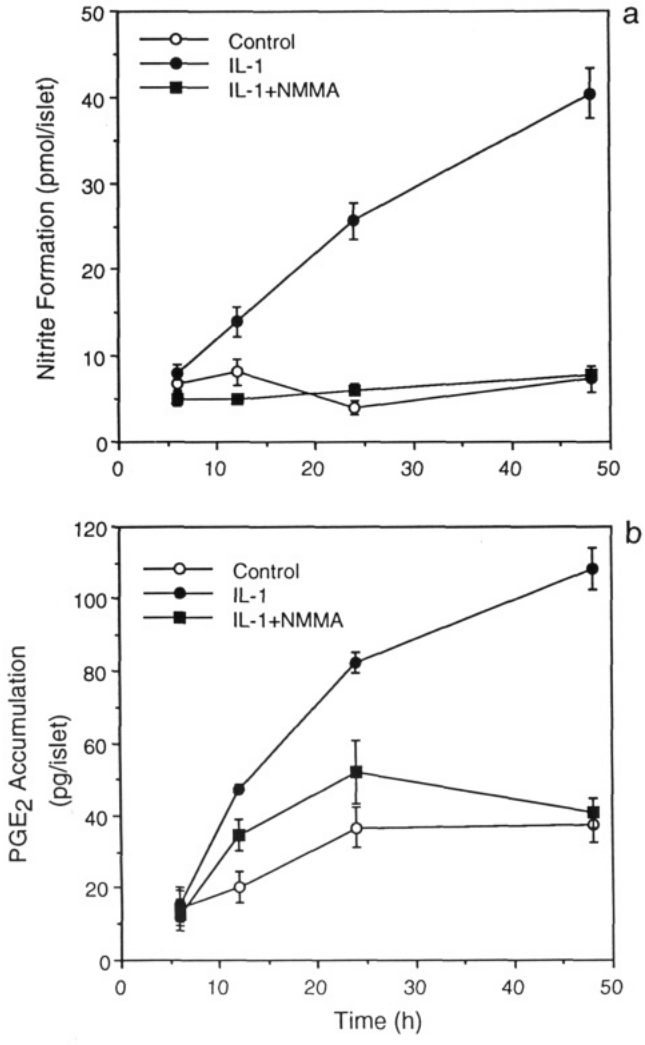

FIGURE 1: IL-1β induction of time-dependent production of both PGE_2 and nitrite by rat islets. Isolated islets were cultured for the indicated times in complete CMRL-1066 in the presence or absence of 5 units/mL IL-1β or IL-1β + 0.5 mM NMMA. At the indicated times, the medium was removed and both nitrite (a) and PGE_2 production (b) were determined. Results are the average ± SEM of two or five individual experiments containing two replicates per condition for both PGE_2 and nitrite, respectively.

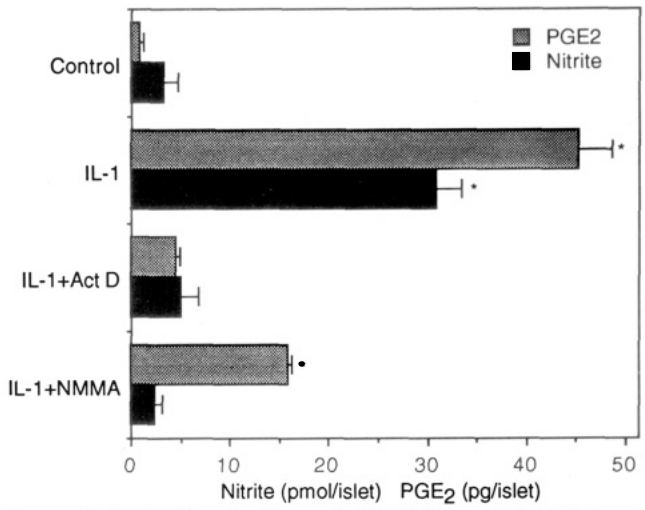

FIGURE 2: NMMA and actinomycin D inhibition of IL-1β-induced nitrite and PGE_2 production by rat islets. Islets were cultured for 18 h in complete CMRL-1066 tissue culture medium, or in CMRL-1066 containing 5 units/mL IL-1β, 1 μM actinomycin D, or 0.5 mM NMMA as indicated. The culture medium was isolated, and both PGE_2 and nitrite production were determined. Results are the average ± SEM of three individual experiments containing three replicates per condition.

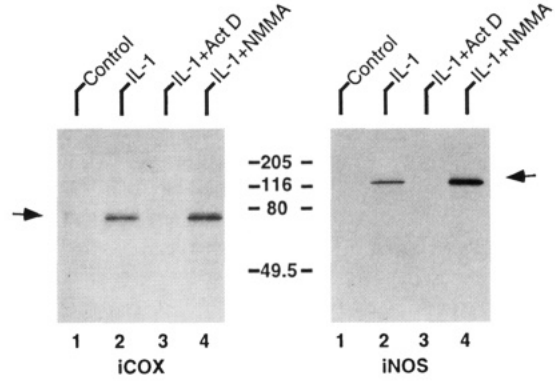

FIGURE 3: Effects of NMMA and actinomycin D on iNOS and iCOX expression by islets. Islets were metabolically labeled with 300 μCi of [^{35}S]methionine in the presence of 5 units/mL IL-1β (lanes 2), IL-1β and 1 μM actinomycin D (lanes 3), or IL-1β and 0.5 mM NMMA (lanes 4) or were untreated (lanes 1), and both iCOX and iNOS were immunoprecipitated from the same islets as stated in Experimental Procedures. Immunoprecipitates were separated by SDS–gel electrophoresis (10%) and visualized by fluorography. Results are representative of two individual experiments.

NMMA, which prevents nitrite and PGE_2 production by islets, does not inhibit the expression of either iCOX or iNOS (lanes 4 for both proteins) by rat islets treated for 18 h with IL-1β. In fact, islet expression of both iCOX and iNOS appears to be enhanced by treatment with NMMA in addition to IL-1β (lanes 4). The higher level of both iCOX and iNOS expression observed in the presence of IL-1β and NMMA is believed to result from NMMA-mediated inhibition of IL-1β-induced iNOS activity. IL-1β-induced nitric oxide formation inhibits cellular respiration and islet function, effects that are prevented by NMMA (Welsh et al., 1991; Corbett et al., 1992a,c). These findings demonstrate that IL-1β induces both iCOX and iNOS expression and that NMMA, which blocks both IL-1β-induced nitrite formation and PGE_2 production, does not inhibit the expression of either enzyme.

Effects of Nitric Oxide on Islet COX Activity. The ability of nitric oxide synthase inhibitors to block IL-1β-induced PGE_2 production without inhibiting the synthesis of iCOX suggests that nitric oxide may directly activate iCOX enzymatic activity. To confirm that nitric oxide is directly activating COX, a whole-cell assay system was used to examine the effects of exogenous arachidonic acid (50 μM) on PGE_2 production by islets treated for 24 h with or without IL-1β or IL-1β + actinomycin D. As shown in Figure 4, treatment of islets for 10 min with 50 μM arachidonic acid stimulates a ~10-fold increase in the level of PGE_2 produced by control islets and islets cultured for 24 h with IL-1β + actinomycin D. Treatment of these islets with NMMA inhibits PGE_2 release by 60% from control and IL-1β + actinomycin D treated groups. The control and IL-1β + actinomycin D treated islets represent the effects of NMMA on constitutive COX (cCOX) activity because iCOX has not been expressed (Figure 3, lanes 1 and 3). Activation of cCOX by nitric oxide is believed to result from arachidonic acid stimulated membrane depolarization and Ca^{2+} influx into islet β-cells (Ramanadham et al., 1992). Ca^{2+} influx results in the activation of Ca^{2+}- and calmodulin-dependent cNOS. Recent studies have established the presence of cNOS in islets (Laychock et al., 1991; Schmidt et al., 1992), and we have demonstrated by immunohistochemical localization that the insulin-secreting β-cell is a source of cNOS (Corbett et al., 1993b).

Figure 4 also demonstrates that nitric oxide activates iCOX activity in islets. Arachidonic acid (50 μM) stimulates a 30%

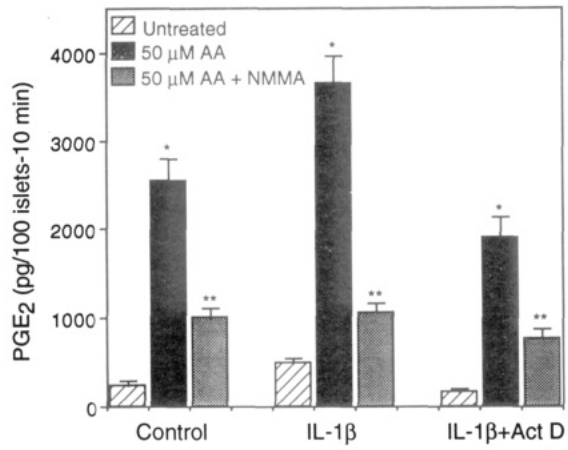

FIGURE 4: NMMA inhibition of arachidonic acid stimulated cCOX and iCOX activity by rat islets. Islets were cultured for 24 h in the presence or absence of 5 units/mL IL-1β or IL-1β and 1 μM actinomycin D. The islets were then isolated and washed, and PGE_2 production was determined following a 10-min incubation in the presence or absence of 50 μM arachidonic acid. Results are the average ± SEM of three individual experiments containing three replicates per condition. Statistical significance ($p < 0.05$) for arachidonic acid stimulated PGE_2 formation compared to untreated islets and NMMA inhibition of arachidonic acid stimulated PGE_2 production is indicated by * and **, respectively.

increase in the level of PGE_2 released from IL-1β-treated islets compared to control islets treated with 50 μM arachidonic acid. NMMA inhibits 70% of this arachidonic acid stimulated PGE_2 production by rat islets treated with IL-1β. These results show that both constitutive and inducible COX enzymatic activities are activated by nitric oxide. NMMA does not appear to directly inhibit the enzymatic activity of either iCOX or cCOX. Salvemini et al. (1993) have demonstrated that the inhibitory effect of NMMA on PGE_2 production by RAW 264.7 is reversed by the addition of excess L-arginine and that nitric oxide donor compounds stimulate PGE_2 production by iCOX, an effect that is prevented by hemoglobin.

In summary, the data presented in this study demonstrate that IL-1β induces the coexpression of both iNOS and iCOX by rat islets, which results in a similar time-dependent accumulation of both nitrite and PGE_2. Nitric oxide stimulates the formation of PGE_2 since NMMA, which completely prevents IL-1β-induced nitric oxide formation, attenuates the time-dependent accumulation of PGE_2. In addition, arachidonic acid stimulates PGE_2 production by rat islets, and this stimulation is inhibited by NMMA. Also, NMMA has no inhibitory effects on the expression of iCOX by rat islets.

Recently, we have obtained evidence that nitric oxide appears to participate in the development of autoimmune diabetes in the non-obese spontaneously diabetic (NOD) mouse (Corbett et al., 1993c). Also, Kleemann et al. (1993) have shown the presence of iNOS mRNA in the pancreata of prediabetic BioBreeding rats. These findings, together with our evidence demonstrating that cytokines induce the coexpression of iNOS and iCOX and that nitric oxide activates COX activity resulting in the production of proinflammatory prostaglandins, suggest that nitric oxide may participate in both the inflammatory response and the β-cell destruction observed in autoimmune diabetes.

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