Nitric oxide and cyclic GMP formation induced by interleukin 1 β in Islets of Langerhans

Evidence for an effector role of nitric oxide in islet dysfunction

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Treatment of pancreatic islets with interleukin 1 (IL-1) results in a time-dependent inhibition of glucose-stimulated insulin secretion which has recently been demonstrated to be dependent on the metabolism of L-arginine to nitric oxide. In this report IL-1 β is shown to induce the accumulation of cyclic GMP (cGMP) in a time-dependent fashion that mimics the time-dependent inhibition of insulin secretion by IL-1 β . The accumulation of cGMP is dependent on nitric oxide synthase activity, since *N*^G-monomethyl-L-arginine (a competitive inhibitor of nitric oxide synthase) prevents IL-1 β -induced cGMP accumulation. cGMP formation and nitrite production induced by IL-1 β pretreatment of islets are also blocked by the protein synthesis inhibitor, cycloheximide. The formation of cGMP does not appear to mediate the inhibitory effects of IL-1 β on insulin secretion since a concentration of cycloheximide (1 μ M) that blocks IL-1 β -induced inhibition of glucose-stimulated insulin secretion and nitric oxide formation does not prevent cGMP accumulation, thus dissociating the two events. By using e.p.r. spectroscopy, IL-1 β is shown to induce the formation of a *g* = 2.04 iron-nitrosyl feature in islets which is prevented by cycloheximide, demonstrating the requirement of protein synthesis for IL-1 β -induced nitric oxide formation. Iron-nitrosyl complex-formation by islets confirms that IL-1 β induces the generation of nitric oxide by islets, and provides evidence indicating that nitric oxide mediates destruction of iron-sulphur clusters of iron-containing enzymes. Consistent with the destruction of iron-sulphur centres is the finding that pretreatment of islets with IL-1 β results in an approx. 60 % inhibition of mitochondrial oxidation of D-glucose to CO₂. Inhibition of islet glucose oxidation appears to be mediated by nitric oxide since both NMMA and cycloheximide prevent IL-1 β -induced inhibition of glucose oxidation. These results show that IL-1 β -induced nitric oxide formation parallels the ability of IL-1 β to inhibit glucose-stimulated insulin secretion by islets, and that protein synthesis is required for IL-1 β -induced nitric oxide formation. These results also suggest that nitric oxide mediates IL-1 β -induced inhibitory effects on the pancreatic β -cell by functioning as an effector molecule responsible for the destruction of iron-sulphur centres of iron-containing proteins, resulting in an impairment of mitochondrial function.

INTRODUCTION

The molecular mechanism by which interleukin 1 β (IL-1 β) inhibits glucose-stimulated insulin secretion is at present poorly understood. Our laboratory has previously reported that RNA transcription and protein translation are required for IL-1 β to exert its inhibitory effects on insulin secretion. The mRNA transcriptional inhibitor, actinomycin D, and the protein translational inhibitor, cycloheximide, have been shown to prevent IL-1 β -induced inhibition of insulin secretion by islets (Hughes *et al.*, 1990). Recently, it has been reported that IL-1 β inhibits insulin secretion by a mechanism that is dependent on the metabolism of L-arginine to nitric oxide and L-citrulline by nitric oxide synthase. IL-1 β -induced inhibition of insulin secretion is blocked by *N*^G-nitro-L-arginine methyl ester (NAME; Southern *et al.*, 1990) as well as *N*^G-monomethyl-L-arginine (NMMA; Corbett *et al.*, 1991). NAME and NMMA are analogues of L-arginine which competitively inhibit nitric oxide synthase (Moncada *et al.*, 1991). IL-1 β has also been shown to induce the formation of nitrite, an end product of the L-arginine-dependent

pathway (Southern *et al.*, 1990; Corbett *et al.*, 1991). E.p.r. spectroscopy has recently been used to confirm the formation of nitric oxide by islets and provide evidence for the destruction of iron-containing enzymes. IL-1 β was shown to induce the formation of an e.p.r.-detectable *g* = 2.04 feature characteristic of iron-nitrosyl complex-formation, and the generation of this IL-1 β -induced complex by islets was blocked by NMMA (Corbett *et al.*, 1991).

Nitric oxide synthase catalyses the mixed functional oxidation of L-arginine to nitric oxide and L-citrulline (Marletta *et al.*, 1988; Palmer *et al.*, 1988; Bredt & Snyder, 1990; Stuehr *et al.*, 1991; Yui *et al.*, 1991). At least two isoforms of nitric oxide synthase have been described. One is constitutive, Ca²⁺- and calmodulin-dependent and found in many tissues including endothelial cells and brain (Bredt & Snyder, 1990; Moncada *et al.*, 1991). The other isoform of the enzyme is inducible by cytokines and lipopolysaccharide, Ca²⁺-independent and was originally isolated from activated macrophages (Stuehr *et al.*, 1991; Yui *et al.*, 1991). Nitric oxide serves numerous biological functions including involvement in signal-transduction mechan-

Abbreviations used: IL-1 β , human recombinant interleukin 1 β ; IL-1 α , human recombinant interleukin 1 α ; NMMA, *N*^G-monomethyl-L-arginine; NAME, *N*^G-nitro-L-arginine methyl ester; KRB, Krebs-Ringer bicarbonate buffer (25 mM-Hepes, 115 mM-NaCl, 24 mM-NaHCO₃, 5 mM-KCl, 1 mM-MgCl₂, 2.5 mM-CaCl₂, pH 7.4); cGMP, cyclic GMP.

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isms mediated by the ability of nitric oxide to activate guanylate cyclase directly resulting in cyclic GMP (cGMP) formation (Moncada *et al.*, 1991). Nitric oxide also functions as an effector molecule associated with immunological cytotoxic actions of activated macrophages (Hibbs *et al.*, 1987, 1990).

The formation of iron–dinitrosyl complexes has been described previously in activated macrophages (Lancaster & Hibbs, 1990) and target cells recognized by activated macrophages (Pellat *et al.*, 1990). The formation of these iron–nitrosyl complexes are believed to be the result of nitric-oxide-mediated destruction of iron–sulphur centres resulting in the loss of iron-containing enzyme activity (Reddy *et al.*, 1983; Lancaster & Hibbs, 1990). Nitric oxide has been implicated as the effector molecule mediating the inhibition of mitochondrial respiration of rat hepatocytes (Stadler *et al.*, 1991) and activated macrophages (Granger & Lehninger, 1982; Drapier & Hibbs, 1986), specifically at iron-containing enzymes (aconitase, NADH–ubiquinone oxidoreductase and succinate–ubiquinone oxidoreductase). The detection of an IL-1 β -induced intracellular iron–dinitrosyl complex by e.p.r. spectroscopy in islets suggests that IL-1 β -induced inhibition of glucose-stimulated insulin secretion may be mediated by the destruction of iron–sulphur centres resulting in an inhibition of mitochondrial iron-containing enzyme activity (Corbett *et al.*, 1991). Consistent with this notion is the observation that IL-1 β -induced inhibition of glucose-stimulated insulin secretion by islets is believed to be the result of a specific impairment of mitochondrial oxidation of D-glucose to CO₂ (Sandler *et al.*, 1987, 1989; Eizirik, 1988). The absolute dependence of glucose-stimulated insulin secretion on the oxidative metabolism of glucose would also confer unique sensitivity of the β -cell to perturbations in mitochondrial function.

The purpose of this investigation was to determine if the synthesis of nitric oxide synthase is induced by treatment of islets with IL-1 β , or if the enzyme is activated by IL-1 β , and also to determine if nitric oxide functions as a signalling or effector molecule mediating the inhibitory effects of IL-1 β on glucose-stimulated insulin secretion. Evidence is presented which indicates that IL-1 β induces the synthesis of nitric oxide synthase by islets, and the resulting formation of nitric oxide functions as an effector molecule which mediates the destruction of iron–sulphur clusters of iron-containing enzymes resulting in an impairment of islet mitochondrial function.

EXPERIMENTAL

Materials

Male Sprague–Dawley rats were purchased from Sasco Inc. (O'Fallon, MO, U.S.A.). Collagenase type P was obtained from Boehringer–Mannheim (Indianapolis, IN, U.S.A.). CMRL-1066 tissue culture medium was from Gibco Laboratories (Grand Island, NY, U.S.A.). NMMA acetate was obtained from Calbiochem (San Diego, CA, U.S.A.), and cycloheximide was from Sigma Chemical Co. (St. Louis, MO, U.S.A.). cGMP, [¹²⁵I] radioimmunoassay kit, D-[U-¹⁴C]glucose (265 mCi/mmol) and [³H]sucrose were from DuPont–NEN Research Products (Boston, MA, U.S.A.). IL-1 α was a gift from Dr. P. T. Lomedico (Hoffman LaRoche, Inc., Nutley, NJ, U.S.A.), and IL-1 β was from Cistron Biotechnology (Pine Brook, NJ, U.S.A.). All other chemicals were from commercially available sources.

Islet isolation

Islets were isolated from male Sprague–Dawley rats (200–250 g) by collagenase digestion as described previously (McDaniel *et al.*, 1983). In brief, on the day before each experiment, pancreases were inflated with Hank's buffered salts solution, and the tissue was isolated, minced, and digested with

3 mg of collagenase/ml for 7 min at 39 °C. Islets were separated on a Ficoll step-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% CO₂ at 37 °C in complete CMRL-1066 media (CMRL-1066 containing 2 mM-L-glutamine, 10% (v/v) heat-inactivated fetal bovine serum, 50 units of penicillin/ml and 50 μ g of streptomycin/ml).

Exposure of islets to IL-1 β

Islets cultured overnight were washed in 2.5 ml of complete CMRL-1066 three times and then counted into Petri dishes containing 1 ml or 2.5 ml of complete CMRL-1066. IL-1 β was added to a final concentration of 5 units/ml (5.7 pM) and the islets were incubated for various periods under an atmosphere of 95% air and 5% CO₂ at 37 °C as indicated in the Figure legends. Complete CMRL-1066 contains 0.3 mM-L-arginine.

Glucose-induced insulin secretion

After the 18 h culture with 5 units of IL-1 β /ml (5.7 pM), cycloheximide or both IL-1 β and cycloheximide, the islets were washed three times (3 ml/wash) in Krebs–Ringer bicarbonate buffer (KRB: 25 mM-Hepes, 115 mM-NaCl, 24 mM-NaHCO₃, 5 mM-KCl, 1 mM-MgCl₂, 2.5 mM-CaCl₂, pH 7.4) containing 3 mM-D-glucose and 0.5% BSA. Groups of 20 islets were counted into 10 mm $\times$ 75 mm siliconized borosilicate tubes and pre-incubated for 30 min in 200 μ l of the same buffer. The pre-incubation buffer was removed and glucose-stimulated insulin secretion was initiated by the addition of 200 μ l of fresh KRB containing either 3 mM- or 20 mM-D-glucose, followed by a 30 min incubation. Both the preincubation and incubation were performed under an atmosphere of 95% air and 5% CO₂ at 37 °C with shaking. Insulin secretion was determined on the incubation buffer by insulin radioimmunoassay.

Measurement of cGMP concentrations

Isolated islets (100) were incubated in complete CMRL-1066 for various periods at 37 °C under 95% air 5% CO₂ in 1 ml Petri dishes containing the indicated concentrations of IL-1 β , NMMA or cycloheximide. The islets were isolated and incubated in 1 ml of 6% (v/v) trichloroacetic acid for 1 h at 4 °C, and then sonicated for 15 s on setting 3 of a Branson Sonifier model 250. The cell lysate was centrifuged at 2500 g for 15 min at 4 °C, and the supernatant was extracted three times with 4 ml portions of water-saturated ether. Samples were freeze-dried to dryness and cGMP was determined using a commercially available cGMP radioimmunoassay kit (DuPont NEN).

Nitrite determination

Groups of 100 islets were cultured for 18 h in 120 μ l of complete CMRL-1066. The culture supernatant was removed and 100 μ l portions were mixed with 100 μ l of Griess reagent (Green *et al.*, 1982) and 300 μ l of distilled water. Nitrite production was determined at an absorbance of 546 nm using a Beckman DU-6 spectrophotometer. In all experiments in which e.p.r. spectroscopy was performed, nitrite release was determined on the media in which the islets were cultured.

E.p.r. spectroscopy

Isolated islets (2500) were cultured in 2.5 ml of complete CMRL-1066 or complete CMRL-1066 containing 5 units of IL-1 β /ml (5.7 pM), or 1 μ M-cycloheximide, or both IL-1 β and cycloheximide for 18 h at 37 °C. The islets were isolated and frozen at –70 °C. E.p.r. spectroscopy was performed on the islets at 77 K using a Varian E-109 spectrometer equipped with a 9.5 GHz microwave bridge. The field was calibrated periodically

Fig. 1. Effects of IL-1 β and NMMA on cGMP formation by islets

Groups of 100 isolated islets were treated for the indicated periods with (●) or without (■) 5 units (5.7 pM) of IL-1 β /ml (a) or 0.5 mM-NMMA (■; b) or IL-1 β and NMMA (●; b) in 1 ml of complete CMRL-1066 medium at 37 °C. After this incubation period, the islets were isolated and cGMP accumulation in the islets was determined by radioimmunoassay. Results are means $\pm$ S.E.M. of three individual experiments containing three replicates in each experiment.

by utilizing a sample of reduced Methyl Viologen. The microwave power was 1 mW, the modulation frequency was 100 kHz, the modulation amplitude was 125×10^{-5} T, and the microwave frequency was 9.105 GHz.

Glucose oxidation and ATP measurements

Glucose oxidation was measured on islets pretreated for 18 h at 37 °C with the indicated concentrations of either IL-1 α or IL-1 β , NMMA, or cycloheximide in 1 ml of complete CMRL-1066 tissue culture media. After pretreatment, the islets were washed three times in KRB containing 3 mM-D-glucose and 0.5% BSA, and islets were randomly counted into microfuge tubes (30 islets/tube). The KRB was removed and replaced with 200 μ l of KRB (without glucose and BSA), and the islets were pre-incubated for 25 min at 37 °C. The islets were isolated by centrifugation, and glucose oxidation reactions were initiated by the addition of 150 μ l of KRB buffer containing 3 mM- or 16.7 mM-glucose and 15 μ Ci of D-[U- 14 C]glucose (265 mCi/mmol). Glucose oxidation, the production of 14 CO $_2$ from 3 mM- or 16.5 mM-D-glucose (supplemented with 15 μ Ci of D-[14 C]-glucose), was determined by incubation of pretreated and control islets for 120 min at 37 °C as described previously (McDaniel *et al.*, 1974). Recovery of 14 CO $_2$ in these studies was more than 90%. ATP concentrations were enzymically measured on islets treated with or without IL-1 α under conditions identical with those used for glucose oxidation, as previously described (Lowry & Passaneau, 1972).

Glucose transport

Islets were pretreated for 18 h with or without IL-1 α as stated above, and D-[14 C]glucose transport by IL-1 α -treated and control islets was measured at 24 °C using a dual-isotope technique with [3 H]sucrose as an extracellular marker (McDaniel *et al.*, 1974).

RESULTS

Effects of IL-1 β on cGMP accumulation by islets

Fig. 1(a) demonstrates that IL-1 β induces the accumulation of cGMP by islets in a time-dependent manner. The initial accumulation of cGMP in islets is apparent after 5 h of treatment with IL-1 β . Continuous treatment of islets for 8 and 18 h with IL-1 β results in further increases in cGMP concentrations. To determine if cGMP accumulation is specific for nitric oxide synthase activity, islets were cultured for various periods with IL-1 β and 0.5 mM-NMMA or NMMA alone. As shown in Fig. 1(b), NMMA completely blocks the IL-1 β -induced accumulation of cGMP, whereas NMMA has no effect on the concentration of

Fig. 2. Effects of cycloheximide on IL-1 β -induced cGMP accumulation and IL-1 β -induced inhibition of glucose-stimulated insulin secretion by islets

(a) Groups of 100 isolated islets were incubated in 1 ml of complete CMRL-1066 tissue culture medium in the presence of 5 units of IL-1 β /ml (5.7 pM), cycloheximide, or IL-1 β and cycloheximide for 18 h at 37 °C as indicated. After the incubation, the islets were isolated and cGMP accumulation was determined by radioimmunoassay. (b) Islets were cultured for 18 h in complete CMRL-1066, or complete CMRL-1066 containing the indicated concentrations of IL-1 β or cycloheximide at 37 °C. After the 18 h culture, the islets were washed three times in KRB containing 3 mM-D-glucose, and groups of 20 islets were counted and transferred to 10 mm $\times$ 75 mm siliconized tubes containing 200 μ l of KRB. Glucose-stimulated insulin secretion was performed as described in the Experimental section. Glucose-induced insulin secretion is expressed as the percentage of a 20 mM-D-glucose control (26.6 ng/20 islets per 30 min). Results are means $\pm$ S.E.M. of three individual experiments containing three to four replicates in each experiment.

cGMP in the absence of IL-1 β . These results suggest that IL-1 β -induced cGMP accumulation is dependent on the activity of nitric oxide synthase.

Effects of cycloheximide on IL-1 β -induced accumulation of cGMP and IL-1 β -induced inhibition of glucose-stimulated insulin secretion by islets

Fig. 2(a) shows that IL-1 β induces the formation of cGMP, and that this increase is not affected by the protein-synthesis inhibitor cycloheximide at a concentration of 1 μ M, but is prevented by a cycloheximide concentration of 10 μ M. In contrast with the effects of cycloheximide on cGMP accumulation,

Table 1. Effects of cycloheximide on IL- β -induced nitrite formation by islets

Groups of 100 islets were incubated for 18 h in 120 μ l of complete CMRL-1066 containing the indicated concentrations of IL-1 β and cycloheximide. The media were removed and nitrite formation was determined as described in the Experimental section. The results are means $\pm$ S.E.M. of four individual experiments containing three replicates in each experiment. Statistical significance was determined by Student's *t* test: *P* < 0.05 compared with control.

Treatment	Nitrite (pmol/islet per 18 h)
Control	2.9 $\pm$ 0.5
1 μ M-Cycloheximide	3.0 $\pm$ 0.6
5.7 pM-IL-1 β	6.4 $\pm$ 1.1*
5.7 pM-IL-1 β + 1 μ M-cycloheximide	3.9 $\pm$ 0.4

Fig. 3. Effects of cycloheximide on the IL-1 β -induced iron-nitrosyl complex-formation and simultaneous formation of nitrite by islets

Isolated islets (at least 2500) were cultured at 37 °C for 18 h in 2.5 ml of complete CMRL-1066, or complete CMRL-1066 containing 5 units of IL-1 β /ml (5.7 pM), 1 μ M-cycloheximide or both IL-1 β and cycloheximide. After culture, islets were isolated and frozen at -70 °C. E.p.r. spectroscopy was performed as described in the Experimental section. Nitrite was determined as described in the Experimental section on 100 μ l portions of the culture supernatant. These data are representative of three individual experiments containing at least 2500 islets per condition. The IL-1 β -induced *g* = 2.04 iron-nitrosyl signal and the *g* = 2.0023 endogenous delocalized electron feature are as indicated by the arrows.

Fig. 2(b) shows that cycloheximide at a concentration of 1 μ M completely prevents IL-1 β -induced inhibition of glucose-stimulated insulin secretion by islets. In the presence of 1 μ M- and 10 μ M-cycloheximide and IL-1 β , insulin secretion is stimulated above that found in control islets, but this stimulation is not statistically significant. Since 1 μ M-cycloheximide blocks IL-1 β -induced inhibition of insulin secretion (Fig. 2b), but does not affect IL-1 β -induced cGMP accumulation (Fig. 2a), cGMP does not appear to mediate the inhibitory effects of IL-1 β on insulin secretion by islets.

We have previously shown that 1 μ M-cycloheximide inhibits approximately 80 % of IL-1 β -induced new protein synthesis, and 10 μ M-cycloheximide blocks over 95 % of islet protein synthesis (Hughes *et al.*, 1990). Results presented in Fig. 2 are consistent with this finding, as inhibition of 80 % of IL-1 β -induced protein synthesis by cycloheximide (1 μ M) would suggest that low concentrations of nitric oxide synthase are expressed, and that the concentration of nitric oxide produced may be sufficient to activate guanylate cyclase resulting in cGMP accumulation.

Fig. 4. Effects of IL-1 α on glucose oxidation and glucose uptake by islets

(a) Glucose oxidation was measured as the production of $^{14}\text{CO}_2$ from 16.5 mM-D-[^{14}C]glucose by islets pretreated for 18 h with or without the indicated concentrations of IL-1 α as described in the Experimental section. (b) D-[^{14}C]Glucose transport by islets incubated for 18 h with (●) or without (□) 2 nM-IL-1 α was measured at 24 °C using a dual-isotope technique as described in the Experimental section. Results are means $\pm$ S.E.M. of 17–18 observations per condition from three separate experiments.

Fig. 5. Effects of NMMA on IL-1 β -induced inhibition of glucose oxidation by islets

Isolated islets were incubated for 18 h with 5 units of IL-1 β /ml (5.7 pM), 0.5 mM-NMMA or IL-1 β and NMMA; the islets were then isolated and the oxidation of D-[^{14}C]glucose to $^{14}\text{CO}_2$ was measured as described in the Experimental section. Results are means $\pm$ S.E.M. of four individual experiments containing at least three replicates per condition.

Effects of cycloheximide on IL-1 β -induced nitrite formation by islets

Cycloheximide at a concentration of 1 μ M also inhibits nitrite formation by islets in response to IL-1 β treatment (Table 1).

Nitrite is an oxidation product of the L-arginine-dependent nitric oxide synthase pathway which is detected spectrophotometrically. These results show that cycloheximide at a concentration that completely prevents the inhibitory effects of IL-1 β on insulin secretion and that has no effect on cGMP accumulation prevents IL-1 β -induced nitrite formation by islets.

Effects of cycloheximide on IL-1 β -induced iron-nitrosyl complex-formation by islets

To establish that IL-1 β induces the formation of both nitric oxide and intracellular targets of its action (cellular iron metabolites), and to examine the effects of cycloheximide on the formation of this free radical, e.p.r. spectroscopy was performed. As shown in Fig. 3, IL-1 β induces the formation of a $g = 2.04$ e.p.r. feature which is characteristic of an intracellular iron-dinitrosyl complex (Hibbs *et al.*, 1990; Lancaster & Hibbs, 1990). The protein synthesis inhibitor cycloheximide (1 μ M) blocks the formation of this IL-1 β -induced e.p.r. signal, but cycloheximide in the absence of IL-1 β has no effect on the formation of this $g = 2.04$ e.p.r. feature (results not shown). The $g = 2.0023$ feature shown in Fig. 3 is due to delocalized electrons found in all cells. In these experiments, a small $g = 2.04$ signal is observed in control islets, and cycloheximide does not appear to block its formation. This feature presumably indicates that islets may contain both the cytokine-inducible isoform of nitric oxide synthase as well as the constitutive isoform of this enzyme. Also shown in Fig. 3 is the simultaneous formation of nitrite in the culture supernatant by the same islets used for e.p.r. spectroscopy. These results confirm that IL-1 β -induced formation of nitric oxide is blocked by the protein-synthesis inhibitor cycloheximide at a concentration (1 μ M) that has no effect of IL-1 β -induced cGMP accumulation, but prevents the inhibitory effects of IL-1 on glucose-stimulated insulin secretion.

Dose-dependent effects of IL-1 α on glucose oxidation and the effects of IL-1 α on glucose uptake by pancreatic islets

Fig. 4(a) demonstrates the dose-dependent effects of IL-1 α on the oxidation of 16.7 mM-D-glucose by islets (120 min) after pretreatment for 18 h with IL-1 α . The oxidation of D-[14 C]glucose to 14 CO $_2$ is significantly affected by pretreatment of islets for 18 h with IL-1 α . Islets pretreated for 18 h with 0.1 nM- or 2 nM-IL-1 α exhibit a 36% decrease in their ability to oxidize 16.5 mM-D-glucose. We have previously reported that pretreatment of islets for 18 h with IL-1 α also results in an inhibition of glucose-stimulated insulin secretion (Comens *et al.*, 1987). These results suggest that IL-1 α -induced inhibition of glucose-stimulated insulin secretion may be a consequence of the ability of IL-1 α to inhibit glucose oxidation.

To determine if the inhibitory effects exerted by IL-1 α on glucose oxidation may be due to an inhibition of facilitated transport of D-glucose by islets, the effects of IL-1 α on glucose uptake were investigated. Fig. 4(b) demonstrates that glucose uptake (pmol/islet) in control islets increases in a time-dependent manner with intracellular equilibrium reached rapidly (2 min). Pretreatment of islets for 18 h with 2 nM-IL-1 α has no influence on glucose uptake. Furthermore, Sandler *et al.* (1989) have reported that IL-1-induced inhibition of glucose oxidation is due to perturbations in mitochondrial function and not to impairment of glucose utilization. The lack of an effect of IL-1 α on facilitated glucose transport is consistent with the observations of Sandler *et al.* (1989).

The effects of IL-1 α pretreatment on islet ATP concentration were also investigated. Measurement of the ATP concentration of islets reveals a 45% decline in the ATP concentration of

Fig. 6. Effects of cycloheximide on IL-1 β -induced inhibition of glucose oxidation by islets

Islets were treated for 18 h with 5 units of IL-1 β /ml (5.7 pM), 10 μ M-cycloheximide or IL-1 β and the indicated concentrations of cycloheximide; they were then isolated and the oxidation of D-[14 C]glucose to 14 CO $_2$ was measured as described in the Experimental section. Glucose oxidation was unaffected by treatment of islets with 10 μ M-cycloheximide (results not shown). Results are means $\pm$ S.E.M. of three individual experiments containing three replicates per condition.

glucose (16.5 mM)-stimulated islets pretreated for 18 h with 2 nM-IL-1 α (5.7 ± 0.5 nmol of ATP/kg of protein; $n = 8$) versus control islets (10.5 ± 1.4 nmol of ATP/kg of protein; $n = 8$). There is also a 32% decrease in the ATP concentration of islets pretreated for 18 h with 2 nM-IL-1 α in 3 mM-glucose (4.5 ± 1.0 nmol of ATP/kg of protein; $n = 9$) versus control islets (6.6 ± 0.6 nmol of ATP/kg of protein; $n = 9$). These results provide further evidence that IL-1-induced inhibition of glucose oxidation results in an impairment of islet oxidative metabolism.

Effects of NMMA on IL-1 β -induced inhibition of glucose oxidation by islets

Fig. 5 demonstrates the effects of the nitric oxide synthase inhibitor, NMMA, on IL-1 β -induced inhibition of glucose oxidation by islets. IL-1 β is shown to inhibit glucose oxidation by 60%, and this inhibition is completely prevented by NMMA. Pretreatment of islets with NMMA in the absence of IL-1 β has no apparent effect on glucose oxidation. These results indicate that IL-1-induced inhibition of glucose oxidation by islets is dependent on the enzymic activity of nitric oxide synthase.

Effects of cycloheximide on IL-1 β -induced inhibition of glucose oxidation by islets

The protein-synthesis inhibitor cycloheximide has previously been shown to block both IL-1 β -induced inhibition of insulin secretion (Hughes *et al.*, 1990; and Fig. 2b) and nitric oxide production by islets (Fig. 3). Fig. 6 demonstrates the protective effects of cycloheximide on IL-1 β -induced inhibition of glucose oxidation by islets. Cycloheximide prevents IL-1 β -induced inhibition of glucose oxidation by islets in a dose-dependent fashion. In these studies islets were pretreated with the indicated concentrations of cycloheximide and 5 units of IL-1 β /ml (5.7 pM) or IL-1 β alone for 18 h and the ability of the islets to oxidize D-[14 C]glucose to 14 CO $_2$ was determined. A concentration of 10 μ M-cycloheximide provides 90% protection against IL-1 β -induced inhibition of glucose oxidation, and 70% of IL-1 β -induced inhibition of glucose oxidation is prevented by 1 μ M-cycloheximide. Cycloheximide at a concentration of 0.1 μ M has no apparent effect on IL-1 β -induced inhibition of glucose oxidation. These results demonstrate that protein synthesis is required for IL-1 β -induced inhibition of glucose oxidation.

DISCUSSION

In this study, IL-1 β is shown to induce the accumulation of cGMP in a time-dependent fashion (Fig. 1) that mimics IL-1 β -induced inhibition of insulin secretion reported previously by our laboratory (Mandrup-Poulsen *et al.*, 1988; Hughes *et al.*, 1990). The salient feature of this time-dependence is that it requires more than 5 h for IL-1 β to induce both an inhibition of insulin secretion and also the accumulation of cGMP by islets. This accumulation of cGMP in response to IL-1 β is completely prevented by pretreatment of islets with NMMA in addition to IL-1 β . NMMA, and NAME, competitive inhibitors of nitric oxide synthase, have also been shown to block IL-1 β -induced inhibition of insulin secretion (Southern *et al.*, 1990; Corbett *et al.*, 1991). The accumulation of cGMP in response to increased concentrations of nitric oxide has been observed in many tissues including endothelial cells, mesangial cells, smooth muscle and hepatocytes making the accumulation of cGMP an important indicator of nitric oxide formation (for review see Moncada *et al.*, 1991).

Nitric oxide appears to function either as a signalling or an effector molecule depending on the isoform of nitric oxide synthase. Nitric oxide produced by the constitutive isoform of nitric oxide synthase activates guanylate cyclase resulting in the formation of cGMP which mediates endothelium-dependent relaxation (Moncada *et al.*, 1991). Nitric oxide produced by the inducible isoform of nitric oxide synthase in macrophages is synthesized in amounts much greater than the concentrations of nitric oxide produced by the constitutive isoform of nitric oxide synthase, and functions as an effector molecule which appears to mediate the cytotoxic effects of macrophages on target cells (Hibbs *et al.*, 1990; Moncada *et al.*, 1991). In the present study, cycloheximide is shown to completely prevent IL-1 β -induced cGMP accumulation at a concentration of 10 μ M, whereas 1 μ M-cycloheximide has no effect on the accumulation of cGMP in islets (Fig. 2a). This protein-synthesis inhibitor is also shown to prevent the inhibitory effects of IL-1 β on insulin secretion by islets at a concentration of 1 μ M (Fig. 2b; and Hughes *et al.*, 1990). Since 1 μ M-cycloheximide protects islets from the inhibitory effects of IL-1 β on insulin secretion but does not prevent cGMP accumulation, it would appear that the formation of cGMP in response to IL-1 β treatment does not mediate the inhibitory effects of IL-1 β on insulin secretion, but is an important indicator of nitric oxide formation. Also Verspohl *et al.* (1988) have reported that atrial-natriuretic-peptide-induced cGMP accumulation in Rin-m5F cells has no apparent effect on insulin secretion, and Laychock (1983) has reported that increased cGMP concentrations stimulated by 8-bromoguanosine 3',5'-cyclic monophosphate potentiate glucose-stimulated insulin secretion by islets. Thus these data suggest that IL-1 β induces the formation of nitric oxide which functions as an effector molecule mediating the inhibitory effects of IL-1 β on insulin secretion, and that cGMP accumulation does not directly mediate the inhibitory effects of IL-1 β on glucose-stimulated insulin secretion, although these results do not unambiguously preclude a role for cGMP. Also, nitric oxide, produced in much larger quantities by the inducible isoform of nitric oxide synthase (as compared with the constitutive enzyme), has been implicated as an effector molecule mediating cellular dysfunction independent of cGMP (Hibbs *et al.*, 1990).

To establish that IL-1 β -induced formation of nitric oxide by islets is dependent on protein synthesis, and to investigate the mechanism by which nitric oxide mediates IL-1 β -induced inhibition of glucose-stimulated insulin secretion, e.p.r. spectroscopy was employed. This technique has been used

previously to establish the formation of nitric oxide by islets treated with IL-1 β (Corbett *et al.*, 1991), activated macrophages (Lancaster & Hibbs, 1990) and the target cells of activated macrophages (Drapier *et al.*, 1991). In the present study IL-1 β is shown to induce the formation of an e.p.r.-detectable $g = 2.04$ iron-dinitrosyl feature in islets, and the formation of this feature is blocked by cycloheximide (Fig. 3). These results establish that IL-1 β induces the formation of nitric oxide and that nitric oxide formation is dependent on protein synthesis.

The mechanism by which pretreatment of islets with IL-1 results in an inhibition of glucose-stimulated insulin secretion is believed to reflect an inhibition of mitochondrial oxidative metabolism of glucose. Pretreatment of islets with IL-1 α or IL-1 β results in an inhibition of the oxidation of D-glucose to CO₂ (Figs. 4–6, and Sandler *et al.*, 1989). In this report evidence is presented which demonstrates that IL-1 α has no effect on glucose uptake (Fig. 4b). Furthermore, Sandler *et al.* (1989) have previously demonstrated that IL-1 β has no effect on glucose utilization (glycolysis). Since IL-1 has no apparent effect on either glucose utilization (Sandler *et al.*, 1989) or the cellular uptake of glucose, it is suggested that the effects of IL-1 on glucose metabolism are due to an impairment of mitochondrial function. Consistent with an inhibition of mitochondrial function is the observation of a 45% decrease in cellular ATP-concentration of islets treated for 18 h with IL-1 α . Also, the inhibitory effects of both IL-1 α and IL-1 β on glucose oxidation by islets were investigated. Both IL-1 α and IL-1 β have been shown previously to bind to an identical IL-1 receptor (Dower *et al.*, 1986), and produce similar inhibitory effects on insulin secretion (Comens *et al.*, 1987; Hughes *et al.*, 1990) and glucose oxidation by islets (Fig. 1a and Fig. 2).

The effector molecule which mediates IL-1 β -induced inhibition of glucose-stimulated insulin is believed to be the free radical, nitric oxide. Cycloheximide at a concentration (1 μ M) that blocks IL-1 β -induced inhibition of glucose-stimulated insulin secretion prevents approx. 70% of IL-1 β -induced inhibition of glucose oxidation. These results suggest that a threshold level of mitochondrial glucose oxidation is necessary for glucose-stimulated insulin secretion. NMMA completely prevents the inhibitory effects of IL-1 β on glucose oxidation (Fig. 5) and also prevents the inhibitory effects of IL-1 β on glucose-stimulated insulin secretion (Corbett *et al.*, 1991). Furthermore, iron-nitrosyl complex-formation is believed to arise from the destruction of iron-sulphur clusters of iron-containing enzymes (Reddy *et al.*, 1983), and these complexes have been observed under conditions where nitric oxide formation results in the inhibition of the catalytic activity of mitochondrial iron-containing enzymes (Drapier & Hibbs, 1986; Hibbs *et al.*, 1990; Stadler *et al.*, 1991). Since IL-1 β induces iron-nitrosyl complex-formation and an impairment of glucose oxidation by islets, and both NMMA and cycloheximide prevent the inhibition of insulin secretion, iron-nitrosyl complex-formation and glucose oxidation, it is proposed that the cellular mechanism by which IL-1 β inhibits glucose-stimulated insulin secretion is via the destruction of iron-sulphur centres of mitochondrial enzymes by nitric oxide. This proposed mechanism establishes a direct connection between the inhibitory effects of nitric oxide on mitochondrial function and glucose-stimulated insulin secretion by pancreatic islets.

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