Insulin-like growth factor I reverses interleukin-1β inhibition of insulin secretion, induction of nitric oxide synthase and cytokine-mediated apoptosis in rat islets of Langerhans

Jon G. Mabley, Véronique Belin, Nerys John, Irene C. Green*

Department of Biochemistry, University of Sussex, Falmer, Brighton BN1 9QG, UK

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Abstract We have previously observed that treatment of rat islets of Langerhans with interleukin-1β for 12 h results in nitric oxide-dependent inhibition of insulin secretion, while 48 h treatment increased rates of islet cell death by apoptosis. Here, we demonstrate that interleukin-1β-mediated nitric oxide formation and inhibition of insulin secretion are significantly reduced by 24 h pretreatment of rat islets of Langerhans with insulin-like growth factor I (IGF-I). IGF-I decreased cytokine induction of nitric oxide synthase in islets. Use of an arginine analogue in culture or IGF-I pretreatment of islets were also effective in protecting islets against cytokine-mediated apoptotic cell death. We conclude that IGF-I antagonises inhibitory and cytotoxic effects of cytokines in rat islets.

Key words: Insulin-like growth factor I; Interleukin-1β; Nitric oxide synthase; Islet of Langerhans; Apoptosis; Insulin secretion

1. Introduction

Insulin-like growth factor I (IGF-I) is a single chain polypeptide of 7–8 kDa [1] which has insulin-like effects on glucose transport and blood glucose levels [1]. Receptors for IGF-I have been observed on rat islet β-cells and α-cells [2]. Short-term treatment of either perfused isolated islets [3] or whole pancreas [4] with IGF-I inhibited insulin secretion but had no effect on glucagon secretion [4]. Recombinant human IGF-I has been shown to reduce but not eliminate insulitis and to delay the onset of diabetes, induced by adoptive T-cell transfer, in NOD mice [5]. The authors speculated that IGF-I may directly promote β-cell regeneration, protect against cytokine attack or reduce nitric oxide formation [5].

Interleukin-1β (IL-1β) induces expression of the calcium-independent isoform form of the enzyme nitric oxide synthase (type II) and increases nitric oxide formation in rat islets of Langerhans [6–8]. IL-1β-mediated nitric oxide affects a number of islet parameters: it inhibits insulin secretion [9,10], glucose oxidation [10], the mitochondrial enzyme aconitase [10], cAMP formation [11] and lowers NAD levels [12]. IL-1β also stimulates islet cGMP formation [11,13] and has islet DNA-damaging effects [14]. IL-1β causes islet cell death by apoptosis [15,16], an effect reversed by an inhibitor of nitric oxide synthase [15]. Increases in cellular cGMP levels and protein kinase G activity have been implicated in nitric oxide-mediated apoptosis signalling in insulin-containing cells [17]. Combinations of IL-1β, interferon-γ, and/or tumour necrosis factor-α are more potent at inhibiting insulin secretion [9,18], lowering cell viability [16,19] and inducing nitric oxide synthase than single cytokines.

Induction of nitric oxide synthase by interleukin-1β or tumour necrosis factor α in rat vascular smooth muscle cells is inhibited by IGF-I [20]. IGF-I has also been shown to be an effective anti-apoptotic agent [21]. Reversal of cytokine-induced impairment of rat islets by anti-inflammatory cytokines has been difficult to demonstrate. The aim of this study was therefore to explore whether IGF-I protected against cytokine-mediated inhibition of insulin secretion, stimulation of nitric oxide formation and cell death by apoptosis in rat islets of Langerhans.

2. Materials and methods

Reagents were obtained from the following sources: tissue culture RPMI 1640 and foetal calf serum were from Gibco BRL (Paisley, UK); rat interferon-γ, specific activity ≥7.5×106 U/mg (Genzyme, UK); N^G-monomethyl arginine (NMMA) (Calbiochem, Nottingham, UK); anti-iNOS (murine macrophage, inducible) rabbit polyclonal antibody (Affiniti Research Products Ltd, UK); acrylamide, arginine hydrochloride, bis-acrylamide, collagenase type XI, leupeptin, pepstatin, phenylmethylsulphonyl fluoride, acridine orange, sodium dodecyl sulphate (SDS), horseradish peroxidase-conjugated goat anti-mouse IgG affinity isolated antibody and dithiothreitol (Sigma Chemicals Poole, UK); L-[U-^{14}C]arginine monohydrochloride (318 mCi/mmol) and enhanced chemiluminescence kit (Amersham, Bucks., UK); Poly-Prep chromatography columns, electrophoresis equipment and Dowex AG50W-x8 cation exchange resin (Bio-Rad Laboratories, Richmond, CA, USA); Immobilon-P PVDF filters (Millipore, UK); interleukin-1β (86/558), tumour necrosis factor-α (88/784) and insulin-like growth factor I (87/518) were from Dr Dennis Schulster at the National Institute of Biological Standards and Controls, UK via a BBSRC CASE-studentship agreement.

2.1. Islet isolation, culture and cytokine treatment

Islets of Langerhans were isolated under aseptic conditions from collagenase-digested pancreata of adult female (180–250 g) or unweaned Sprague-Dawley rats [11,18]. Batches of 100–400 islets were cultured in RPMI 1640 medium containing 5.5 mM glucose, penicillin (50 U/ml), streptomycin (50 μg/ml) and 5% foetal calf serum for 24 h before experimental treatments. Cytokine treatment was carried out by addition of 10 μl of a ×100-fold concentrated cytokine into 1 ml of fresh culture medium. Where IGF-I pretreatment was used, IL-1β or combined cytokines were added to the same IGF-I-containing culture dish for a further 24–48 h. After treatment the islets were removed from culture and analysed for insulin secretory responsiveness, nitric oxide synthase activity or expression, and apoptotic nuclear bodies.

2.2. Islet insulin secretion response

Islets were removed from culture and preincubated for 1 h in physiological buffer containing 2 mM glucose [11]. The insulin secretory

*Corresponding author. Fax: (44) (1273) 678433.
E-mail: i.c.green@sussex.ac.uk

Abbreviations: IGF-I, insulin-like growth factor I; IL-1β, interleukin-1β; TNF-α, tumour necrosis factor-α; IFN-γ, interferon-γ; TGFβ1, transforming growth factor β1; iNOS, inducible nitric oxide synthase

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response was measured from groups of three islets incubated in 0.6 ml buffer containing 20 mM glucose for 30 min at 37°C. At the end of the incubation a 0.3 ml aliquot was removed and assayed for secreted insulin. Acid ethanol (conc. HCl:water:ethanol 0.45:7:23 v/v/v) (0.3 ml) was added to the islet residue and left overnight at 4°C to extract insulin from β-cell granules; extracts were then radioimmunoassayed for insulin. Insulin was assayed using rat insulin standard, guinea pig insulin antiserum and ^{125}I-labelled bovine insulin [11]. Insulin secreted was expressed as a percentage of islet insulin content, to take account of possible changes in insulin stores or islet cell number after cytokine treatment.

2.3. Nitric oxide synthase assay

The assay is based on the conversion of radiolabelled arginine to labelled citrulline and nitric oxide and has been described previously [22].

2.4. Detection of nitric oxide synthase by Western blotting

Following treatments in culture, rat islets were sonicated in 100 μl of ice-cold Tris-HCl buffer (pH 7.6, 0.05 M) containing EDTA (0.5 mM), EGTA (0.5 mM), leupeptin (1 μM), pepstatin A (1 μM), phenylmethylsulphonyl fluoride (0.1 mM) and dithiothreitol (1 μM) using an MSE 150 W ultrasonic disintegrator MK.2 at an amplitude of 15 μm for 15 s [22]. The sonicated samples were centrifuged at 105 000×*g* for 1 h at 4°C. The supernatant was mixed 1:1 (v/v) with double strength sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, bromophenol blue) and boiled for 3 min to denature the proteins. The protein content of the samples was determined [8] and gel lanes were equiloaded. The samples were separated on a 10% polyacrylamide separating gel at 150 V for 30 min. The separated proteins were blotted onto a PVDF membrane (pore size 0.45 μm) at 25 V for 105 min using a semi-dry transfer system. The PVDF membrane was blocked with 5% (w/v) milk protein in TBS/Tween (Tris-base (10 mM), sodium chloride (100 mM) and 0.1% (v/v) Tween 20; pH 7.4) for 1 h at room temperature followed by incubation overnight at 4°C in a solution of the primary antibody, an anti-iNOS (murine macrophage, inducible) rabbit polyclonal antibody, diluted in TBS/Tween at 1:1000. Following thorough washing in TBS/Tween the PVDF membrane was incubated for 1 h with an anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase before being visualised using an enhanced chemiluminescence kit according to the manufacturer's instructions. The optical intensity of the iNOS band was determined using a Sharp high resolution colour scanner and Imagemaster software (Pharmacia Biotech), the band in growth factor pretreated or control islets was compared to that in islets treated with IL-1β and expressed as a percentage of iNOS expression in the IL-1β treated islets.

2.5. Use of acridine orange to visualise apoptotic cells

For determinations of cell death using acridine orange cytochemistry collagenase-isolated neonatal rat islets were treated in culture medium with or without IGF-I for 24 h prior to treatment with either IL-1β alone or IL-1β combined with TNF-α and IFN-γ for 48 h. Islets were also treated with combined cytokines in arginine-free medium supplemented with the arginine analogue, NMMA (1 mM). Islets were further trypsinised to disperse into single cells which were centrifuged at 500×*g* for 2 min and resuspended in 20 μl of culture medium. 10 μl of homogeneous cell suspension was mixed with an equal volume of acridine orange solution in culture medium, final concentration acridine orange was 10 μg/ml, transferred to a microscope slide and viewed on a Zeiss microscope using a narrow band fluorescence filter. Nuclei were counted and analysed immediately after acridine staining [16]; those showing apoptotic figures – DNA condensed into rounded, brightly fluorescing fragments – were recorded and the percent apoptosis calculated [16].

2.6. Statistical analysis

The data are presented as means ± S.E.M. The significance of differences between treatments was determined by Student's unpaired *t*-test or one-way analysis of variance.

3. Results

Treatment of isolated rat islets of Langerhans for 24 h with IL-1β (100 pM) resulted in an inhibition of glucose-stimulated insulin secretion (Fig. 1). Treatment of islets with IGF-I (10 nM), simultaneously with IL-1β (100 pM), failed to restore insulin secretion inhibited by IL-1β (Fig. 1). However, 24 h pretreatment of islets with 10 nM IGF-I protected against IL-1β-mediated inhibition of insulin secretion (Fig. 1); a lower dose of IGF-I (1n M) had no beneficial effect (control insulin secretion, expressed as a percent of insulin stored in same group of islets 3.1 ± 0.2; IL-1β: 1.5 ± 0.1; IL-1β+IGF-I (1 nM): 1.7 ± 0.3; IGF-I (1 nM): 3.3 ± 0.8; $n = 4$). IGF-I treatment, on its own, for 24 h or 48 h had no effect on subsequent insulin secretion from rat islets (Fig. 1). Neither growth factor nor cytokine treatment had any significant effect on rat islet insulin content (J.G. Mabley, unpublished observations). Nitric oxide synthase induction and activity were determined by Western blotting and enzyme activity assay. IGF-I (10 nM) pretreatment reduced the IL-1β-mediated rise in nitric oxide synthase conversion of arginine to citrulline plus nitric oxide (Fig. 2A) and decreased expression of inducible nitric oxide synthase (Fig. 2A,B). IGF-I (10 nM) treatment, alone, did not affect expression of nitric oxide synthase but increased arginine conversion significantly, although to less than half the level seen with IL-1β alone (Fig. 2A,B). Treatment with IL-1β (50 pM) for 48 h increased islet cell death (Fig. 3), the level of apoptosis was greatly enhanced when IL-1β was combined with TNF-α (50 pM) and IFN-γ (2 U/ml) (Fig. 3). Treatment of rat islets with combined cytokines in arginine-free medium supplemented with the arginine analogue NMMA (1 mM) prevented induction of apoptosis (Fig. 3). Pretreatment with IGF-I (10 nM) for 24 h prior to cytokine addition had no significant effect on the variable level of apoptosis induced by

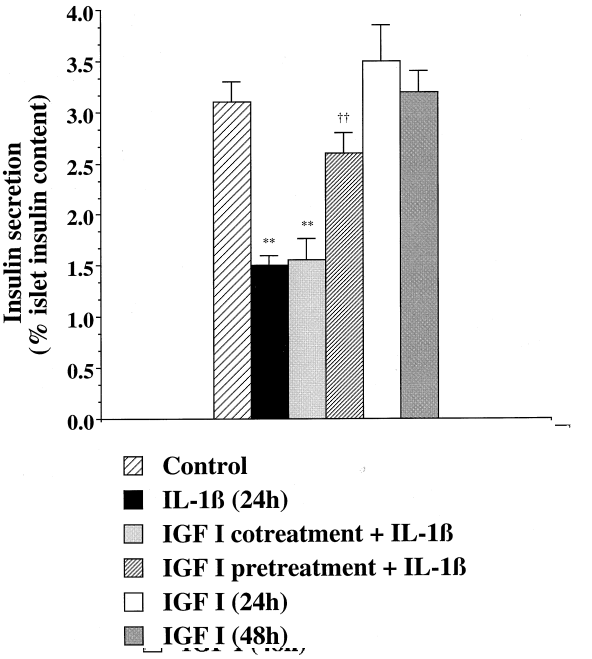

Fig. 1. Pretreatment, but not simultaneous treatment, of rat islets of Langerhans with IGF-I (10 nM) prevents IL-1β (100 pM)-mediated inhibition of glucose-induced insulin secretion. IGF-I alone for 24 or 48 h has no effect. Insulin secretion from control rat islets was 1.1 ± 0.14 ng insulin/islet/30 min ($n = 4$). Values are mean ± S.E.M. of insulin secretion expressed as a percentage of islet insulin content from 3–6 separate isolates (6–8 observations per isolate), using Student's *t*-test $^{**}P < 0.01$ vs. control and $^{\dagger\dagger}P < 0.01$ vs. IL-1β.

IL-1β alone but protected rat islets from apoptosis induced by combined cytokines (Fig. 3), IGF-I alone had no effect.

4. Discussion

We have demonstrated that IGF-I blocks IL-1β's inhibitory action on glucose-stimulated insulin secretion from rat islets of Langerhans. This protection is evident only if islets have been pretreated – rather than co-treated – with IGF-I. In our experiments, exposure of rat islets for either 24 or 48 h to IGF-I had no effect on insulin secretion, although there are reported observations that 2 h treatment of either perfused islets or pancreas with IGF-I caused an inhibition of insulin secretion [3,4]. Pretreatment of rat islets in culture with concentrations of IGF-I lower than 10 nM did not oppose IL-1β's inhibitory effects in current experiments, where foetal calf serum concentrations of 5% were used throughout. We have examined the efficacy of IGF-I pretreatments in culture media

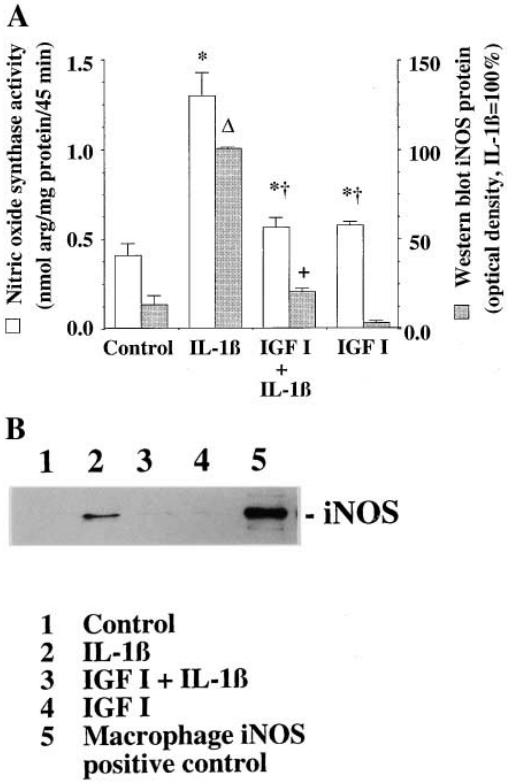

Fig. 2. Pretreatment of islets with IGF-I attenuated the IL-1β increased activity and expression of inducible nitric oxide synthase. Groups of islets were pretreated with IGF-I (10 nM) for 24 h prior to addition of IL-1β (100 pM) for a further 24 h. Nitric oxide synthase enzyme activity in islet cytosol (A) was determined in a 45 min in vitro assay; values are mean ± S.E.M. nmole arginine converted/mg islet protein for 3–4 islet isolates (2 observations per isolate). Statistical significance were determined using one-way analysis of variance; $^{*}P<0.05$ vs. control, $^{\dagger}P<0.05$ vs. control and IL-1β. The intensity of nitric oxide synthase protein bands visualised by ECL development of Western blots from treated islets was determined and expressed as a percentage that observed with IL-1β treatment (A), values are mean ± S.E.M. $n=4$ separate experiments $^{\Delta}P<0.05$ vs. control and $^{+}P<0.05$ vs. IL-1β by Student's t-test (A). Pretreatment of islets with IGF1 prevented expression of IL-1β-induced iNOS shown by Western blotting (B); a protein extract of macrophage iNOS supplied by Affiniti with the iNOS polyclonal antibody is shown for comparison.

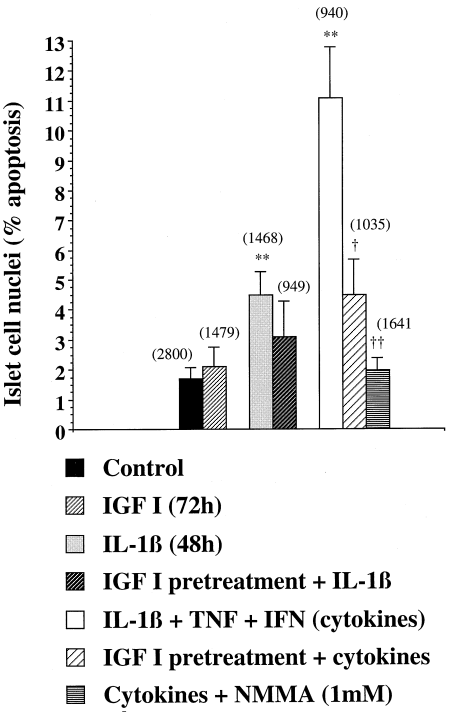

Fig. 3. Treatment of neonatal rat islets with IL-1β (50 pM) alone or combined with TNF-α (50 pM) and IFN-γ (2 U/ml) for 48 h increased apoptosis observed using acridine orange cytochemistry. An arginine analogue, NMMA (1 mM), prevented cytokine-mediated islet cell death. Pretreatment with IGF-I (10 nM) for 24 h also significantly protected rat islets from induction of apoptosis by combined cytokines. The number of nuclei observed is written above each treatment column. Values are mean ± S.E.M. of percent of nuclei showing apoptotic bodies (4–7 experiments), using Student's t-test $^{**}P<0.01$ vs. control; $^{\dagger\dagger}P<0.01$ vs. combined cytokine treatment.

containing 1% and 0.1% serum, and found that concentrations of 1 nM or 0.1 nM IGF-I significantly reversed IL-1β inhibition of insulin secretion under these conditions [23]. It is not known whether IGF-I binding proteins in foetal calf serum are responsible for serum dependence of protective effects, although binding proteins are known to regulate the plasma level, half life and biological activity of IGF-I [1,24].

IL-1β's inhibition of rat islet insulin secretion in the short term is mediated, to a significant extent, by induction of nitric oxide synthase and the formation of nitric oxide [9]. Pretreatment of rat islets with IGF-I inhibited the IL-1β-mediated increase in cytosolic conversion of arginine to citrulline and nitric oxide shown here. Other growth factors lower the activity of inducible nitric oxide synthase by decreasing the availability of the substrate arginine, through upregulation of arginase activity [25], or restricting essential co-factors such as tetrahydrobiopterin [26]. A role for arginase has been proposed in cytokine and prostaglandin suppression of nitric oxide synthase activity [27]. The low level of arginine conversion in untreated islets is decreased further by valine, an inhibitor of arginase (J.G. Mabley, unpublished observations), and IGF-I alone increased islet arginine conversion, which could indicate an increase in arginase activity [22]. However, it is unlikely that IGF-I lowers nitric oxide synthase activity via modulation of arginase since it inhibited the expression of nitric oxide synthase, as determined by Western blotting. The elevation of cytosolic arginine conversion in IGF-I-treated islets, compared to control islets, may also be

attributable to IGF-I's ability to stimulate constitutive nitric oxide synthase [28] which is found, not only in the insulin-containing cells of the islet [8], but also in endothelial cells lining the capillary blood vessels of islets [29].

A number of cytokines which prevent induction of nitric oxide synthase in other cell types have proved ineffective in protecting rat islets from IL-1β's effects. Interleukin-4 [30], interleukin-10 [31] and interleukin-13 [32] were shown to be unable to reverse either IL-1β-mediated inhibition of insulin secretion or stimulation of nitric oxide formation in rat islets. However, transforming growth factor β1 (TGFβ1), another well-known inhibitor of nitric oxide synthase induction [33], inhibits cytokine-mediated induction of nitric oxide synthase in the insulin-containing cell line, RINm5F, and protects against inhibition of insulin secretion, decreased protein synthesis and loss of cell viability [34]. TGFβ1 pretreatment of rat islets of Langerhans also prevents IL-1β's inhibition of insulin secretion, but its effects on nitric oxide synthase induction in islets are equivocal [35].

Induction of apoptosis in rat islet cells by combined cytokine treatment is prevented by an arginine analogue. This confirms our previous findings that nitric oxide generated chemically induces loss of islet cell viability and apoptosis [16], and suggests that cytokine-mediated generation of nitric oxide may signal islet cell death. IGF-I can activate phosphatidylinositol-3-kinase which in turn activates the serine-threonine kinase protein kinase B/Akt [36], which has been shown to prevent apoptosis [36] and regulate neuronal survival [37]; mechanisms other than inhibition of nitric oxide formation may mediate IGF-I's anti-apoptotic effect in rat islets of Langerhans. An interesting link between activation of phosphatidylinositol 3-kinase and inhibition of iNOS transcription by interleukin-13 was recently observed in an epithelial cell line [38].

In conclusion, IGF-I may antagonise the inhibitory effect of IL-1β on insulin secretion or cytokine-mediated apoptosis by lowering induction of nitric oxide synthase and nitric oxide formation.

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