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Inhibition of poly (ADP-ribose) synthetase by gene disruption or inhibition with 5-iodo-6-amino-1,2-benzopyrone protects mice from multiple-low-dose-streptozotocin-induced diabetes

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1 Activation of poly(ADP-ribose) synthetase (PARS, also termed polyADP-ribose polymerase or PARP) has been proposed as a major mechanism contributing to β-cell destruction in type I diabetes. In the present study, we have investigated the role of PARS in mediating the induction of diabetes and β-cell death in the multiple-low-dose-streptozotocin (MLDS) model of type I diabetes.

2 Mice genetically deficient in PARS were found to be less sensitive to MLDS than wild type mice, with a lower incidence of diabetes and reduced hyperglycemia.

3 A potent inhibitor of PARS, 5-iodo-6-amino-1,2-benzopyrone (INH_2BP), was also found to protect mice from MLDS and prevent β-cell loss, in a dose-dependent manner. Paradoxically, in the PARS deficient mice, the compound increased the onset of diabetes.

4 *In vitro* the cytokine combination; interleukin-1β, tumor necrosis factor-α and interferon-γ inhibited glucose-stimulated insulin secretion from isolated rat islets of Langerhans and decreased RIN-5F cell viability. The PARS inhibitor, INH_2BP, protected both the rat islets and the β-cell line, RIN-5F, from these cytokine-mediated effects. These protective effects were not mediated by inhibition of cytokine-induced nitric oxide formation.

5 Inhibition of PARS by INH_2BP was unable to protect rat islet cells from cytokine-mediated apoptosis.

6 Cytokines, peroxynitrite and streptozotocin were all shown to induce PARS activation in RIN-5F cells, an effect suppressed by INH_2BP.

7 The present study provides evidence for *in vivo* PARS activation contributing to β-cell damage and death in the MLDS model of diabetes, and indicates a role for PARS activation in cytokine-mediated depression of insulin secretion and cell viability *in vitro*.

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Abbreviations: cyclic GMP: cyclic guanosine 3′ 5′ monophosphate; IFN: interferon-gamma; INH_2BP: 5-iodo-6-amino-1,2-benzopyrone; iNOS: inducible nitric oxide synthase; MLDS: Multiple-low-dose-streptozotocin; MTT: 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide; NO: nitric oxide; PARS: poly (ADP-ribose) synthetase; TNF: tumor necrosis factor

Introduction

Type I diabetes is characterized by the selective destruction *via* an autoimmune process of the insulin-secreting β-cells in the pancreatic islets of Langerhans. Streptozotocin is a specific β cell toxin and can be used to chemically induce diabetes in rats and mice. Streptozotocin is taken up by the β-cells through the glucose transporter Glut-2 (Schnedl *et al*., 1994) where it decomposes intracellularly causing DNA damage; directly, by alkylation (Delaney *et al*., 1995) and indirectly, *via* generation of NO (NO) (Turk *et al*., 1993), resulting in β-cell death by necrosis (Like *et al*., 1978). There are two streptozotocin-induced animal models of diabetes, administration of a single large dose of streptozotocin induces diabetes within 48 h by directly destroying the β-cells, and multiple-low-dose-streptozotocin (MLDS) administration which causes β-cell damage resulting in an immune cell response directed towards the β-cells. MLDS is characterized by a progressive hyperglycemia and an insulitis similar to that observed in recent onset type I diabetics (Like & Rossini, 1976; Rossini *et al*., 1978). Islet infiltrating immune cells produce cytokines and nitrogen and oxygen free radical species which cause β-cell destruction (Rabinovitch *et al*., 1992). Cytokine treatment of islets leads to β-cell expression of the inducible isoform of NO synthase (iNOS) (Green *et al*., 1994) and subsequent production of NO and related free radical species such as peroxynitrite. Cytokine-induced NO formation in β-cells inhibits insulin secretion (Southern *et al*., 1990), decreases

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cellular ATP levels (Sandler *et al*., 1990), increases cyclic GMP levels (Green *et al*., 1993), DNA damage (Delaney *et al*., 1993) and cell death (Di Matteo *et al*., 1997). Chemically generated free radicals such as NO, superoxide and peroxynitrite (a potent reactive oxidant formed from the rapid reaction of NO and superoxide) (di Matteo *et al*., 1997; Hadjivassiliou *et al*., 1998) have all been shown to functionally inhibit β-cells.

Single strand breaks in DNA results in the activation of the nuclear DNA repair enzyme, poly (ADP-ribose) synthase (PARS) – also known as poly (ADP-ribose) polymerase (PARP) – which transfers ADP-ribose units to nuclear proteins. This process is an energy consuming cycle which decreases intracellular AND^+ resulting in depletion of the cellular ATP pool (Berger, 1985), slowing the rate of glycolysis and mitochondrial respiration leading to cellular dysfunction and necrosis (Szabó & Dawson, 1998a; Szabó, 2000). Interference with PARS activity can also induce a pleiotropic cellular response traceable to a series of protein–protein and protein–DNA interactions (Kun, 1998). There is evidence for the activation of PARS playing a role in the development of diabetes and the destruction of the β-cells. Inhibitors of PARS, nicotinamide and 3-aminobenzamide, have been reported to reduce the incidence of MLDS induced diabetes (Masiello *et al*., 1990; Rossini *et al*., 1977). Both these compounds have also been shown to protect islets *in vitro* from cytokine-mediated toxicity (Akabane *et al*., 1995; Hoorens & Pipeleers, 1999).

In other cell systems the combination of NO and superoxide leading to the formation of peroxynitrite has been shown to be cytotoxic *via* multiple mechanisms including PARS activation (Szabó & Dawson, 1998a). Peroxynitrite has been shown to cause cellular injury and DNA damage to both rat (Hadjivassiliou *et al*., 1998) and human (Hadjivassiliou *et al*., 1998) islets of Langerhans. The involvement of NO in the pathogenesis of type I diabetes has been demonstrated using NO synthase inhibitors which delay the onset and reduce the incidence of diabetes in the nonobese diabetic (NOD) mouse (Southan *et al*., 2000) and BB rat (Wu, 1995) models of diabetes. In the NOD mouse increased expression of iNOS in the pancreas has been observed (Rabinovitch *et al*., 1996) along with nitrotyrosine positive β-cells indicative of cellular production of peroxynitrite (Suarez-Pinzon *et al*., 1997). Knockout mice lacking iNOS have also been shown to be less susceptible to MLDS induced diabetes (Flodstrom *et al*., 1999).

The PARS inhibitors used to date have been shown to have other protective actions in cells including inhibition of iNOS expression (Andrade *et al*., 1996) and hydroxyl radical scavenging (Wilson *et al*., 1984). To specifically evaluate the role of PARS in type I diabetes we utilized a mouse phenotype which has the PARS gene disrupted ($PARS^{-/-}$) and studied the susceptibility of these mice to MLDS-induced type I diabetes. We also examined the effectiveness of a novel PARS inhibitor, 5-iodo-6-amino-1,2-benzopyrone (INH_2BP), which has no free radical scavenging activities (Szabó *et al*., 1998b) in the MLDS model of diabetes and on the deleterious effects of cytokines on islets of Langerhans and the rat insulinoma cell line RIN-5F, *in vitro*.

Methods

In vivo *studies*

Experimental protocol PARS $^{-/-}$ or $^{+/+}$ mice (Wang *et al*., 1995), 129/SvxC57BL6 background, were treated with streptozotocin (40 mg kg^{-1} dissolved in citrate buffer, pH 4.5) or vehicle (citrate buffer) i.p. for 5 consecutive days $\pm$ INH_2BP (60 mg kg^{-1} day^{-1}) orally starting on day 1. Male BALB/c mice were treated orally for 1 week prior to injection of streptozotocin with INH_2BP at either 20 or 60 mg kg^{-1} day^{-1}. Treatment with INH_2BP was given twice a day orally by gavage throughout the study. Blood glucose was monitored over the following 21 days using a one-touch blood glucose meter (Lifescan). Blood glucose was measured on days 1, 7, 14 and 21 from blood obtained from the tail vein. Hyperglycemia was defined as non-fasting blood glucose level higher than 200 mg dL^{-1}. In both cases the cumulative incidence of diabetes was calculated as a percentage of hyperglycemic mice per treatment group at each time point.

Pancreatic insulin content Samples of pancreas were removed on day 21 and weighed before being placed into 6 ml of acid ethanol (23 : 7 : 0.45, ethanol:dH_2O:HCL), homogenized and incubated for 72 h at 4°C. The samples were centrifuged and the insulin concentration of the supernatant determined using a commercially available ELISA kit (Alpco).

In vitro *studies*

Isolation and culture of islets of Langerhans Rat islets of Langerhans were isolated under aseptic conditions from collagenase-digested pancreata of adult female rats (175–200 g). Batches of 500 islets were cultured in RPMI 1640 media containing 5.5 mM glucose, penicillin (50 U ml^{-1}), streptomycin (50 μg ml^{-1}) and 5% foetal calf serum for 48 h prior to being divided into experimental groups.

Culture of the β-cell line, RIN-5F RIN-5F cells were obtained from the ATCC and maintained in RPMI 1640 media containing 5.5 mM glucose, penicillin (50 U ml^{-1}), streptomycin (50 μg ml^{-1}) and 10% foetal calf serum. The cells were plated into either 12 (4×10^5 cells $well^{-1}$), 24- (2×10^5 cells $well^{-1}$) or 96- (6×10^4 cells $well^{-1}$) well plates and grown to confluence before being used experimentally.

Experimental treatments

A combination of IL-1β, TNF-α and IFN-γ was used in all *in vitro* experiments. Isolated islets were treated with IL-1β (5×10^{-1} M), TNF-α (5×10^{-1} M) and IFN-γ (5 U ml^{-1}) whereas the RIN-5F cells were treated with higher concentrations of cytokines, IL-1β (1×10^{-10} M), TNF-α (1×10^{-10} M) and IFN-γ (10 U ml^{-1}). For the insulin secretion studies and the PARS activation assay the islets or cells were treated for 24 h. For the cell viability and apoptosis studies the cells were treated for 48 h. In all cases the PARS inhibitor, INH_2BP, was added simultaneously with the cytokines. When using peroxynitrite the RIN-5F cells were treated with INH_2BP for 10 min prior to the addition of peroxynitrite (750 μM) for a further 15 min before PARS activity was measured. When treating with streptozotocin (3 mM) for both the cell viability assay and

the PARS assay the INH_2BP was added simultaneously and the cells incubated for 24 h.

Islet insulin secretion response

Batches of 100 isolated rat islets were treated for 24 h with the cytokine combination $\pm INH_2BP$. The culture media was removed and frozen for subsequent determination of nitrite/nitrate levels while the islets were pre-incubated in Gey and Gey physiological buffer (Gey & Gey, 1936) containing 2 mM glucose for 1 h at 37°C. The islets were then washed in fresh 2 mM glucose containing buffer before groups of six islets were handpicked into 1 ml of Gey and Gey buffer containing 20 mM glucose and again incubated for 1 h at 37°C. At the end of the incubation 0.4 ml was removed and assayed for insulin using a commercially available ELISA kit from Alpco. Results were expressed as ng insulin/islet/h.

Measurement of nitrite/nitrate levels

Serum nitrite/nitrate levels were determined by converting the nitrate to nitrite using the enzyme nitrate reductase followed by addition of Griess reagent to colormetrically quantify the nitrite concentration (Green *et al*., 1982). The serum was diluted 1 : 5 in PBS before a 25 μl aliquot was added to a mixture of 25 μl nitrate reductase (1 U 1.5 ml^{-1}) and 25 μl of NADPH (0.134 mg ml^{-1}), both prepared in 40 mM TRIS, pH 7.6. The samples were then incubated at room temperature for 3 h. Following this period 100 μl of Griess reagent (1 : 1 mix of 1% sulphanilamide in 5% phosphoric acid and 0.1% naphyly-ethylenediamine) was added and incubated for a further 10 min at room temperature, the absorbency of the samples was measured at 540 nm with a 650 nm reference. The concentration of nitrite/nitrate was determined from a standard curve of sodium nitrate and calculated as μM nitrite/nitrate.

Measurement of apoptosis

Apoptosis of islet cells following treatment with cytokines was determined using a commercial Cell Death Detection ELISA Plus kit (Roche). The apoptosis assay uses mouse monoclonal antibodies against DNA and histones to specifically determine if mono- and oligonucleosomes are present in the cytoplasmic fraction of cell lysates, a marker of apoptosis. Isolated rat islets of Langerhans were treated with the cytokine combination $\pm INH_2BP$ for 48 h. Groups of 20 islets were then incubated for 2 h in lysis buffer before being centrifuged at $200 \times g$ for 10 min. The cytoplasmic fraction of the sample was then assayed as per the manufacturer's instructions. Results are expressed as an apoptotic index number where the level of apoptosis in the untreated islet cells was given an index of one and the apoptosis in all other treatments was compared to the untreated cells.

Assessment of cell viability

RIN-5F cell viability was determined by the reduction of yellow MTT into a purple formazan product by mitochondrial dehydrogenases of metabolically active cells. RIN-5F cells were grown on 96-well plates until they reached confluency. RIN-5F cells were treated for 48 h with a combination of cytokines $\pm INH_2BP$ or for 24 h with streptozotocin (3 mM) $\pm INH_2BP$. Following this treatment period the media was removed and 200 μl MTT (1 mg ml^{-1}) added. After 3 h the MTT solution was carefully removed and the purple crystals were solubilized in 100 μl of DMSO. The DMSO was transferred to an ELISA plate and absorbance measured at 550 nm with a 620 nm reference (Di Matteo *et al*., 1997). The absorbances obtained from treated cells were expressed as a percentage of absorbances obtained from untreated cells.

PARS assay

RIN-5F cells were treated with either a combination of cytokines $\pm INH_2BP$ for 24 h, peroxynitrite (750 μM) $\pm INH_2BP$ for 15 min or streptozotocin (3 mM) $\pm INH_2BP$ for 24 h. For the measurement of PARS activity (Szabo *et al*., 1998b), the media was removed and replaced with 0.5 ml HEPES (pH 7.5) containing 0.01% digitonin and [^{3}H]-AND (0.5 μCi ml^{-1}) for 20 min. The cells were then scraped from the wells and placed in an Eppendorf tubes containing 200 μl of ice-cold 50% TCA (w v^{-1}), the tubes were then placed at 4°C. After 4 h the tubes were centrifuged at $1800 \times g$ for 10 min and the supernatant removed, the pellet was washed twice with 500 μl ice-cold 5% TCA. The pellet was solubilized in 250 μl NaOH (0.1 M) containing 2% SDS overnight at 37°C, the PARS activity was then determined by measuring the radioactivity incorporated using a Wallac scintillation counter. The solubilized protein (250 μl) was mixed with 5 ml of scintillant (ScintiSafe Plus, Fisher) before being counted for 10 min. Results are expressed as per cent increase over activity measured in untreated cells.

Protein synthesis assay

RIN-5F cell *de novo* protein synthesis was determined as incorporation of [^{3}H]-amino acids into newly synthesized, perchloric acid (PCA) precipitable protein. RIN-5F cells were treated with the cytokine combination $\pm INH_2BP$ in media containing a mixture of [^{3}H]-amino acids (100,000 d.p.m. $well^{-1}$) for 24 h. Following treatment the media was removed and the cellular protein precipitated by adding 800 μl ice-cold 5% PCA to each well and leaving the plate on ice for 30 min. The PCA was then removed and the protein solubilized in 200 μl sodium hydroxide (0.1 M) for 30 min at room temperature (Di Matteo *et al*., 1997). [^{3}H]-amino acid incorporation into protein was measured by liquid scintillation counting and results expressed as a percentage of the d.p.m.'s obtained from untreated cells.

Statistical analysis

The data are presented as mean $\pm$ s.e.mean, statistical analysis was preformed either using the χ^2 test or ANOVA with a P value of less than 0.05 considered significant.

Results

Effect of MLDS in $PARS^{+/+}$ and $^{-/-}$ mice

Treatment of $PARS^{+/+}$ mice with MLDS resulted in a progressive hyperglycemia (Figure 1). On Day 21, the

streptozotocin treated PARS$^{+/+}$ mice had a significantly higher blood glucose level compared to vehicle treated mice (Figure 1A). PARS$^{-/-}$ mice also developed a progressive hyperglycemia following streptozotocin treatment, but this was significantly lower compared to PARS$^{+/+}$ mice (Figure 1B), with the median blood glucose on day 21 being 314 and 142 in the PARS$^{+/+}$ and $^{-/-}$ mice respectively. On day 21 significantly fewer PARS$^{-/-}$ mice were diabetic (blood glucose greater than 200 mg dL^{-1}) compared to the wild-type animals; 24% compared to 81% (Figure 1C,D). Treatment of PARS$^{+/+}$ mice with the PARS inhibitor INH_2BP (60 mg kg^{-1} day^{-1}), protected the mice against both the streptozotocin induced hyperglycemia (Figure 1A) and induction of diabetes (Figure 1C). Interestingly and unexpectedly, the reverse was found in the PARS$^{-/-}$ mice: treatment with INH_2BP (60 mg kg^{-1} day^{-1}) increased the incidence of diabetes from 24 to 60% (Figure 1D) and though the mean blood glucose levels on day 21 were not significantly different (Figure 1B) the median blood glucose increased from 142 to 224 mg dL^{-1} with INH_2BP treatment.

Streptozotocin treatment of PARS$^{+/+}$ mice dramatically reduced pancreatic insulin content by 70%; treatment with the PARS inhibitor partially reversed this effect (Figure 2a). There was no significant decrease in pancreas insulin content of PARS$^{-/-}$ mice treated with streptozotocin, unlike in mice treated with both streptozotocin and INH_2BP where the pancreas insulin content was reduced by more than 80% (Figure 2b).

Effect of the PARS inhibitor, INH_2BP on MLDS induced diabetes in male BALB/c mice

The PARS inhibitor INH_2BP dose dependently attenuated the increase in mean blood glucose levels in response to streptozotocin (Figure 3A). There was also a significant decrease in the percentage of mice having a blood glucose greater than 200 mg dL^{-1} on day 21, from 83 to 58% in the

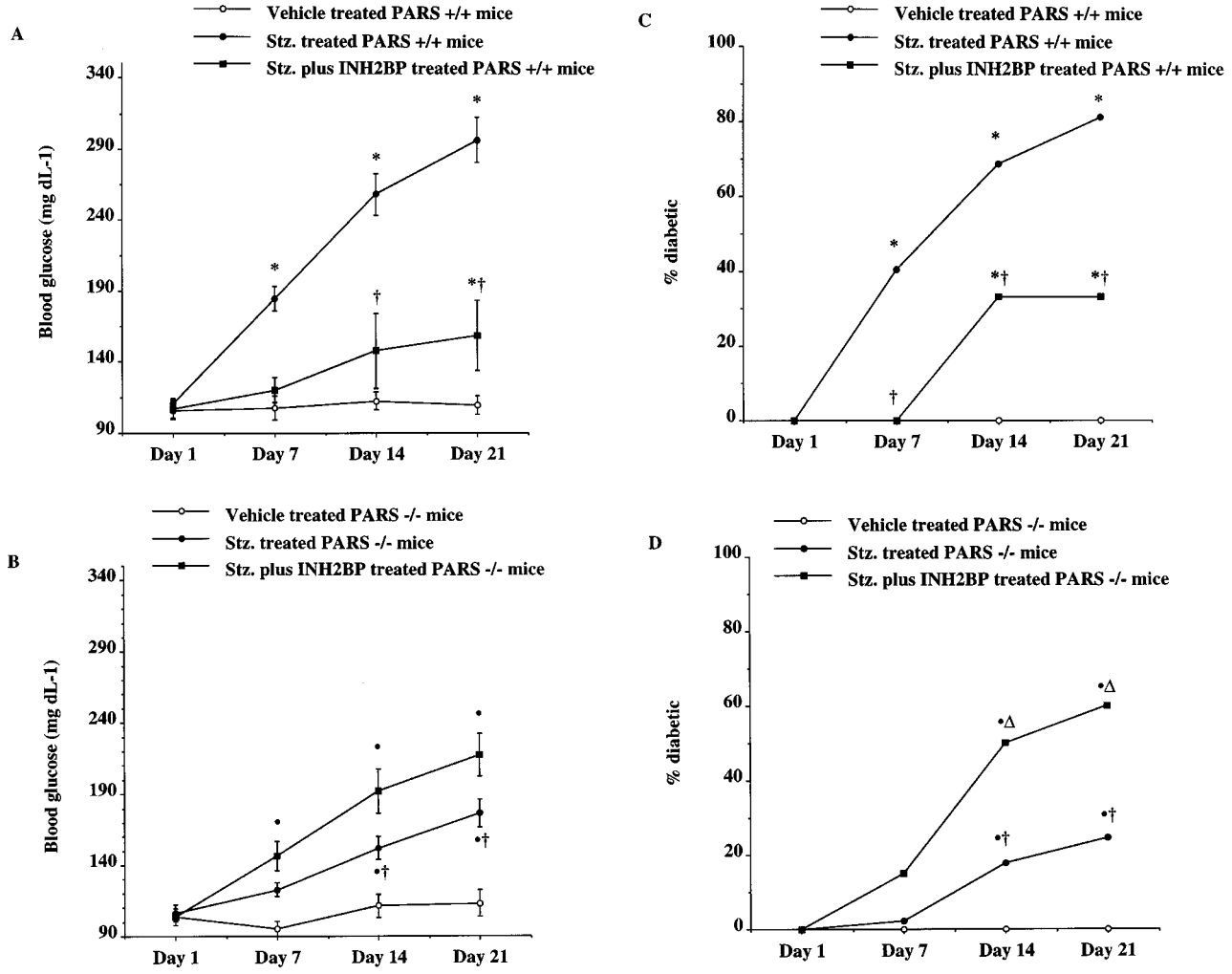

Figure 1 Effect of MLDS ± INH_2BP (60 mg kg^{-1} day^{-1}) on blood glucose levels and diabetes incidence in PARS$^{+/+}$ mice (A,C) or PARS$^{-/-}$ mice (B,D). Blood glucose levels are shown as shown as mean ± s.e.mean of mg glucose dL^{-1} ($n=9$ for vehicle treated mice, $n=20$ for mice of both phenotypes treated with MLDS and INH_2BP and $n=40$ for mice treated with MLDS alone), incidence of diabetes expressed as a cumulative percentage of mice with a blood glucose level greater than 200 mg dL^{-1} over the 21 day period. Statistical analysis was performed using ANOVA and χ^2 where $P<0.05$ was considered significant. *$P<0.05$ *vs* untreated PARS$^{+/+}$ mice; †$P<0.05$ *vs* streptozotocin treated PARS$^{+/+}$ mice, ·$P<0.05$ *vs* untreated PARS$^{-/-}$ mice and Δ$P<0.05$ *vs* streptozotocin treated PARS$^{-/-}$ mice.

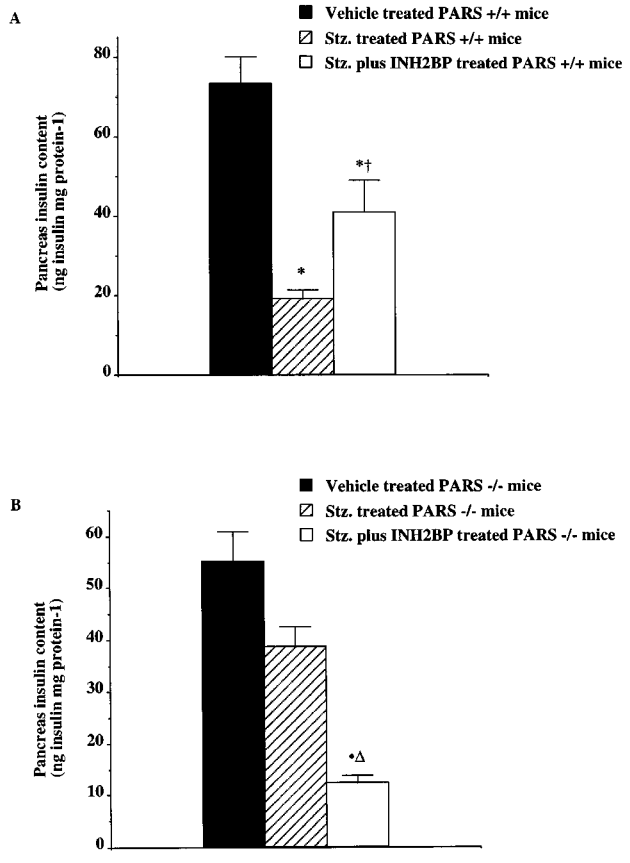

Figure 2 Pancreas insulin content on day 21 in $PARS^{+/+}$ (A) and $PARS^{-/-}$ (B) mice following MLDS$\pm$$INH_2BP$ (60 mg kg^{-1} day^{-1}). Results expressed as ng insulin mg^{-1} pancreas protein and shown as mean$\pm$s.e.mean ($n=9$ for vehicle treated mice, $n=20$ for mice of both phenotypes treated with MLDS and INH_2BP and $n=40$ for mice treated with MLDS alone). Statistical analysis was carried out using ANOVA, $*P<0.05$ *vs* Untreated $PARS^{+/+}$ mice; $\dagger P<0.05$ *vs* streptozotocin treated $PARS^{+/+}$ mice, $\bullet P<0.05$ *vs* untreated $PARS^{-/-}$ mice and Δ $P<0.05$ *vs* streptozotocin treated $PARS^{-/-}$ mice.

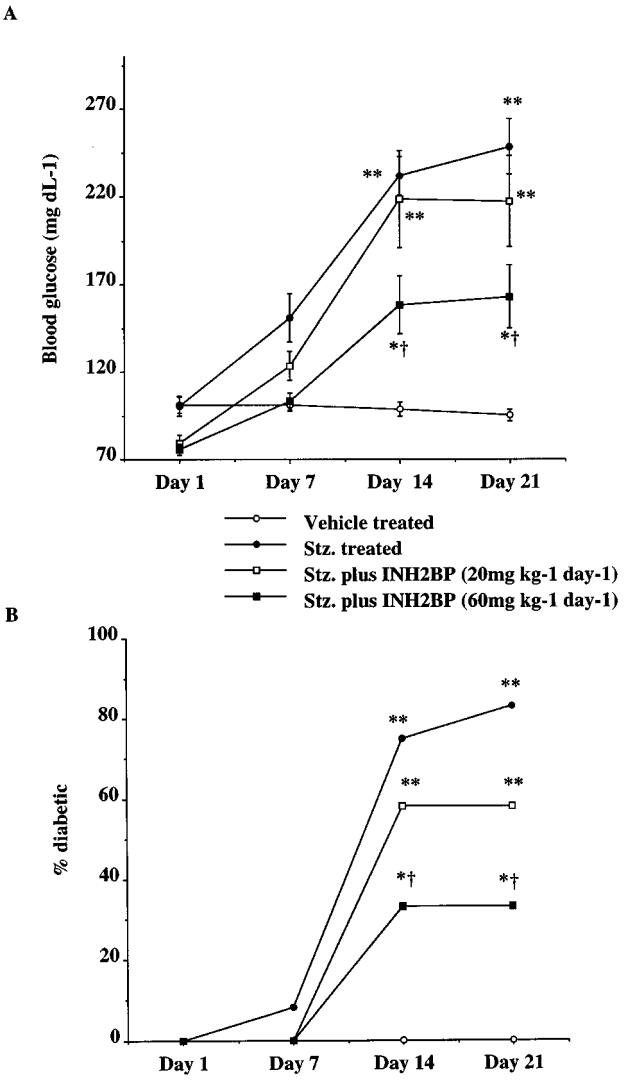

Figure 3 Dose dependent effect of INH_2BP on (A) hyperglycemia shown as mean$\pm$s.e.mean mg glucose dl^{-1} diabetes incidence, and (B) diabetes incidence shown as cumulative percentage of mice with blood glucose greater than 200 mg dL^{-1} in male BALB/c mice following treatment with MLDS (12 mice per experimental group). Statistical analysis was performed using ANOVA and χ^2 test where $P<0.05$ was considered significant. $*P<0.05$ and $**P<0.01$ *vs* untreated mice and $\dagger P<0.05$ *vs* streptozotocin treated mice.

group treated with 20 mg kg^{-1} day^{-1} and 33% in the group treated with 60 mg kg^{-1} day^{-1} (Figure 3B). On Day 21, the serum nitrite/nitrate levels were increased in mice treated with streptozotocin compared to vehicle treated mice, INH_2BP had no effect on nitrate levels (Figure 4A). Pancreatic insulin content was reduced in the BALB/c strain of mouse following streptozotocin treatment and this was prevented by both 20 and 60 mg kg^{-1} day^{-1} INH_2BP (Figure 4B).

In vitro *effects of INH_2BP on insulin secretion, nitric oxide formation and apoptosis in isolated islets of Langerhans*

Exposure of cultured rat islets to IL-1β, TNF-α and IFN-γ for 24 h resulted in an inhibition of the insulin secretory response to 20 mM glucose (Figure 5A). Treatment for 24 h with INH_2BP alone had a small but significant inhibitory effect on insulin secretion from rat islets in response to 20 mM glucose (Figure 5A). However, INH_2BP, when applied simultaneously with the cytokine combination, dose dependently reversed the inhibition of insulin secretion (Figure 5A) to the levels observed with INH_2BP treatment alone. Combined cytokines increased islet NO formation (Figure 5B), an effect which was not influenced by simultaneous treatment with INH_2BP. Treatment of rat islets for 48 h with combined cytokines increased the level of apoptosis; INH_2BP had no effect either alone or in combination with cytokines on the levels of apoptosis observed (Figure 5C).

INH_2BP effects on RIN-5F cells

Treatment of RIN-5F cells for 48 h with combined cytokines reduced cell viability by 30% which was reversed dose dependently by simultaneous addition of INH_2BP (Figure 6A). Treatment of RIN-5F cells with cytokines for 24 h decreased protein synthesis (Figure 6B and effect which was not prevented by INH_2BP but actually synergistically enhanced as INH_2BP alone decreased protein synthesis

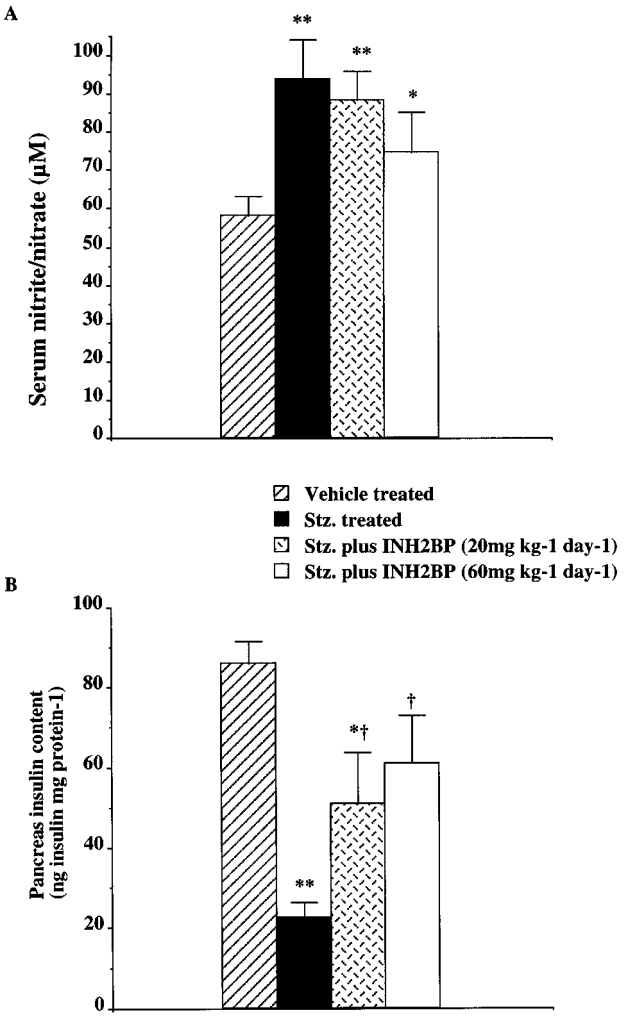

Figure 4 Effect of MLDS on (A) serum nitrite levels (μM) and (B) pancreas insulin content (ng insulin mg^{-1} pancreatic protein) on day 21 in male BALB/c mice. Data shown as mean$\pm$s.e.mean from 12 mice per experimental group. Statistical analysis was performed using ANOVA where $^{*}P<0.05$ and $^{**}P<0.01$ *vs* untreated mice and $\dagger P<0.05$ *vs* streptozotocin treated mice.

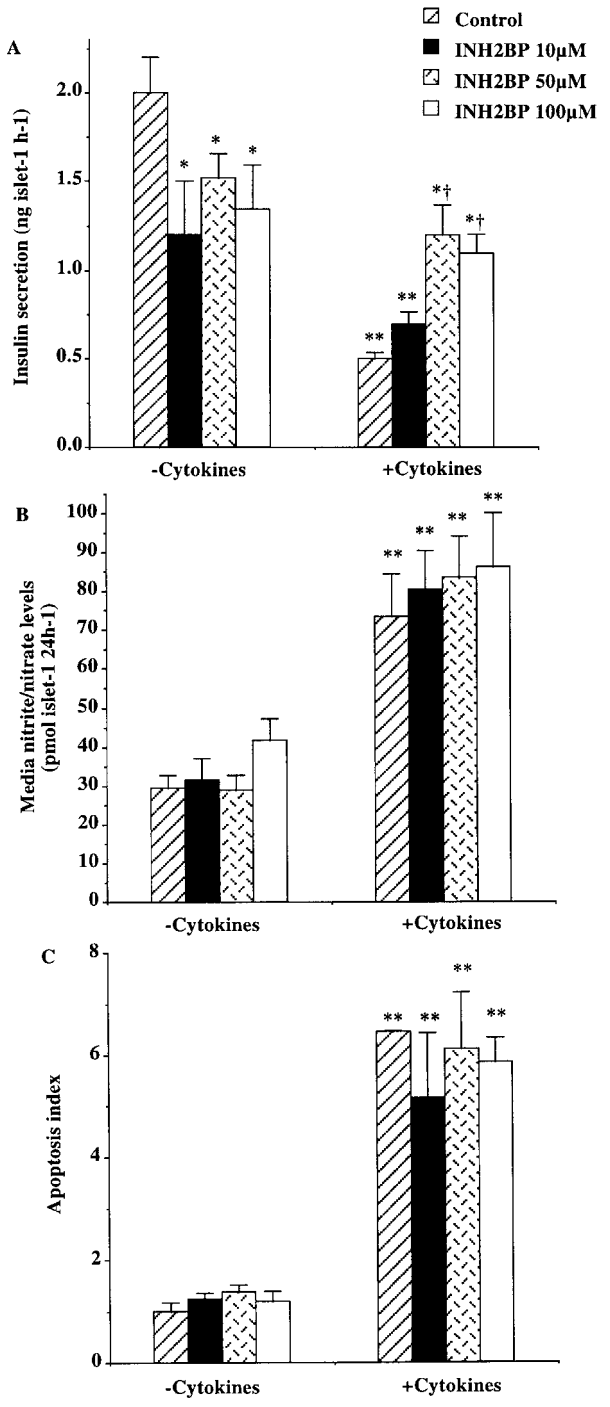

Figure 5 *In vitro* effects of INH_2BP on isolated rat islets of Langerhans treated with cytokines. (A) Acute 20 mM glucose-stimulated insulin secretory response of islets following 24 h treatment with cytokines$\pm INH_2BP$. (B) Effect of INH_2BP on 24 h cytokine-stimulated NO formation. (C) Effect of INH_2BP on 48 h cytokine-induced apoptotic cell death in rat islets of Langerhans. Data is expressed as mean$\pm$s.e.mean from three separate islet isolations. Statistical analysis was performed using ANOVA where $^{*}P<0.05$ and $^{**}P<0.01$ *vs* untreated islets and $\dagger P<0.05$ *vs* cytokine treated islets.

(Figure 6B). INH_2BP treatment, as previously observed in the islet experiments, had no effect on the cytokine-mediated increased NO formation (Figure 6C).

Cytokines and peroxynitrite activate PARS in RIN-5F cells

Combined cytokine treatment resulted in a significant increase in PARS activity above that seen in the control, INH_2BP prevented this increase in PARS activity by cytokine treatment (Figure 7A). Application of peroxynitrite (750 μM) to RIN-5F cells for 15 min resulted in a more pronounced increase in PARS activity, which was dose dependently inhibited by INH_2BP (Figure 7B).

Streptozotocin decreases cell viability and activates PARS in RIN-5F cells

Treatment of RIN-5F cells with streptozotocin (3 mM) for 24 h significantly reduced cell viability, as measured by the MTT assay, to 66$\pm$2.7% ($n=3$, $P<0.05$ *vs* vehicle treated cells). Simultaneous treatment with INH_2BP dose dependently prevented this loss of cell viability with RIN-5F cell

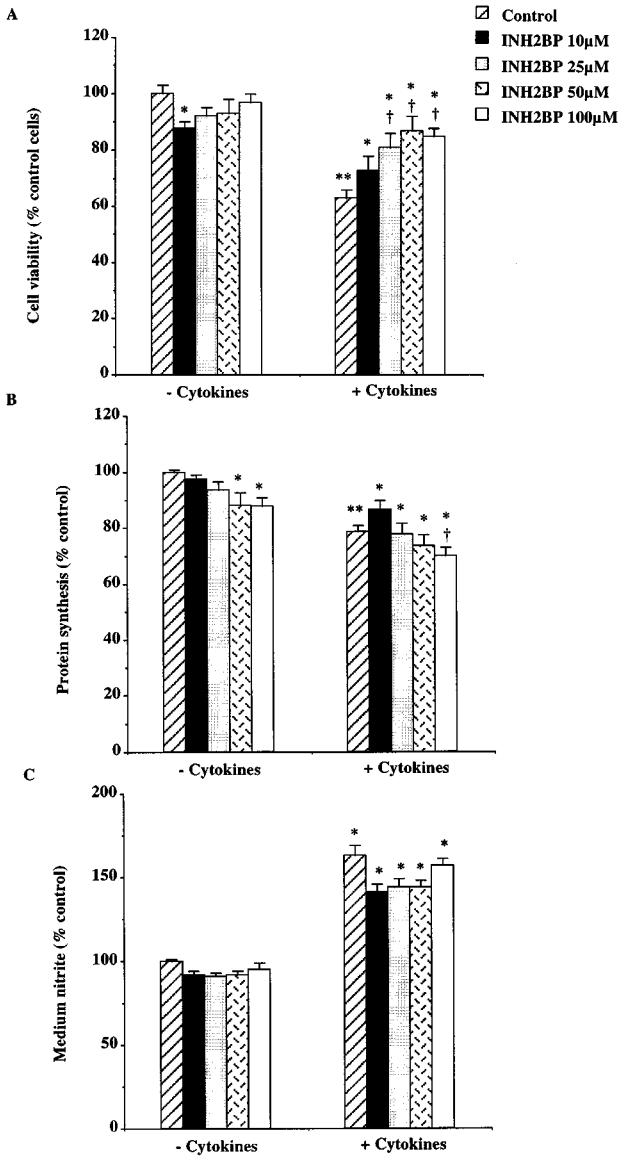

Figure 6 Effect of INH_2BP on (A) 48 h cytokine-mediated decrease in cell viability, (B) 24 h cytokine-mediated decrease in protein synthesis and (C) 48 h cytokine-induced NO formation in RIN-5F cells. Data is expressed as a percentage of untreated cells and displayed as mean ± s.e.mean from 3–4 separate experiments. Statistical significance was determined using ANOVA where $^*P<0.05$ and $^{**}P<0.01$ *vs* untreated cells and $\dagger P<0.05$ *vs* cytokine treated cells.

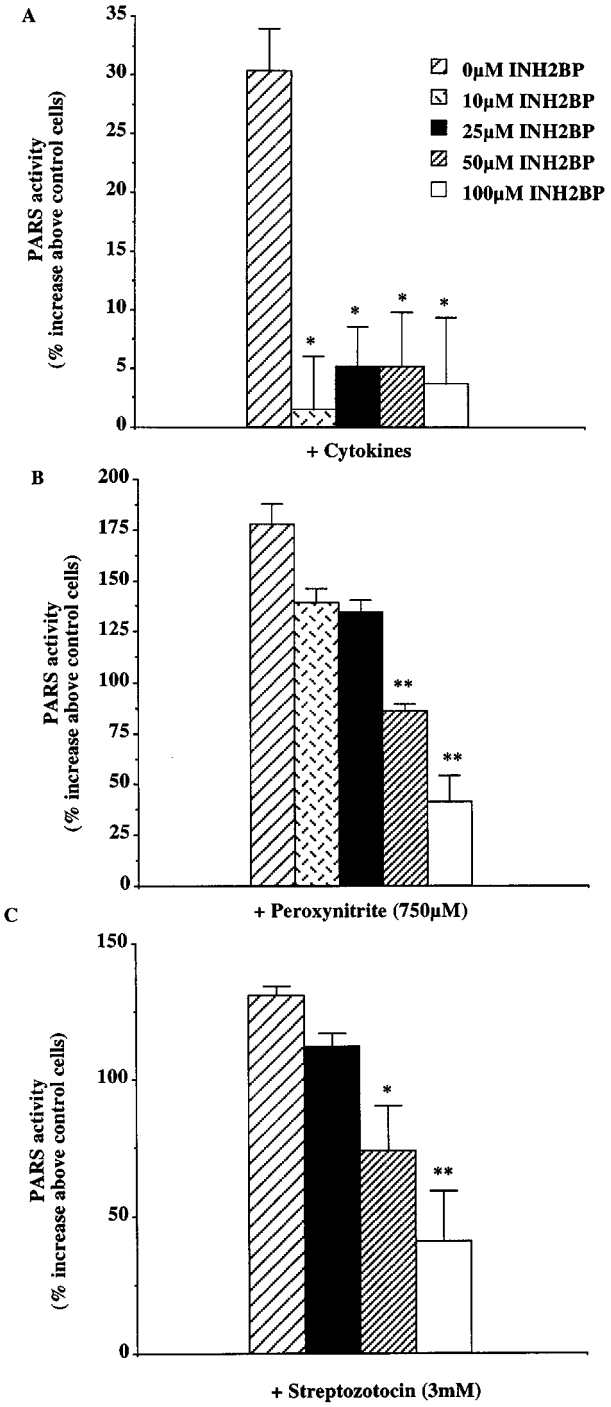

Figure 7 Cytokines (A), peroxynitrite (B) and streptozotocin (C) activate PARS in RIN-5F cells: inhibition by INH_2BP. Data is expressed as percentage increases in c.p.m. over untreated ß-cells and plotted as mean ± s.e.mean from three separate experiments. Statistical significance was determined using ANOVA where $^*P<0.05$ and $^{**}P<0.01$ *vs* untreated RIN-5F cells and $\dagger P<0.05$ *vs* RIN-5F cells treated with either cytokines, peroxynitrite or streptozotocin.

viability being reduced to 60 ± 1.8%, 94 ± 2.8 ($P<0.01$ *vs* streptozotocin treated cells) and 91 ± 1.7% ($P<0.01$ *vs* streptozotocin treated cells), with simultaneous addition of 25, 50 and 100 μM INH_2BP respectively. Treatment with streptozotocin (3 mM) for 24 h also activated PARS (Figure 7C), an effect reversed by INH_2BP.

Discussion

Our data suggests that activation of the enzyme poly (ADP-ribose) synthetase plays a significant role in the development of type I diabetes: inhibiting PARS by either gene disruption or by using a specific inhibitor protected against MLDS induced diabetes in mice, a model of type I diabetes in humans. Previously only the less potent PARS inhibitors such as 3-aminobenzamide and nicotinamide (Masiello *et al.*, 1985) have been used to protect against Type I diabetes.

However, both agents have additional pharmacological characteristics, including scavenging of hydroxyl radicals (Wilson *et al*., 1984; Szabó *et al*., 1999a) and inhibition of NO generation (Andrade *et al*., 1996), effects which may play significant additional roles in their mechanism of action.

In the current study we have found that $PARS^{-/-}$ mice are less susceptible to MLDS-induced diabetes, having a lower incidence of diabetes and hyperglycemia demonstrating for the first time that PARS activation specifically plays a role in this animal model of Type I diabetes. A novel, potent inhibitor of PARS, INH_2BP (Szabó *et al*., 1998b), which lacks oxyradical or peroxynitrite scavenging activities, also proved to be effective in protecting both strains of mice, the 129SVxC57BL6 $PARS^{+/+}$ and BALB/c, from MLDS-induced diabetes and hyperglycemia. We also confirmed a previously reported difference in sensitivity to streptozotocin between the two strains (Cardinal *et al*., 1999). The current studies are in line with a series of recent reports, which were published during the completion of the current studies, demonstrating that mice lacking functional PARS enzyme are protected against streptozotocin-induced diabetes (Burkart *et al*., 1999; Pieper *et al*., 1999). It is noteworthy, nevertheless, that in the above listed studies, a single high dose of streptozotocin was administered, and the protection obtained in the PARS deficient mice was more pronounced than the protection seen in the current study. The explanation for this difference may lay in the disparity between the models of diabetes employed: the MLDS model has a smaller direct oxidant component and a higher immune component, in which the role of PARS activation may be relatively smaller. We propose that $PARS^{-/-}$ mice are protected from the single large dose of STZ by preventing the massive β-cell necrosis directly induced by this chemical agent. β-cell destruction in the MLDS-model of type I diabetes may be more relevant to the human condition, as there is a significant immune cell component in the destructive process (Rossini *et al*., 1977) with increased expression of cytokines (both Th1 and Th2) in the pancreas (Herold *et al*., 1996).

During the course of our study, however, an interesting and unexpected phenomenon was noted. $PARS^{+/+}$ mice treated with streptozotocin show the classic progressive hyperglycemia and increase in diabetes incidence, effects attenuated by PARS gene disruption or inhibition by INH_2BP. Treatment of $PARS^{-/-}$ mice with the PARS inhibitor INH_2BP, however, increased their sensitivity to streptozotocin, with treated mice showing increased blood glucose and incidence of diabetes comparable to untreated $PARS^{-/-}$ mice. We do not believe that a direct cytotoxic effect of INH2BP can contribute to this action, as INH_2BP itself is not diabatogenic in normal mice and, even at very high *in vitro* doses, only moderately cytotoxic to islets. Recently novel isoforms of PARS have been identified (Babiychuk *et al*., 1998; Smith *et al*., 1998) and Pieper *et al*. (1999) found significant residual PARS activity in most tissues of $PARS^{-/-}$ mice probably related to the existence and catalytic activity of these minor PARS isoforms. The $PARS^{-/-}$ mice used in this study are deficient in poly (ADP-ribose) synthetase-1 (PARS-1), with the other isoforms still present and functional (Wang *et al*., 1995; Vodenicharov *et al*., 2000). It is conceivable that application of a PARS inhibitor to $PARS^{-/-}$ mice reduces the residual PARS activity to critically low levels impairing the cells ability to respond to stress. The result of this increased damage would be a larger number of β-cells undergoing necrosis, thereby reducing β-cell mass and enhancing immune activation to such an extent as to offset the protective effect of disrupting the PARS-1 gene. It is evident from our data that many more β-cells have been destroyed following streptozotocin treatment in the INH_2BP treated $PARS^{-/-}$ mice, since the insulin content of the pancreas was reduced by more than 80%, compared to no reduction in the untreated $PARS^{-/-}$ mice. The dramatic effect of the specific β-cell toxin, streptozotocin, on the insulin content of the pancreas of $PARS^{-/-}$ treated with a specific PARS inhibitor mice may suggest the presence of residual pancreatic PARS activity which is essential to β-cell survival.

Inhibition of iNOS activity has been shown to protect from MLDS-induced diabetes (Flodstrom *et al*., 1999; Southan *et al*., 2000). Recently $PARS^{-/-}$ mice were reported to be protected from systemic inflammation induced with high doses of endotoxin, an effect which was mediated by a down regulation of iNOS expression and subsequent NO formation an effect related to inhibition of activation of NF-κB (Oliver *et al*., 1999). MLDS induced a significant increase in serum levels of nitrite/nitrate indicative of an increase in NO formation, probably resulting from the inducible isoform of NO synthase being expressed in immune and β-cells, which was unaffected by PARS inhibition. The unaffected serum nitrite/nitrate levels by the PARS inhibition indicates that the immune system is still activated and hence inhibition of PARS is not fully effective in preventing streptozotocin-mediated damage of the β-cells. Apoptosis has been reported to be a major pathway of β-cell death in the development of IDDM in the MLDS model (O'Brien *et al*., 1996). Since inhibition of PARS in the current study failed to prevent apoptosis in islet cells treated with cytokines *in vitro*, it is likely that the protection of β-cells *in vivo* is mediated by an inhibition of necrosis, possibly thereby reducing immune cell activation and infiltration into the pancreatic islet. PARS inhibitors have been reported to reduce immune cell infiltration into tissues in various animal models of inflammation including arthritis and peritonitis (Szabó & Dawson, 1998a).

The PARS inhibitor INH_2BP, was able to partially reverse cytokine mediated inhibition of glucose stimulated insulin secretion from isolated rat islets of Langerhans. Treatment with INH_2BP alone had a small inhibitory effect on glucose-stimulated insulin secretion, simultaneous treatment of islets with INH_2BP and cytokines dose dependently reversed the cytokine-mediated inhibition of insulin secretion, returning it to the levels observed with INH_2BP alone. This effect was not related to an effect on cytokine-mediated induction of NO synthase, since only minor effects on medium nitrite/nitrate levels were observed. Recently PARS activation has been shown to promote the activation of NF-κB (Oliver *et al*., 1999), a transcription factor proposed to be essential for induction of NO synthase in islet β-cells (Flodstrom *et al*., 1996). However, it appears that the promotion of NF-κB activation by PARS is more related to the physical presence of the enzyme (through molecular scaffolding functions, and physical association), rather than its catalytic activity, and can be inhibited in PARS deficient cells, but less so by pharmacological PARS inhibitors (Oliver *et al*., 1999;

Soriano *et al*., 2001). The lack of effect of INH_2BP on NO formation further strengthens this notion. In previous studies, nicotinamide has been shown to have either no effect (Bolaffi *et al*., 1994) or a partial protective effect (Buscema *et al*., 1992) against cytokine-inhibited stimulated insulin release from islets. Nicotinamide was able to reverse the inhibitory effects of IL-1β on accumulated insulin release (Andersen *et al*., 1994). The effect of nicotinamide was only seen at millimolar concentrations (Andersen *et al*., 1994), and might be, partially or fully, related to the compound's antioxidant effects. In contrast, INH_2BP exerted its effects at the micromolar level. Pro-inflammatory cytokines have previously been shown to reduce ATP levels in islets (Sandler *et al*., 1990) which may be caused by activation of PARS. INH_2BP may therefore prevent the decrease intracellular ATP levels and thereby reverse the cytokine-mediated inhibition of insulin secretion.

INH_2BP prevented the loss of cell viability in the rat insulinoma cell line, RIN-5F, but had no effect on cytokine-mediated apoptosis of islet cells. Similar effects were also observed with nicotinamide (Bolaffi *et al*., 1994; Hoorens & Pipeleers, 1999). The lack of protection from cytokine-mediated apoptosis is not surprising, INH_2BP or PARS deficiency does not inhibit apoptosis in many other cellular systems (Virag *et al*., 1998). In fact, PARS activation may shift the pattern of cell death from necrosis towards apoptosis (Nicotera *et al*., 1998; Virag *et al*., 1998), because by maintaining cellular energy balance, it provides energy for the cell to complete the process of apoptosis, which is an energy demanding process (Virag *et al*., 1998; Nicotera *et al*., 1998). Recently another PARS inhibitor, nicotinamide, was been shown to only protect human islets from chemically induced necrosis and not apoptosis (Hoorens & Pipeleers, 1999). INH_2BP failed to prevent the inhibition of protein synthesis by cytokines, and, in fact, when applied alone, had a small but significant inhibitory effect, which synergized with the cytokine-mediated inhibition of protein synthesis. PARS has been proposed to play a role in mediating gene expression (Szabó & Dawson, 1998a; Oliver *et al*., 1999), and it is conceivable that PARS also plays such a role in the β-cell.

Based on the above data, we hypothesize that one of the mechanisms of protection against diabetes by PARS inhibition is by protecting against β-cell necrosis. This protection is likely to be against both the direct effect of streptozotocin and/or against immune cell mediated necrosis induced by peroxynitrite or cytokines. Indeed INH_2BP protects the RIN-5F cells from both the loss of cell viability induced by streptozotocin as well as preventing activation of PARS induced either with streptozotocin, cytokines or peroxynitrite. Islet cell necrosis has been observed in animal models of diabetes and in early onset type I diabetic humans. Furthermore, necrosis has been proposed to be the trigger for the immune system to invade the islets *via* the immune system being exposed to antigens from inside the β-cell, leading to the destruction of the β-cells by an autoimmune process. In the MLDS model it is proposed that each injection of STZ damages a fraction of the β-cells, probably by necrosis, eventually leading to a local inflammation and infiltration by mononuclear cells (Kolb & Krönke, 1993), which culminates in widespread β-cell destruction. Therefore PARS inhibition reduces the β-cell damage and hence will slow down islet infiltration and immune cell attack on the β-cells, preventing or delaying β-cell death. The current study demonstrates that PARS inhibition, possibly in combination with other therapies which inhibit β-cell apoptosis, may be a promising strategy to delay or prevent development of Type I diabetes.

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