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IL-1 β Receptor Blockade Protects Islets Against Pro-inflammatory Cytokine Induced Necrosis and Apoptosis

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Abstract

Pro-inflammatory cytokines (PIC) impair islet viability and function by activating inflammatory pathways that induce both necrosis and apoptosis. The aim of this study was to utilize an in vitro rat islet model to evaluate the efficacy of a clinically approved IL-1 receptor antagonist (Anakinra) in blocking PIC induced islet impairment. Isolated rat islets were cultured for 48h $\pm$ PIC (IL-1 β , IFN γ , and TNF α and $\pm$ IL-1ra then assayed for cellular integrity by flow cytometry, MAPK phosphorylation by proteome array, and gene expression by RT-PCR. Nitric oxide (NO) release into the culture media was measured by Griess reaction. Islet functional potency was tested by glucose stimulated insulin secretion (GSIS) and by transplantation into streptozotocin-induced diabetic NOD.scid mice. Rat islets cultured with PIC upregulated genes for NOS2a, COX2, IL6, IL1b, TNFa, and HMOX1. IL-1ra prevented the PIC induced upregulation of all of these genes except for TNFa. Inhibition of PIC induced iNOS by NG-monomethyl-L-arginine (NMMA) only blocked the increased expression of HMOX1. IL-1ra completely abrogated the effects of PIC with respect to NO production, necrosis, apoptosis, mitochondrial dysfunction, GSIS, and in vivo potency. IL-1ra was not effective at preventing the induction of necrosis or apoptosis by exogenous NO. These data demonstrate that Anakinra is an effective agent to inhibit the activation of IL-1 β dependent inflammatory pathways in cultured rat islets and support the extension of its application to human islets in vitro and potentially as a post transplant therapy.

Keywords

Islet; Cytokines; Apoptosis; Flow Cytometry; IL-1ra

INTRODUCTION

Isolated pancreatic islet transplantation offers the benefit of improved glycemic control for a subgroup of patients with type 1 diabetes and hypoglycemia unawareness. However, the success of this therapy is currently limited by the efficiency of islet isolation from the pancreas and the durability of the islets after transplantation. The majority of islet transplant patients reaching insulin independence will require restoration of exogenous insulin therapy within 5 years to restore normoglycemia (Shapiro et al., 2006). The loss of islet mass and function can be attributed to a multitude of compounding factors including ischemia induced at procurement and during organ transportation, the stresses of the islet isolation procedure,

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and the microenvironment of the engraftment site within the liver. Within moments after implantation within the liver, a cascade of non-specific inflammatory events are initiated which include activation of blood-mediated coagulation pathways (Bennet et al., 2000) and release of pro-inflammatory cytokines (PIC) primarily from macrophage and endothelial cells (Barshes et al., 2005; Kaufman et al., 1990; Nagata et al., 1990; Stevens et al., 1994).

A significant body of research has demonstrated that rodent and human islets are highly sensitive to the actions of interleukin-1 β (IL-1 β), tumor necrosis factor alpha (TNF α), and interferon gamma (IFN γ) (Corbett et al., 1993a; Corbett et al., 1993b; Xenos et al., 1994). The release of cytokines by macrophages resident within the islet is known to occur during in vitro culture (Arnush et al., 1998). In addition, in vivo macrophage production of PIC has been shown in rats after islet transplant under the kidney capsule and within the liver (Montolio et al., 2007; Bottino et al., 1998). In vitro culture of islets or beta cell lines with exogenous IL-1 β , TNF α , and IFN γ is an established model by which apoptotic and necrotic pathways of cell death are induced. The effector mechanisms by which these cytokines induce beta cell death encompass both nitric oxide (NO) dependent and independent pathways (Eizirik and Mandrup-Poulsen, 2001). In rodent islets, IL-1 β alone can induce iNOS expression and the resultant NO production impairs beta cell function by oxidation of mitochondrial aconitase which leads to diminished glucose oxidation and ATP production (Scarim et al., 1997). Steer and Corbett have shown that this NO-mediated pathway of beta cell death is primarily necrotic indicated by release of the high mobility group box 1 protein (HMGB1) (Steer et al., 2006). In contrast, human islets appear to be more resistant to cytokine induced NO production and the primary mechanism of dysfunction may be the result of mitochondrial reactive oxygen species (ROS) production and activation of pro-apoptotic caspase enzymes (Cnop et al., 2005). In either case, the prevention of PIC induced beta cell impairment and death is of primary importance for the successful culture and transplantation of islets.

A variety of specific and non-specific inhibitors of reactive nitrogen and oxygen species have been evaluated for their effects on islets in culture and in vivo post transplant. Inhibition of iNOS by culture with NG-monomethyl-L-arginine (NMMA) or aminoguanidine can block NO production by both rodent and human islets (Corbett et al., 1993c; Misko et al., 1993). Treatment with N-acetyl cysteine or glutathione peroxidase can enhance the antioxidant capacity of islet beta cells (Kaneto et al., 1999; Tanaka et al., 2002). While these methodologies have been observed to provide protection from glucotoxicity and cytokine induced impairment of rodent and human islets, we hypothesized that a better strategy would be to block the initiation of the inflammatory pathways triggered by the interaction of IL-1 β with its cellular receptor.

In this study we evaluated the effectiveness of the IL-1 receptor antagonist (Kineret[®], (Anakinra)) in blocking the pro-apoptotic and -necrotic effects of exogenous IL-1 β , TNF α , and IFN γ on cultured rat islets. Other studies have successfully targeted the interaction of IL-1 β with its receptor on islet beta cells using the IL-1ra (Corbett and McDaniel, 1995; Rydgren et al., 2006; Sandberg et al., 1994; Tellez et al., 2005; Welsh et al., 1995) However, unlike the IL-1ra used in previous studies, Anakinra is a U.S. FDA approved drug which has been evaluated as an anti-inflammatory therapy in clinical trials of human subjects with rheumatoid arthritis (Botsios et al., 2007) and type 2 diabetes (Larsen et al., 2007). It is a recombinant, nonglycosylated form of the human interleukin-1 receptor antagonist (IL-1ra) consisting of 153 amino acids with a molecular weight of 17.3 kilodaltons. The biologic activity of Anakinra derives from its ability to competitively inhibit IL-1 binding to the interleukin-1 type I receptor (IL-1RI). Rat islets cultured $\pm$ PIC $\pm$ IL-1ra were compared with respect to MAPK pathway activation, inflammatory gene expression, nitrite formation, mitochondrial integrity and function, viability, apoptosis, glucose stimulated insulin

secretory capacity and in vivo function after transplant into streptozotocin-induced diabetic immunodeficient mice.

MATERIALS AND METHODS

Rat islet isolation and culture

Rat islets were isolated from male Lewis rats (Harlan Sprague Dawley, Indianapolis, IN) according to guidelines established and approved by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. Animals were anesthetized by 3% isoflurane or injected i.p. with Ketamine/Xylazine [80/8 mg/kg]. Digestion solution containing collagenase type V (Sigma-Aldrich, St Louis, MO) and DNase (Roche Diagnostics, Indianapolis, IN) was injected *in situ* via the pancreatic common bile duct. Islets were purified by centrifugation on a discontinuous Ficoll gradient (Mediatech Inc., Manassas, VA). Purified islets were cultured $\pm$ cytokines (1000 U/mL TNF α , 1000 U/mL IFN γ , 50 U/mL IL1 β ; R&D Systems, Inc., Minneapolis, MN); IL-1 β receptor antagonist (10 μ g/ml AnakinraTM, Amgen, Thousand Oaks, CA); DEANO (500 μ Mol diethylamine nitric oxide, Invitrogen, Eugene, OR) or NMMA (2 mMol N-monomethyl-L-arginine, EMD Biosciences, Inc., San Diego, CA) for 48 hours in CMRL 1066 media (Mediatech) containing 10% fetal bovine serum at 37°C in a 5% CO₂ incubator.

Multi-parametric flow cytometry assessments

Islets were dispersed into single cell suspensions by incubation with trypsin [0.05%] and EDTA [0.53 mM] for 5 minutes at 37°C followed by passage through a narrow gauge pipet tip. Dispersed cells were suspended in Krebs buffer (KRB) containing 3.3 mM glucose and 0.25% bovine serum albumin and stained separately with probes for apoptosis (Annexin V PE, Invitrogen, Carlsbad, CA and Caspace FITC (VAD-FMK), Promega, Madison, WI), and mitochondrial membrane potential (JC-1, Invitrogen) for 30 minutes in a 37°C incubator with 5% CO₂. After staining, cells were washed twice in KRB and analyzed for fluorescence using a BD LSR IITM flow cytometer (Becton Dickinson, Franklin Lakes, NJ). ToPro3 or Sytox Blue (Invitrogen) was added to samples just prior to data acquisition to identify necrotic and apoptotic cells. Data were analyzed using Flo Jo (Treestar Inc. Ashland, OR) and GraphPad Prism software (GraphPad, San Diego, CA). For the kinetic assessment of glucose response, data was acquired at a rate of ~300 events/s for 3 minutes at a glucose concentration of 3.3 mM, glucose was then added to the sample to increase the concentration to 16.7 mM. Data was then acquired for 11 minutes to assess changes in endogenous NAD(P)H auto-fluorescence detected by UV excitation at 350 nm and emission at 450nm (Hanson et al., 2009).

Adenine nucleotide quantification by HPLC

Total islet cellular extracts were prepared by phenol:chloroform:isoamyl alcohol extraction followed by a two step ether extraction of residual phenol. Islet extracts were run on a Discovery C-18 column (Sigma) on a HP1100 series quad pump reverse phase HPLC with a variable wavelength UV detector set to 254nm. Analysis of chromatograms was performed using Chemstation software (Agilent Technologies, Waldbronn, Germany) with peak identification by co-elution with known chemical standards.

Glucose stimulated insulin secretion assays

Islets were handpicked into oxygen (95% O₂/5% CO₂) saturated basal Krebs-Ringer Bicarbonate Buffer (KRB, 137 mM NaCl, 4.7 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 2.5 mM CaCl₂·2H₂O, 25 mM NaHCO₃, 0.25% BSA, 3.3 mM glucose) followed by incubation at 37°C for 30 min. in a 5% CO₂/95% air incubator. Groups of 8 – 10

islets from the equilibration cultures were then transferred to fresh oxygen saturated KRB containing either 3.3 mM or 16.7 mM glucose and incubated an additional 60 min. in a 37°C water bath with gentle shaking. Secreted insulin in the media was measured by ELISA (Millipore, Billerica, MA) and values normalized to extracted islet DNA (Quant-iT™ Picogreen, Invitrogen).

MAPK proteome array

Islets were treated in vitro $\pm$ cytokines $\pm$ IL-1ra for one hour. Islets were collected by centrifugation and washed twice with PBS. Phosphorylated proteins were detected using the Proteome Profiler MAPK array (R&D Systems) following manufacturer's instructions. Membranes were visualized with Biomax XAR film (Kodak, Rochester, NY), films were scanned, and pixel density measured using Totallab (Nonlinear Dynamics, Durham, NC).

Quantitative RT-PCR

RNA was extracted islets using the RNeasy miniprep kit (Qiagen, Chatsworth, CA) with on-column DNase digestion following manufacturers suggested protocol. RNA was reverse-transcribed using Omniscript reverse transcriptase (Qiagen). Quantitative PCR was performed using Taqman Universal PCR Master Mix and Taqman gene expression assays (Applied Biosystems, Foster City, CA) with rat gene specific primers for NOS2a, COX2, IL-6, IL-1b, TNFa, HMOX1 and HPRT (Applied Biosystems) on a GeneAmp 5700 Sequence Detection System (Applied Biosystems). Fold change was calculated using the $\Delta\Delta C_t$ method relative to untreated with actin (ACTB) as the endogenous control.

Nitrite determination

Nitrite production was determined by mixing 50 μ l of cell culture medium with 50 μ l of Griess reagent. The absorbance at 540 nm was measured by plate reader, and nitrite concentrations were calculated from a sodium nitrite standard curve.

In vivo islet functional assessment

NOD.scid male mice, purchased from The Jackson Laboratory (Bar Harbor, MA), were used as islet recipients following guidelines established by the University of Wisconsin-Madison Institutional Animal Care and Use Committee. Diabetes was induced following an overnight fast by single intraperitoneal injection of 150 mg/kg streptozotocin (Sigma-Aldrich Corp.). Glucose was measured daily using an Ascensia Elite glucometer (Bayer, Burr Ridge, IL). Mice were considered diabetic if nonfasting blood glucose (BG) was >350 mg/dL for three consecutive days. Mice were anesthetized by 2% isoflurane and islets were implanted underneath the kidney capsule. BG was measured for a total of 30 days after transplantation. An intraperitoneal (IP) glucose tolerance test (IPGTT) was performed 30 days post-transplant by giving an IP glucose injection (3g/kg) and blood samples for insulin measurement and blood glucose were taken at times 0, 20, 24, 28, 30 and 60 min after glucose administration. Insulin secretion was assayed by ELISA (Crystal Chem Inc., Downers Grove, IL). The restoration and maintenance of normoglycemia due to islet graft function was proven by removal of the graft-bearing kidney with restoration to the hyperglycemic state within 2 days.

Statistical analysis

All results are presented as mean $\pm$ SE. Statistical analyses of data was performed using GraphPad Prism Software (GraphPad Software, Inc., San Diego, CA) and Microsoft Excel (Microsoft, Redmond, WA) software. Statistical significance was defined as a p value <0.05 .

RESULTS

IL-1ra blocks PIC induced NO release by rat islets in vitro

A key effector molecule induced by PIC is nitric oxide (NO), which has been shown to induce islet necrosis and apoptosis. To determine if IL-1ra (Anakinra) could inhibit PIC induced NO release, rat islets were cultured alone or in combination with the IL-1ra [10 µg/ml] and the PIC cocktail of IL-1 β [50 U/ml], TNF α [1000 U/ml] and IFN γ [1000 U/ml]. Media from rat islets cultured $\pm$ PIC and IL-1ra was assayed by Griess reaction to quantify nitrite an indicator of NO (Fig. 1). PIC induced significant NO release by islets which was completely blocked by co-culture with IL-1ra.

IL-1ra provides protection against PIC induced islet impairment

Having shown that IL-1ra prevented PIC induced NO release by rat islets in vitro, we sought to quantify the effects on islet cell viability. Rat islets were cultured for 48h alone and in combination with IL-1ra and the PIC cocktail. Islet cell integrity was measured by flow cytometry with necrotic cells identified by staining with a membrane impermeable DNA binding probe and apoptotic cells identified by staining for phosphatidyl serine translocation with Annexin V and active pro-apoptotic caspase enzymes with the pan caspase substrate VAD-FMK. PIC treated islets showed diminished viability and increased percentages of necrotic and apoptotic cells (Fig. 2a). Apoptotic cells were primarily Annexin V positive and lacking in staining for active caspase enzymes. Co-culture with IL-1ra provided complete protection from PIC – induced necrosis and apoptosis. To determine if NO was the primary mediator of islet impairment, rat islets were cultured for 48h alone or in combination with the NO donor DEANO in the absence of PIC. Flow cytometry analysis of rat islet cells cultured with DEANO showed increased necrosis and apoptosis, which was not prevented by the presence of IL-1ra (Fig. 2b). In parallel experiments, rat islets were cultured for 48h with PIC and the iNOS inhibitor NG-monomethyl-L-arginine (NMMA) (Fig. 2c). Islet cell viability, necrosis, or apoptosis did not differ significantly from untreated control islets or islets treated with PIC and IL-1ra when cultured with NMMA.

PIC induce mitochondrial dysfunction

Assays of mitochondrial function were performed to better characterize the cellular impairments induced by PIC and the protective effects of IL-1ra. Rat islets were cultured for 48h $\pm$ PIC and IL-1ra and then assessed by flow cytometry for mitochondrial transmembrane potential ($\Delta\Psi_m$) using the probe JC-1. Glucose stimulated metabolic activity was also quantified by flow cytometry by kinetic detection of the reduction of NAD(P)H in response to 16.7 mM glucose (Hanson et al., 2009; Patterson et al., 2000). PIC treatment significantly increased the percentage of islet cells with $\Delta\Psi_m$ disruption and lowered the area under the curve (AUC) for glucose stimulated NAD(P)H reduction (Fig. 3a and b). Moreover, measurement of adenine nucleotides from cultured islet by HPLC showed lower ATP/ADP ratios as a result of PIC treatment (Fig. 3c) when compared to untreated or IL-1ra treated islets. Rat islets cultured with IL-1ra and PIC did not differ from control untreated islets with respect to percentages of cells with polarized $\Delta\Psi_m$, glucose-stimulated NAD(P)H reduction, or ATP/ADP ratios.

MAPK pathway activation by PIC

The MAP kinase family of cell signaling molecules respond to diverse extracellular stimuli including PIC. PIC trigger a stress-induced cascade of protein kinase activation that results in phosphorylation of p38 MAPK and SAPK/JNK, leading to activation of cellular processes that can induce apoptotic cell death (Saldeen et al., 2001). To better understand the inflammatory signaling pathways activated by PIC and the protective effects of IL-1ra, rat

islets were cultured for one hour after addition of PIC, and protein lysates were analyzed using a protein array for phosphorylated signaling molecules including AKT, ERK, JNK, MSK2 and p38 MAPK (Fig. 4). As previously shown (Abdelli et al., 2004), JNK, p38 MAPK and ERK were all phosphorylated in purified islets and PIC treatment induced additional phosphorylation of JNK2, and p38 α MAPK. In addition, MSK2, downstream of p38 MAPK also showed increased phosphorylation upon exposure to PIC. Rat islets cultured with IL-1ra + PIC did not show increased phosphorylation of JNK2, p38 MAPK or MSK2.

Activation of inflammatory gene expression

Alone or in combination with IFN γ and TNF α , IL-1 β has been shown to stimulate β -cell expression of iNOS and the resulting production of nitric oxide (NO). As shown by MAPK array, treatment of rat islets with PIC resulted in the phosphorylation of p38 and JNK kinases which are associated with NO induction. Cultured rat islets showed strong induction of the NOS2a gene and of multiple genes associated with inflammation (COX2, IL6, IL1b, and TNF α) (Fig. 5). Additionally, the HMOX1 gene was up-regulated presumably in response to increased oxidative stress in cytokine treated islets. IL-1ra treatment abrogated PIC induced inflammatory gene expression and NO production. Induction of inflammatory gene products, including COX2, NOS2a, IL6 and IL1b, was not blocked by inhibition of NO production by NMMA. In contrast, both IL-1ra and NMMA blocked induction of the HMOX1 gene.

Islet function in vitro and in vivo is maintained with IL-1ra treatment

Having demonstrated the protective effects of IL-1ra against PIC-induced islet impairment in vitro, we sought to evaluate the functional potency of similarly treated rat islets. Rat islets cultured for 48hrs following our standard protocol (Untreated, IL-1 ra alone, PIC alone, PIC + IL-1 ra), were tested for glucose stimulated insulin secretion (GSIS) by static incubation assay (Fig. 6). Calculation of GSIS stimulation indices demonstrated a deficit in islet glucose responsiveness following PIC treatment that was prevented by IL-1ra, although the difference did not reach statistical significance. However, examination of the actual secreted insulin levels showed that the reduced S.I. value of PIC treated islets was the result of deregulated insulin release at 3.3 mM glucose rather than reduced 16.7 mM glucose stimulated insulin release. Evaluation of rat islet functional potency in vivo was performed by transplant under the kidney capsule of streptozotocin-induced diabetic immunodeficient NOD.scid mice (Figure 7a). Recipients of PIC treated islets showed delayed graft function and were unable to restore stable glycemic control (blood glucose <200 mg/dL). The recipients of untreated, IL-1 ra alone, and PIC + IL-1 ra treated islets showed rapid restoration of normoglycemia and stable blood glucose control over the duration of the experiment (28 days). Intraperitoneal glucose tolerance tests (IPGTT) were performed to further evaluate the in vivo function of transplanted islets (Fig. 7b). NOD.scid mice transplanted with PIC treated islets were unable to normalize blood glucose within 90 minutes post glucose challenge (data not shown) and showed a reduced AUC for secreted insulin when compared to untreated islets. The high degree of intra- and inter-animal variability prevented the differences from reaching statistical significance. Recipients of untreated, IL-1ra treated, or PIC + IL-1ra treated islets showed equivalent glucose clearance (data not shown) post challenge and AUC for secreted insulin.

DISCUSSION

Pro-inflammatory cytokines including IL-1 β , TNF α , and IFN γ are principle inducers of effector molecules which cause the destruction of pancreatic islets both in vitro and in vivo. Preventing the deleterious effects of PIC is essential to improving the efficacy of isolated

islet transplantation as a curative therapy for type I diabetes. Mono-therapies that are focused on blocking iNOS, quenching ROS, or inhibiting pro-apoptotic caspase enzymes may be inefficient or incomplete in the protection provided to islets against PIC (Kato et al., 2003; Papaccio et al., 2005; Pedulla et al., 2007; Emamaullee et al., 2007). We hypothesized that competitively inhibiting the interaction of IL-1 β with its cellular receptor could provide a more complete level of protection to isolated islets from PIC induced apoptosis and necrosis. The objective of the study was to utilize the rat islet culture model with exogenous PIC treatment to elucidate the mechanisms of islet impairment and determine if IL-1ra blockade using a clinically approved agent (Anakinra) would provide a protective benefit.

As has been shown previously by others, we found that treatment of isolated rat pancreatic islets with PIC adversely affected viability and glucose-induced insulin secretion in vitro and in vivo. The mechanisms of islet death showed elements of both necrosis (loss of membrane integrity indicated by ToPro-3 staining) and apoptosis (Annexin V staining). A sensitive indicator of cell viability is the integrity and function of the mitochondria as indicated by transmembrane polarity, glucose induced electron transport chain activity and ATP production. By all of these measures, PIC treated islets demonstrated mitochondrial dysfunction. However, very few apoptotic or necrotic cells showed evidence of active caspase enzymes by flow cytometry using the pan-caspase substrate VAD-FMK. In addition, we were not able to detect significant release of cytochrome c into the cytoplasm of PIC treated islets by Western blotting (data not shown). These data are consistent with the observation that PIC can induce apoptosis that may not require complete mitochondrial membrane collapse, release of cytochrome c, and activation of effector caspases 3, 7, and 9 (Ferraro-Peyret et al., 2002; Roue et al., 2003; Irawaty et al., 2002). NO has been shown to cause endoplasmic reticulum stress in islets that leads to apoptosis that is caspase -12 dependent (Oyadomari et al., 2002; Contreras et al., 2003; Cardozo et al., 2005). The VAD-FMK probe used in this study has binding affinity for caspases-2, 3, 6, 7, 8, 9 and 10 but not 12 (McStay et al., 2008; Roy et al., 2008). It is also possible that our 48h culture period was insufficient to allow for detection of significant activation of effector caspases in non-necrotic islet cells. Exogenous NO in the form of DEANO added to rat islet cultures mimicked the effects of PIC and IL-1ra provided no protective benefit. In addition, inhibition of PIC induced iNOS activity by NMMA provided protection from necrosis and apoptosis that was equivalent to controls. Together, these data further support the primacy of the IL-1 β – NO-necrosis axis in rat islets.

PIC treatment was shown to activate the pro-inflammatory MAPK signaling pathway, upregulate multiple pro-inflammatory genes including iNOS, and lead to dramatic production of NO as indicated by increased nitrite levels in the culture media. Clearly, treatment of rat islets in vitro with PIC results in NO production which is associated with mitochondrial dysfunction and initiation of pathways that lead to cell death, some via apoptotic mechanisms. IL-1ra in this model system was completely effective in preventing all of the measured indicators of islet cellular impairment. The presence of NMMA in culture with PIC did not prevent the upregulation of COX2, NOS2A, IL-6, IL-1b, or TNFa. It is possible that with increased time in culture islet cells not killed by NO, could be induced down apoptotic pathways mediated by continued endogenous cytokine production. Our results indicate that prevention of the initiation of the inflammatory pathways is a more effective protective strategy than quenching the toxic effector molecules.

Rat islets cultured with IL-1ra (Anakinra) alone showed no impairment of either viability or function. Interestingly, rat islets treated with IL-1ra alone in vitro consistently showed a slight improvement in viability and function, although not reaching the level of statistical significance. These observations likely reflect the presence of IL-1 β secreting intra-islet macrophages (Arnush et al., 1998; Corbett and McDaniel, 1995). A surprising observation

was that IL-1ra treatment was sufficient to protect against a cocktail of 3 cytokines (IL-1 β , TNF α , and IFN γ). While TNF α and IFN γ both have been reported to have detrimental effects upon islets, our data indicates that their role in islet health is secondary to the contribution of IL-1 β . Alternately, the effects of these cytokines on islet health may be in part or almost entirely mediated through the generation of IL-1 β .

The data presented here demonstrate that IL-1ra (Anakinra) is an effective treatment against the insults of multiple pro-inflammatory cytokines. Rat islets in culture were protected by IL-1ra with respect to both viability and function. Furthermore, there is also evidence that IL-1ra treatment was beneficial in blocking the effects of endogenously produced IL-1 β induced during islet isolation and culture. Studies are currently underway in our laboratory to evaluate the ability of IL-1ra (Anakinra) to improve rat islet engraftment within an intrahepatic allotransplant model. In addition, we are investigating the utilization of IL-1ra (Anakinra) as an additive to human pancreas organ preservation solutions, and isolated islet culture media. The translation of our findings to human islets could lead to dramatic improvements in pre-transplant islet viability and subsequent islet engraftment and long term function post-transplant.

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Fig. 1.

IL-1ra inhibits PIC induced NO release by rat islets. Rat islets were cultured for the indicated time periods either alone or in the presence of pro-inflammatory cytokines (PIC= IL-1 β , IFN γ , and TNF α) $\pm$ IL-1ra. Media was then assayed by Griess reaction for the presence of nitrite as an indicator of NO formation. Data for in vitro NO release are the mean $\pm$ SE of n=4 and statistical significance was determined by two-way ANOVA. *p<0.05 vs untreated, ** p<0.01 vs cytokine treated.

Fig. 2.

IL-1ra provides complete protection from PIC induced endogenous NO but not exogenous NO. Flow cytometry analysis of islets dispersed into single cells was performed following 48h culture $\pm$ PIC and IL-1ra (a), $\pm$ the NO donor DEANO [500 μ M] and IL-1ra (b), and $\pm$ PIC and the iNOS inhibitor NMMA. Necrotic cells were identified by staining with the membrane impermeable DNA binding dye ToPro3 or Sytox blue (c). In (a) and (b) apoptotic cells were identified by negative or dull staining for ToPro3 but positive staining for externalized phosphatidyl serine by Annexin V and/or active caspase enzymes using the pan-caspase substrate VAD-FMK. Viable cells were defined as negative for ToPro3, Annexin V, and VADFMK. For (c) necrotic cells were identified as Sytox Blue bright, apoptotic cells as Sytox Blue dull, and viable cells as Sytox Blue negative. Data shown for are the mean $\pm$ SE of n=5 (a and b) of n=3 (c). Statistical analysis was performed by one-way ANOVA with * indicating a $p < 0.05$ vs all other groups.

Fig. 3.

IL-1ra treatment abrogates mitochondrial dysfunction caused by PIC. Rat islets cultured for 48h $\pm$ PIC and IL-1ra were evaluated for changes in mitochondrial integrity and function. The percentage of islet cells with disrupted $\Delta\Psi_m$ was quantified by flow cytometry analysis of dispersed islet cells stained with JC-1 (a). Data shown are the mean $\pm$ SE n=5, for the percentage of islet cells with polarized $\Delta\Psi_m$ indicated by high JC-1 red/JC-1 green fluorescence. Glucose induced mitochondrial respiratory activity was measured by kinetic flow cytometry assay of the conversion of NAD(P) to NAD(P)H indicated by the UV-laser excited autofluorescence of the reduced pyridine nucleotides (b). Data shown are the mean $\pm$ SE n=5, for the calculated area under the curve of NAD(P)H reduction in response to an increase from 3.3 mM to 16.7 mM glucose over the first 10 minutes. Total islet cell adenine nucleotide levels were measured by HPLC analysis of extracts (c).

Fig. 4.

MAPK pathway activation by PIC and the protective effects of IL-1ra. Rat islet total protein extracts were prepared 1hr after placing in culture $\pm$ PIC and IL-1ra. Phosphorylated proteins were measured by Proteome Profiler MAPK Array (R&D Systems). Data shown are the pixel densities of detected protein specific chemiluminescence and are representative of three separate experiments.

Fig. 5.

Inflammatory gene expression profiles following PIC treatment of rat islets and the effect of IL-1ra and NMMA. Total RNA was extracted from rat islets cultured for 48h $\pm$ PIC, IL-1ra, and the iNOS inhibitor NMMA reverse transcribed into cDNA and subjected to RT-PCR to quantify the expression levels of genes related to inflammation. Data shown are the fold change of mRNA relative to untreated islet mRNA and statistical significance was determined by one-way ANOVA on Δ CT values with * indicating a $p < 0.05$ when compared to control untreated islets.

Fig. 6.

IL-1ra treatment prevents deregulated glucose-induced insulin secretion by rat islets. Glucose stimulated insulin secretion was assessed by static incubation of hand-picked islets in quintuplicate with insulin measured by ELISA and values normalized to DNA content. The Stimulation Index (SI) (inset graph) was calculated as DNA normalized insulin secretion in response to 16.7 mM glucose/DNA normalized insulin secretion in response to 3.3 mM glucose. Data are mean $\pm$ SE.

Fig. 7.

IL-1ra preserves the in vivo functional potency of PIC treated rat islets. Streptozotocin-induced diabetic NOD.scid mice were transplanted with rat islets (500 IEQ each mouse, n=4 per group) cultured for 48h either alone or with, IL-1ra, PIC, or IL-1ra + PIC (a). Data are the mean $\pm$ SE of daily blood glucose values for mice transplanted with islets from the respective treatment groups. Statistical analysis was performed by two-way ANOVA with * indicating a p<0.01 vs all other groups. In vivo assessment of islet graft function was tested by glucose tolerance test (IPGTT) performed by i.p. injection 3 g/kg glucose followed by measurement of plasma glucose and insulin (b). Glucose-stimulated insulin secretion was computed as the area under the curve (AUC) in response to glucose challenge from T₀ to T₆₀ minutes.