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Investigations of Strategies to Counteract Proinflammatory Cytokines in Experimental Type 1 Diabetes

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Abstract

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Type 1 diabetes (T1D) is a chronic autoimmune disease targeted against the pancreatic β-cells. Proinflammatory cytokines are considered to play a major role in the destruction of the insulin-producing β-cells. This thesis studied strategies to counteract proinflammatory cytokines in experimental T1D. Both animal models for T1D as well as β-cell preparations exposed *in vitro* to putative noxious conditions were examined.

In the first study we observed that cytokine treatment of mouse pancreatic islets lacking inducible nitric oxide synthase (iNOS) induced a prolongation of the early stimulatory phase of glucose stimulated insulin secretion. Various experiments led to the conclusion that this prolonged stimulatory effect may involve the DAG/PLD/PKC pathway.

Next, we transplanted mouse islets deficient in iNOS to spontaneously diabetic NOD mice. We observed a normalization of hyperglycemia but not a delayed allograft rejection compared to transplanted wild type islets. Thus, absence of iNOS in the graft was not sufficient to prolong allograft survival.

In paper III we found that sustained glucose stimulation of rat pancreatic islets was coupled to a decreased conversion of proinsulin to insulin. Islet treatment with IL-1β was also coupled to a decreased proinsulin conversion. Islet proconvertase activity may be a target in islet damage.

In paper IV prolactin (PRL) was administered to mice in the multiple low dose streptozotocin model and we observed that PRL enhanced a Th2 response. This may contribute to the protective action by PRL in this model of autoimmune T1D.

Finally, by examining β-cells overexpressing Suppressor of cytokine signalling 3 (SOCS-3) it was found that this could inhibit IL-1β induced signalling through the NF-κB and MAPK pathways. SOCS-3 overexpression also inhibited apoptosis induced by cytokines in primary β-cells. Lastly, we demonstrated that SOCS-3 transgenic islets were protected in an allogeneic transplantation model.

Keywords: Type 1 diabetes, proinflammatory cytokines, SOCS-3, pancreatic islets, inducible nitric oxide synthase, insulin secretion, NOD mice, islet transplantation, proinsulin conversion, streptozotocin, prolactin

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“The reasonable man adapts himself to the world; the unreasonable one persists in trying to adapt the world to himself. Therefore, all progress depends on the unreasonable man. “

- George Bernard Shaw

List of Papers

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals.

I Andersson AK*, Börjesson A*, Sandgren J and Sandler S. Cytokines affect PDX-1 expression, insulin and proinsulin secretion from iNOS deficient murine islets. *Mol Cell Endocrinol* 2005; 240: 50-57.

II Börjesson A, Andersson AK and Sandler S. Survival of an islet allograft deficient in iNOS after implantation into diabetic NOD mice. *Cell Transplantation* 2006; 15: 769-775.

III Börjesson A and Carlsson C. Altered proinsulin conversion in rat pancreatic islets exposed long-term to various glucose concentrations or interleukin-1β. *J Endocrinol* 2007; 192: 381-387.

IV Lau J*, Börjesson A*, Holstad M and Sandler S. Prolactin regulation of the expression of TNF-α, IFN-γ and IL-10 by splenocytes in murine multiple low dose streptozotocin diabetes. *Immunol Lett* 2006; 102: 25-30.

V Rønn SG*, Börjesson A*, Bruun C, Heding PE, Frobøse H, Mandrup-Poulsen T, Karlsen AE , Rasschaert J, Sandler S and Billestrup N. Suppressor of cytokine signalling-3 expression inhibits cytokine-mediated destruction of primary mouse and rat pancreatic islets and delays allograft rejection. *Diabetologia* 2008; 51: 1873-1882.

*Shared contribution as first author

Contents

Abbreviations

APC	Antigen presenting cell
DAG	Diacylglycerol
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FasL	Fas ligand
FCS	Fetal calf serum
G6PDH	Glucose-6-phosphate dehydrogenase
IFN-γ	Interferon gamma
IL-1β	Interleukin-1 beta
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factor
JAK	Janus activated kinase
KRBH	Krebs-Ringer bicarbonate HEPES
MHC	Major histocompatibility complex
MAPK	Mitogen-activated protein kinase
MLDSTZ	Multiple low dose streptozotocin
NF-κB	Nuclear factor kappa B
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOD	Non-obese diabetic
PC	Proinsulin convertase
PDX-1	Pancreatic and duodenal homeobox gene 1
PKC	Protein kinase C
PLD	Phospholipase D
PRL	Prolactin
RT-PCR	Real time polymerase chain reaction
SEM	Standard error of the mean
SOCS	Suppressors of cytokine signalling
STAT	Signal transducers and activators of transcription
STZ	Streptozotocin
TNF-α	Tumour necrosis factor alpha
Wt	Wild type

Introduction

Diabetes mellitus is one of the most common endocrine disorders and is classified into two main types. Type 1 diabetes (T1D) is insulin-dependent diabetes mellitus (IDDM) and is also called juvenile-onset diabetes. Type 2 (T2D) diabetes is noninsulin-dependent diabetes mellitus (NIDDM) and is also called adult-onset diabetes. Type 1 diabetes can be classified as a chronic autoimmune disease while Type 2 diabetes is caused by impaired ability to secrete enough insulin together with peripheral resistance to insulin.

It has been postulated that T1D gradually develops (1). This process is characterised by a number of events during the progressive β-cell loss (figure 1). Type 1 diabetes becomes clinically manifest when endogenous insulin production no longer is sufficient to control the glucose metabolism. The peak of onset is in early puberty and the first acute symptoms include thirst, polyuria, fatigue and weight-loss. For the patients, the disease results in a life-long requirement of insulin injections. Still, the precise mechanism of disease onset remains to be clarified and it is considered that the etiology of T1D is multifactorial.

Genetic studies of T1D indicate that the susceptibility to the disease is in part inherited, with the genes for major histocompability complex (MHC) on chromosome 6 being particularly important (2,3). Large geographic differences in prevalence of T1D are found (4) and it is known that there has been a conspicuous increase in incidence during the past 50 years (5). This has led to the suggestion that environmental factors have critical roles in the etiology of T1D. Viral infections (6), dietary proteins (7) and stress (8) have been suggested to be such factors that may initiate or trigger the process leading to β-cell destruction.

The appearance of circulating antibodies directed against various islet cell antigens are found in T1D patients and is an early indication of emerging destruction of pancreatic β-cells (9). The most characteristic hallmark in the pathogenesis of T1D is the selective destruction of the pancreatic β-cells, mediated mainly by infiltrating T-lymphocytes and macrophages (10).

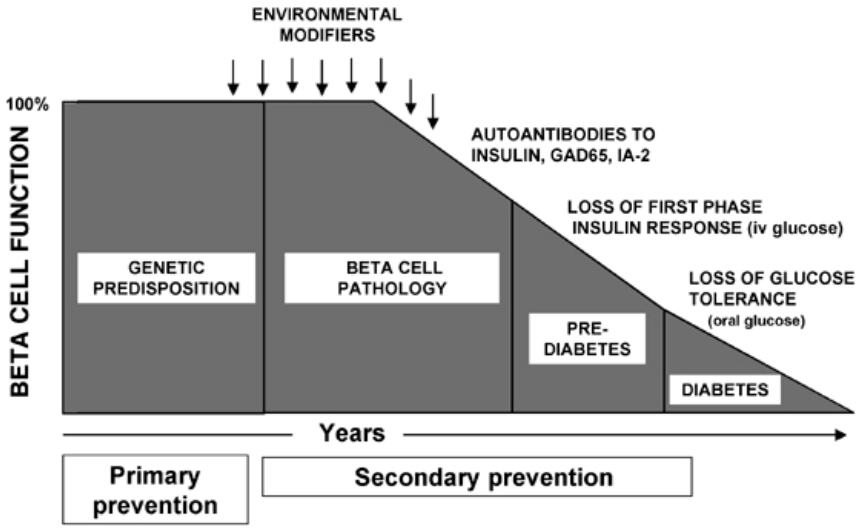

Figure 1. It has been postulated that T1D gradually develops in a number of stages with increasing β-cell loss. Individuals at risk for developing T1D with underlying islet inflammation can be identified by the presence of circulating autoantibodies to specific islet antigens. The subclinical phase of T1D, before severe insulin deficiency leads to symptoms of hyperglycemia, provides a window for secondary prevention (modified from ref. 1).

Pancreatic islet physiology

In rats the pancreas contains approximately 4000-5000 islets of Langerhans (11,12). The islets are dispersed throughout the pancreas and the size of an islet is 25-300 μm (13). In the pancreatic islets there are four main types of hormone producing cells: insulin-secreting β-cells (60-70%); glucagon-secreting α-cells (25%), somatostatin-secreting δ-cells (10%) and pancreatic polypeptide-secreting PP-cells (1-5%). Recently, a fifth cell type, the ghrelin-secreting epsilon-cells have been described (14). The pancreatic islets also contain nerves, fibroblasts, macrophages, dendritic cells and endothelial cells. The pancreatic islets develop from the gut endoderm (15) and transcription factors including PDX1, PAX4 and PAX6 are described to have central roles in determination of the cell types and their functions (16).

The pancreatic islets are coordinated by neural and hormonal networks and secrete hormones in an oscillatory manner. Nutrient metabolism and incretin hormones trigger insulin secretion. Important cellular components and messengers involved in insulin secretion include glucose transporters, glucokinase, ATP/ADP-ratio, mitochondrial metabolism, ATP-sensitive potassium channels, cAMP and Ca^{2+}.

In the pancreatic β-cells proinsulin is formed from preproinsulin by elimination of the signal peptide. Proinsulin is thereafter converted to insulin. The conversion occurs in the secretory granules via activities of the two endoproteases PC1 and PC2, and the exoprotease CPH (17). The conversion of proinsulin to insulin is regulated partly through effects on the biosynthesis of PC1 and PC2 (18-20). It is known that proinsulin only is about 10% as biologically active as insulin. Elevated levels of proinsulin are found both at the onset of T1D (21) and in T2D (22) and cytokines are proposed to cause increased ratios of secreted proinsulin (23).

Insulitis and β-cell destruction

In T1D the inflammatory process referred to as insulitis occurs around and within the pancreatic islets (24). The insulitis and destruction of β-cells persist for a long period prior to the diagnosis of T1D (25). Access to pancreatic biopsies from newly diagnosed T1D patients is limited but it is established that there is inflammation in pancreatic islets from newly diagnosed T1D patients (26,27). Available data from research on insulitis in T1D are mostly obtained from animal models e.g. the multiple low dose streptozotocin (MLDSTZ) model, non-obese diabetic (NOD) mice and BioBreeding (BB) rat.

Based on studies in NOD mice it is suggested that insulitis develops in stages. An initial benign form of insulitis is followed by a more efficient β-cell destruction, insulin deficiency and hyperglycemia (28,29). Antigen presenting macrophages and dendritic cells appear early during the insulitis and later CD4+ and CD8+ T-cells appear (30). Also B-lymphocytes are present but are not considered to be involved in the destruction of the β-cells (31).

The T-cell mediated destruction of β-cells is characteristic for T1D and mediated by release of cytotoxic molecules, including cytokines, granzyme B or perforin, or by direct delivery of cell-death signals via the Fas pathway (32,33). Putative mechanisms of β-cell destruction are shown in figure 2.

Cytokines

For long, cytokines were considered to be mediators merely involved in the regulation of the immune system. Today, it is known that virtually every cell in the body can respond to or even produce cytokines, with the pancreatic β-cell being no exception (34).

Some cytokines are classified as Th1 or Th2 based on the T-helper (CD4+) lymphocytes that produce them (35). In mice Th1 cytokines include IL-2, IFN-γ, TNF-β and IL-12 while Th2 cytokines include IL-4, IL-5 and

IL-10 (36). Th1 cytokines activate both T-cells and macrophages and promote cellular immune responses. Th2 cytokines produce reactions that favour humoral, IgE-mediated allergic and mucosal immune reactions.

The β-cell is a cell-type particularly sensitive to cytokines, probably through insufficient expression and/or regulation of protective mechanisms blocking signals from a cytokine exposure (37,38). The original observation that cytokines cause β-cell damage was published two decades ago (39). It is established that the proinflammatory cytokines IL-1β, IFN-γ and TNF-α are cytotoxic to β-cells, in pM-nM concentrations and may cause β-cell death in both rodent (alone or in combination) and human pancreatic islets (in combination) (40,41).

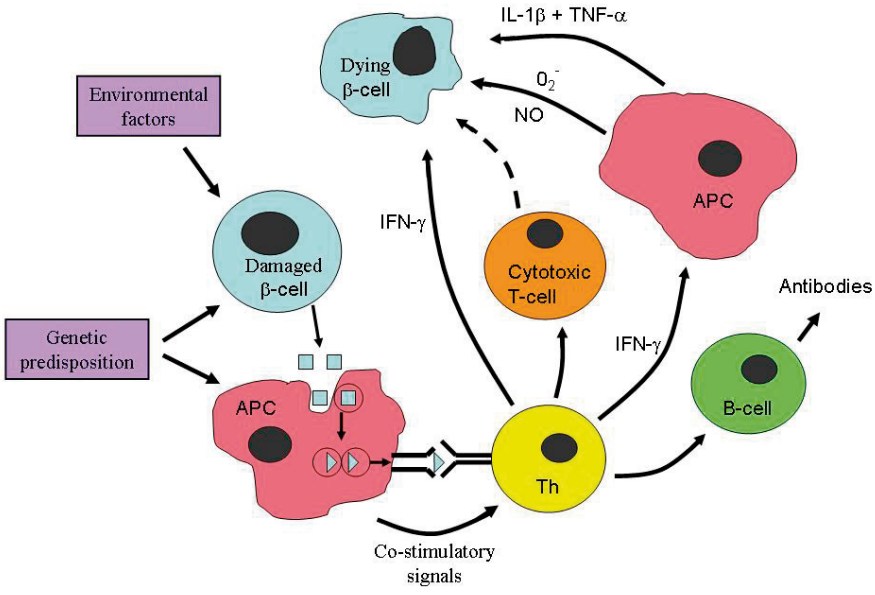

Figure 2. Adapted from the Copenhagen model of type 1 diabetes (ref. 10). One or several environmental factors are suggested to cause damage to the β-cells and this leads to release of β-cell proteins. Antigen presenting cells (APCs) engulf such proteins, process them and present peptides by MHC class II. APCs will produce and secrete cytokines and co-stimulatory signals. If Th-lymphocytes specifically recognize the antigenic peptide, it leads to transcription of cytokine genes. IFN-γ will stimulate the APC to increase MHC class II expression and secretion of IL-1β and TNF-α. Other APC cells in the islet are also induced to secrete cytokines. IL-1β, potentiated by TNF-α and IFN-γ is cytotoxic to β-cells through induction of free radical (NO, O_2^-) formation in the islet. Cytokines also induce changes in expression of many proteins in the β-cells. For example, the interaction between Fas on β-cells and Fas ligand on infiltrating lymphocytes leads to β-cell apoptosis.

IL-1β

The cytokine IL-1β was described as an important mediator of β-cell destruction already twenty years ago (42,43). It was later observed that receptors for IL-1β are present on pancreatic β-cells (44). Important signalling pathways that are activated by IL-1 receptors are mitogen activated protein kinases (MAPKs), stress activated protein kinase (JNKs) and tyrosine kinases. The cell signalling leads to induction of different transcription factors including nuclear factor kappa B (NF-κB), activator protein 1 (AP-1) and interferon regulatory factor 1 (IRF-1) (45-47). Activation of the transcription factor NF-κB is involved in IL-1β mediated induction of iNOS and subsequent NO production may play an important role for the deleterious effects of the cytokine. Inhibition of iNOS has been described to lead to partial protection against IL-1β (48-51). Factors induced by IL-1β also include PKC activation via PLD induction and DAG formation (52,53).

From *in vitro* studies of rodent islets it is established that this cytokine may cause impairment of β-cell function and β-cell death. In rat islets, IL-1β inhibits insulin secretion, insulin biosynthesis and oxidative metabolism (54). IL-1β exposure also causes DNA damage and reduces cell viability of rat islets (55,56). When mouse islets are exposed to IL-1β, insulin secretion is impaired and glucose oxidation can be affected, however often not to the same extent as in rat islets (57,58). Human islets are more resistant than rodent islets to IL-1β exposure, but it is established that the combination of IL-1β, IFN-γ and TNF-α causes impairment of β-cell function and β-cell death in human islet cells (59,60).

IFN-γ

Another proinflammatory cytokine considered to be involved in β-cell impairment and destruction is IFN-γ. This cytokine mediate its effects via the signalling pathways of Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) (61). In islet cells, IFN-γ is described to upregulate MHC I and induce IRF-1 expression (62,63).

IFN-γ was observed to cause inhibitory effects on isolated rat islets (64). When murine IFN-γ was added to murine islets, an impairment of insulin secretion was observed (65). Moreover, an IFN-γ induced inhibition of glucose oxidation rate, without any effect on insulin secretion was detected in another study (66). In human islets, IFN-γ alone has been described to cause minor toxic effects and suppressed insulin secretion (59,67,68).

Thus, some studies have described an inhibitory effect by IFN-γ alone, but in most studies the combination of IFN-γ + IL-1β and/or TNF-α is needed to compromise β-cell function (41).

TNF-α

It is described that TNF-α and IL-1β signalling converge on the NIK/IKK/NF-κB and MAPK pathways (37). TNF-α alone can cause impairment of islet functions *in vitro*. However, the combination of TNF-α and IL-1β + IFN-γ seems to be needed to cause β-cell death (41,64).

In animal studies it has been shown that the effects of TNF-α inhibition is dependent upon the timing of administration. Anti-TNF-α treatment increased diabetes incidence in adult NOD mice, but reduced the incidence in neonates (69). Therefore, it is possible that TNF-α promotes the early development of autoimmunity but suppresses an already established diabetogenic process.

IL-10

IL-10 is a potent anti-inflammatory cytokine. The cytokine is known to block activation and subsequent affector function of macrophages and dendritic cells, such as secretion of proinflammatory cytokines and T-cell stimulation (70). The effects of IL-10 administration on the development of T1D in NOD mice seem to be dependent on the time and mode of application. It has been showed that NOD mice can be protected against diabetes development with a non-pathogenic recombinant adeno-associated virus (rAAV) vector encoding IL-10 (71,72). Moreover, syngeneic islet grafts were protected in diabetic NOD recipients immunized with rAAV-IL-10 (73).

SOCS-3

Suppressors of cytokine signalling (SOCS) are intracellular proteins that control signals from cytokine receptors and are essential for normal immune physiology. Eight members of the SOCS family have been identified (SOCS1-SOCS7 and CIS) (74). A low basal expression of SOCS proteins can be detected in various tissues. Most SOCS proteins are induced by cytokines and act in a classical negative feedback loop to inhibit cytokine signal transduction (75,76).

SOCS-1 inhibits IFN-γ signaling and a β-cell specific SOCS-1 overexpression has been shown to protect pancreatic β-cells from T-cell mediated destruction (77). Diabetes incidence was also reduced in rat insulin promoter (RIP)-SOCS1-NOD mice (78) and allogeneic transplantation of SOCS-1 over-expressing mouse islets leads to prolonged graft survival (79).

In β-cell lines SOCS-3 has been shown to protect against cytokine mediated β-cell destruction by suppressing both IFN-γ and IL-1β signalling (80). SOCS-3 has also been found to suppress IL-1β mediated NF-κB and MAPK activation, via inhibition of the TGFβ activated kinase (81). Moreover, it is reported that SOCS-3 influences the expression of several IL-1β induced inflammatory and pro-apoptotic genes in β-cell lines (82).

Prolactin

Prolactin (PRL) is a pleiotropic hormone that is mainly produced in the pituitary gland. The most commonly known functions of PRL are stimulation of mammary gland development and initiation and maintenance of lactation (83). PRL is structurally similar to members the cytokine/hematopoietic family. Receptors for PRL are widely distributed throughout many different tissues and the hormone is involved in different functions in the body (84). For example, PRL plays an important role in modulating the immune response in animals and humans (85).

PRL can stimulate the release of both Th1 and Th2 cytokines. The hormone increases the production of IFN-γ from lymphocytes and induce the expression of IL-2 receptors on the surface of lymphocytes (86,87). Moreover, PRL can activate Th2 lymphocytes and stimulate autoantibody production (88).

iNOS and NO

The nitric oxide synthase (NOS) enzymes are known to catalyse a reaction of L-arginine, NADPH and oxygen to the free radical NO, citrulline and NADP. Three distinct isoforms of NOS have been identified: nNOS being the isoform first found and predominating in neuronal tissue, iNOS being the isoform which is inducible in a wide range of cells and tissues and eNOS being the isoform first found in vascular endothelial cells. These isoforms have also been differentiated on the basis of their constitutive (eNOS and nNOS) versus inducible (iNOS) expression, and their Ca^{2+}-dependence (eNOS and nNOS) or Ca^{2+}-independence (iNOS) (89).

The iNOS isoform is found in different tissues and NO produced by iNOS can be of importance in defence against invading microorganisms (90). The iNOS isoform has been found both in rodent (91,92) and human (68) pancreatic β-cells and NO is produced in these cells in response to cytokines. It is described that NO produced by iNOS is involved in rodent β-cell destruction (93,94).

This thesis describes experiments in mice with an inactivation (knockout) of the iNOS gene. Previous studies have shown that islets from the iNOS-/- mice do not produce NO after exposure to IL-1β or IL-1β + IFN-γ (95). It has also been demonstrated that the iNOS-/- mice are resistant against multiple low dose streptozotocin (MLDSTZ) induced diabetes (96).

Islet transplantation

The islet cell transplantation is an option for treating patients with brittle T1D. Since development of the Edmonton protocol (97), the outcome of clinical transplantation of human islets has been improved. However, long-term insulin independence has not been achieved in the majority of cases (98). The clinical use of islet transplantation is limited due to various problems posttransplantation such as poor revascularization of transplanted islets and immune destruction of islet grafts. In animal models different strategies have been tested to counteract immune destruction of transplanted islets, e.g. encapsulating islets before transplantation and modulating the gene expression profile of islets before transplantation. Modulation of islet gene expression can be both gene upregulation and gene knockdown approaches.

The liver is the predominating implantation site for islets in humans. In animals a variety of implantation sites have been successfully tested such as spleen, omentum and testes (99-101). One of the most extensively used sites in rodent studies is the renal subcapsular space. In this site, islets are easily retrieved for histological study, and it is possible to confirm graft function by removing the islet-bearing kidney.

Studies of transplantation of islets directly into spontaneously diabetic NOD mice may contribute to the knowledge of islet allotransplantation as a possible treatment of T1D. It has been suggested that NOD mice will destroy transplanted islet tissue by a recurrence of the disease process that destroyed the original islets (102). The situation with islet transplantations into diabetic NOD mice is similar to that of transplantation of human islets where, in addition to allograft rejection the transplanted islets are also subjected to an autoimmune attack, since spontaneously diabetic recipients already are primed to destroy islet β-cells.

Aims

The specific aims of this thesis were to:

- Characterise the mechanisms behind the altered kinetics of glucose stimulated insulin secretion from cytokine exposed pancreatic islets deficient in iNOS.

- Test whether transplantation of pancreatic islets deficient in iNOS can permit increased islet graft survival.

- Examine if rat pancreatic islets may adapt to changes in glucose concentration or IL-1β by altering the rate of conversion of proinsulin to insulin.

- Investigate whether prolactin can regulate the production of cytokines by splenocytes isolated from mice treated with multiple low doses of STZ.

- Study the effect of Suppressor of Cytokine Signalling (SOCS)-3 in primary rodent β-cells and in transplantation into diabetic animal models.

Materials and methods

Animals

All the animal experiments in this thesis were conducted in accordance with international guidelines and approved by the local animal ethics committees.

The mice deficient in iNOS used in study I and II were generated from embryonic stem cells with a targeted disruption of the iNOS gene (103). Breeding pairs of mice were provided by JS Mudgett (Merck Research Laboratories, NJ, USA), JD MacMicking and C Nathan (Cornell University, NY, USA). The mice were then bred at the Rudbeck Laboratory, Uppsala. Homozygous iNOS mice were maintained by interbreeding the F2 generation.

The NOD mice studied in paper II and V were originally obtained from Clea Company, Aobadi, Japan and bred at Biomedical Centre, Uppsala. The NOD mouse strain is inbred and derived from the ICR mouse. The incidence of spontaneous diabetes is 60-80% in females, but much lower in males. The onset of diabetes is often at 12-14 weeks of age but can occur at 25 weeks or even later (29,104).

Pancreatic islets were isolated from adult Sprague-Dawley (Biomedical Centre, Uppsala) rats in paper III. This strain is an outbred and well-characterised animal model often used in experimental diabetes research. In paper V islets were isolated from neonatal rats as previously described (105). These islets were then dispersed into single cells and used to evaluate the protective effect of a SOCS-3 encoding adenovirus construct against cytokine exposure.

In paper V mice on a C57BL/6 background with β-cell specific SOCS-3 expression (106) and a SOCS-3 encoding adenovirus construct were used to characterise the protective effect of SOCS-3 in mouse and rat islets exposed to cytokines. Moreover, SOCS-3 transgenic islets were transplanted into spontaneously diabetic NOD mice. In addition, pancreatic islets were transplanted into alloxan-induced diabetic BALB/c mice (B&K, Sollentuna, Sweden). Alloxan is widely used to cause β-cell death and diabetes in experimental animals. The cytotoxic effect of alloxan is mediated by the production of highly reactive hydroxyl radicals (107).

The Multiple Low Dose Streptozotocin Model

Streptozotocin (STZ) is a substance with diabetogenic properties in rodents. STZ is transported into the β-cells via the glucose transporter GLUT2, causes DNA damage and subsequent β-cell death (107). Injecting mice or rats with a single high dose STZ results in a β-cell specific toxic effect leading to hyperglycemia. Injecting mice with five daily low doses of STZ results in a delayed hyperglycaemia with infiltration of immune cells in the pancreatic islets after ~10 days (108).

In study IV, adult male C57BL/Ks mice (Biomedical Centre, Uppsala) were injected intraperitoneally with either 40 mg/kg body weight streptozotocin (Sigma Chemicals, St Louis, MO, USA) or saline once a day for five consecutive days. STZ was dissolved in saline immediately before administration. The mice were given a second intraperitoneal injection of either prolactin (Sigma) or saline 30 min after the first injection. The prolactin injections were given daily throughout the study.

Islet isolation and culture

To isolate pancreatic islets from adult mice or rats a collagenase procedure (109) was used and islets were picked by hand with a braking pipette. Groups of 100-150 islets were precultured for 3-7 days before islet treatment or transplantation. The culture medium was RPMI 1640 (Sigma) medium (11 mM glucose) supplemented with L-glutamine (Sigma), benzylpenicillin (Roche Diagnostics, Bromma, Sweden), streptomycin (Sigma) and foetal calf serum (Sigma). The culture medium was changed every second day. For further details, see paper I, II, III and V. In study V, human islets were obtained from Rudbeck Laboratory, Uppsala University Hospital. The human islets were cultured in RPMI 1640 (Sigma) containing 5.6 mmol/l glucose. For further details see paper V.

Islet treatment

In study I mouse islets were exposed to human IL-1β (PeproTech, London, U.K.) at a concentration of 25 U/ml or a combination of human IL-1β (25 U/ml) and murine IFN-γ (1000 U/ml; PeproTech). The used concentrations are established as suitable when trying to create a condition that leads to impairment of pancreatic β-cell functions. In study III rat islets were exposed to human IL-1β alone at an activity of 25 U/ml. In study V islets were in addition to IL-1β and IFN-γ also exposed to TNF-α (for further details see paper V).

Glucose stimulated proinsulin and insulin secretion

In study I islets were transferred to flat bottom multi-well plates containing RPMI 1640 (+ foetal calf serum, L-glutamine and antibiotics) supplemented with 16.7 mM glucose. Islets were incubated with or without the addition of IL-1β. Medium was changed after 2, 4, 6 and 20 h and analysed for insulin using a rat insulin ELISA (Mercodia, Uppsala). For analysis of proinsulin released to the medium islets were incubated for 6 or 24 h with or without addition of IL-1β or IL-1β + IFN-γ. Thereafter, glucose stimulated proinsulin and insulin secretion was performed as described below for study II.

In study II we examined the secretory function of the two types of islets by glucose stimulated insulin release. Islets were transferred to 100 µl KRBH and incubated for 30 min at 1.7 mM glucose and then transferred to 16.7 mM glucose for 90 min. After the period in 16.7 mM glucose islets were ultrasonically disrupted and insulin extracted. Insulin release to the KRBH media and insulin in the extracts was measured by ELISA (Mercodia, Uppsala). (Paper II).

For insulin release experiments in study V, triplicates of ten islets each were transferred to glass vials containing 250 µl KRBH supplemented with 10 mmol/l HEPES (KRBH) and 2 mg/ml BSA. During the first hour of incubation the medium contained 1.7 mmol/l glucose. The medium was then removed and replaced by 250 µl KRBH supplemented with 16.7 mmol/l glucose for the second hour. Islets were then ultrasonically disrupted and insulin extracted. Insulin release to the KRBH media and insulin in the extracts was measured by ELISA.

Glucose oxidation

In study V islet glucose oxidation rates in groups of 10 islets were determined according to a previously described method (110). Islets were incubated in glass vials containing KRBH supplemented with D-[U-^{14}C]glucose (Amersham International, Amersham, UK) and non-radioactive glucose to give a final concentration of 16.7 mM. Incubation conditions were 37°C (O_2/CO_2, 95:5) in a slow shaking water bath (30 strokes/min) for 90 min. The glucose oxidation was terminated with Antimycin A (Sigma) and Na_2HPO_4 (pH 6) was added to release the CO_2 formed. To trap the CO_2, Hyamine 10-x (New England Nuclear, Boston, MA, USA) was added and the glass vials were further incubated for 2 h. Ultimate Gold scintillation liquid (Packard Instrument, Meriden, CT, USA) was then added in order to measure the radioactivity by liquid scintillation counting. Glass vials without islets were used as blanks.

Measurement of newly synthesized proinsulin/insulin

In study III pulse-chase labelling of islet proteins was achieved by incubating islets with radioactive labelled leucine for 30 min at 37°C in 16.7 mM glucose. After washing, one islet sample was immediately frozen. Two of the samples were further incubated for 45 or 90 min in 16.7 mM glucose.

Labelled islets were sonicated in glycine buffer and after addition of a solution of antibovine insulin antisera (Biomakor, Rehovot, Israel), the samples were shaken and left for 1 h at room temperature. Subsequently, 100 ml protein A-Sepharose CL-4B suspended in glycine buffer, was added and the samples gently shaken for 15 min. After sedimentation by gentle centrifugation, the pellet was rinsed with glycine buffer and frozen at -70°C.

The antibody precipitated proteins were dissociated from the protein A complex and separated on a gradient gel of 10-15% acrylamide/0.5-0.75% methylenebisacrylamide with 6 M urea. Densitometry was used to determine the intensities of the spots. For further details see paper III.

Islet transplantation

At transplantation in study II and V recipient diabetic NOD mice or Balb/c mice were anesthetized by intraperitoneal injection of Avertin (a 2.5 % solution of 10 g 97 % 2,2,2-tribromo-ethanol in 10 ml 2-methyl-2-buthanol; Kemila, Stockholm, Sweden) (111). For NOD recipients, groups of 600 precultured islets were packed in a braking pipette and implanted beneath the left kidney capsule. For BALB/c recipients, groups of 300 islets were packed and implanted beneath the kidney capsule. The result of islet transplantation was followed by monitoring blood glucose and body weight. The procedure is described more thoroughly in paper II and V.

Morphological examination of grafts

In study II it is shown that transplantation of 600 wt or iNOS-/- islets could reverse the hyperglycemia in diabetic NOD recipients. When blood glucose increased above 12.0 mM after the preceding normalisation, mice were sacrificed by cervical dislocation. The islet graft attached to the kidney capsule was removed and fixed for histology or placed in RNA*later* buffer.

Retrieved grafts were fixed in 10% formalin, embedded in paraffin. Embedded grafts were sectioned at 5 µm, mounted on glass slides and stained with haematoxylin-eosin and/or insulin antibodies. The mononuclear cell infiltration and insulin content was ranked according to arbitrary classes. For further details see paper II.

RNA isolation, cDNA conversion and RT-PCR

In paper I-IV, islet samples, islet grafts and spleen cells were lysed using buffer RLT (Qiagen, KEBO, Spånga, Sweden) with addition of 1% β-mercaptoethanol. Total RNA was isolated from islets and grafts using RNeasy Kits from Qiagen. Samples were treated with DNase (Qiagen) to digest genomic DNA. By using Reverse Transcription System (Promega, SDS, Falkenberg, Sweden) cDNA was synthesised from the mRNA.

The Lightcycler instrument (Roche) combined with SYBR Green I (Roche) was use to amplify and analyse generated cDNA. PCR amplifications were performed in a volume of 10 µl. The amplification was performed by denaturing for 15 s at 95°C, annealing for 10 s at 60°C and elongation for 15 s at 72°C for 45 cycles. Cycle threshold (Ct) values were obtained for individual samples using the second derivative maximum method and used for relative comparisons (112,113).

In paper V, the RNA extraction was performed by the RNAzol method (Cinna Biotecx, Houston, TX, USA). The cDNA synthesis was done using a TaqMan Gold RT-PCR kit (Perkin-Elmer, Boston, MA, USA). The real-time PCR reactions in paper V were performed as described in the sequence detector manual (ABI Prism 7700; AB Applied Biosystems, Foster City, CA, USA).

Spleen cell preparation

In study IV, spleens were placed in cold Hanks' solution (SBL Vaccin, Stockholm, Sweden) with addition of 50 U/ml benzylpenicillin (Roche) and 0.05 mg/ml streptomycin (Sigma), and punctured repeatedly with a pair of forceps in order to release the spleen cells. The cell suspension was centrifuged and the cell pellet resuspended in 0.19 M NH_4Cl at 4°C to lysate the red blood cells. Next, the macrophage content was depleted by incubating the cell suspension for 60 min in tissue culture dishes containing RPMI 1640 and 10% foetal calf serum (Sigma), 100 U/ml benzylpenicillin (Roche) and 0.1 mg/ml streptomycin (Sigma) at 37°C (air + 5% CO_2) to allow these cells to attach to the culture dishes. Finally, the free-floating cells were transferred to new culture dishes containing RPMI 1640 (Sigma) and 10% foetal calf serum (Sigma) and maintained for 24 h.

A fraction of the spleen cells were collected in a pellet, the medium removed and subsequently the cells were suspended in redistilled water and sonicated. The DNA content in the homogenate was then measured fluorophotometrically.

Flow cytometry

In study IV, we analysed intracellular cytokine proteins in splenocytes. We used freshly isolated spleen cells preparations depleted of erythrocytes and macrophages, from six to seven animals in each treatment group (NaCl + NaCl, NaCl + PRL, STZ + NaCl, STZ + PRL). To promote accumulation of cytokine proteins and thereby obtain an enhanced staining signal, cells were incubated in RPMI 1640 (Sigma) supplemented with 1 μg/ml Brefeldin A (Sigma) for 5 h. Cells were fixed in PBS + 4% paraformaldehyde for five min. Permeablization of cells was performed during 10 min using PBS with addition of 2% rat serum, 0.1% BSA and 0.1% Triton (Sigma). FITC-conjugated antibodies for murine TNF-α, IFN-γ and IL-10 were purchased from Becton-Dickinson (Stockholm, Sweden). Cells were incubated with antibodies (0.5 μg/μl) for 30 min (4°C and dark). Thereafter, cells were analyzed with a FACS Calibur flow cytometer (Becton-Dickinson) and data were analyzed using Cell Quest software (Becton-Dickinson).

Cytokine ELISA

In study IV we analysed TNF-α, IFN-γ and IL-10 concentrations in supernatant samples using DuoSet ELISA (R&D Systems, Minneapolis, MN, USA). Absorbance values were read using a Labsystems integrated EIA Management System iEMS ELISA reader and analyzed with DeltaSoft3 2.22 EMSsoftware (Dr. E. Bechtold and BioMetallics©). The detection levels for TNF-α, IFN-γ and IL-10 were 31.3, 15.6 and 13.7 pg/ml, respectively.

Measurement of accumulated nitrite

Nitrite is a stable product of NO and oxygen, often used to indicate NO formation in biological fluids. Nitrite reacts with primary aromatic amines in an acidic solution and diazonium salts are formed, which will react with certain compounds to form intensely coloured azo-dyes. In study V, NO was measured as accumulated nitrite in the culture medium using the Griess reagent (0.5 % naphtyletylenediamine dihydrochloride, 5 % sulfanilamide and 25 % H_3PO_4) (114). The light absorbance of the reaction products were then measured by a spectrophotometer and the nitrite concentrations were calculated by the use of standard curves.

Cell viability and apoptosis assays

In paper V analyses of cell viability and apoptosis are described. These experiments were performed by co-authors at Steno Diabetes Centre, Gentofte, Denmark.

The MTT (diMethylThiazol-diphenylTetrazolium bromide) assay (Promega, Madison, WI, USA), measures mitochondrial impairment and was used to analyze cell viability/toxicity in isolated transgenic SOCS-3 islets and wt islets exposed to cytokines.

Life Death detection assay (Roche Diagnostics, Indianapolis, IN, USA) was used to measure apoptosis in cytokine exposed transgenic and wt islets. The assay was performed as specified in the supplier manual.

Moreover, the ApopTag Apoptosis Detection Kit (TUNEL-assay) (Intergen Company, Purchase, NY, USA) was used in paper V to determine apoptosis in SOCS-3 adenovirus-transduced primary β-cells. The assay was performed according to the manual of the manufacturer.

Western blotting

The western blotting in paper V was also performed by co-authors at Steno Diabetes Centre. Cells were lysed for 1 hour in ice cold lysis buffer and Western blotting was performed as described (81) with the following antibodies: Flag IgG (Sigma Aldrich), p-JNK/JNK IgG, p-p38/p38 IgG, p-ERK/ERK (Cell Signalling Technologies, Cambrigde, MA, USA), and IκB IgG (Active Motif, San Francisco, CA, USA). The used secondary antibodies were either rat or mouse anti-rabbit IgG Ab conjugated with horseradish peroxidase (Cell Signalling Technologies).

Statistics

Data in this thesis are presented as means ± SEM and compared using Student's unpaired t-test or paired t-test. In paper IV, flow cytometry data were analyzed using Mann-Whitney rank sum test and in paper V the Kaplan-Meier log-rank test was used in the transplantation studies. A p-value of less than 0.05 was considered as statistically significant.

Results and discussion

Paper I

Cytokines affect PDX-1 expression, insulin and proinsulin secretion from iNOS deficient murine islets.

Mol Cell Endocrinol 2005; 240: 50-57

It is established that IL-1β acutely augments the glucose stimulated insulin secretion (52,115). We have found that mouse iNOS-/- islets exposed to IL-1β for 48 h show changes in the insulin secretory response, with an increase in insulin output and followed by a suppressed response in insulin secretion after a glucose challenge (116). Presently we aimed to further characterise the mechanisms behind the altered kinetics of glucose stimulated insulin secretion from cytokine exposed pancreatic islets deficient in iNOS.

We found that the early stimulatory phase of insulin secretion was delayed and prolonged when iNOS-/- islets were exposed to IL-1β. Interestingly, the stimulatory effect of IL-1β was inhibited by IFN-γ in these islets. In wt islets, the stimulatory effect had almost vanished after 6 h of IL-1β exposure. After 6 h of IL-1β exposure to wt islets, it is likely that NO formed as a consequence of iNOS induction has started to affect cellular processes and impair the β-cell function (117,118). In iNOS-/- islets, there is no increase in NO formation, and therefore the stimulatory phase of IL-1β exposure might be sustained.

Previous studies of cytokine exposed rodent islets have suggested that PLD1 induction, DAG formation and PKC activation may be involved in the early stimulatory phase of insulin secretion after cytokine exposure (52,53). In order to examine if the DAG/PKC/PLD pathway is involved in the early stimulatory phase of insulin secretion from wt and iNOS islets, we examined mRNA expression of PLD1a and PLD1b after cytokine exposure to wt and iNOS-/- islets. The time-point 6 h was chosen since after 6 h of cytokine exposure, iNOS is expressed and NO formation has begun affect cellular processes in wt islets. After 24 h of cytokine treatment, insulin secretion is likely to be impaired.

In the present investigation we detected an increase in PLD1a expression from iNOS-/- islets after 6 h of exposure to IL-1β or IL-1β+IFN-γ. Both

PLD1a and PLD1b mRNA expressions were increased, in absence of NO formation, from cytokine treated iNOS-/- islets after 24 h. The observed increase in PLD1a and PLD1b mRNA expression from iNOS-/- islets after cytokine exposure supports the view that the DAG/PLD/PKC pathway may participate in the increased insulin secretion observed from iNOS-/- islets acutely after exposure to IL-1β. However, the changes in insulin secretion were not strictly correlated with the changes in PLD expression, indicating involvement of other mechanisms.

To study the role of proinsulin convertases, which could influence proinsulin conversion in cytokine exposed iNOS-/- islets, convertase mRNA expression and proinsulin secretion were examined. We found that IL-1β induced suppression of PC1 mRNA expression was at least partly NO-dependent, since PC1 expression was not reduced in iNOS-/- islets after 24 h of IL-1β exposure. IL-1β induced suppression of PC2 mRNA expression was, however, not NO dependent.

The reduction in PC1/PC2 expression after cytokine treatment was reflected as an increase in proinsulin secretion especially from IL-1β treated wt islets. When cytokines were combined, proinsulin secretion was markedly reduced from wt islets. The reduction in proinsulin secretion from cytokine exposed wt islets is probably the result of an overall suppression of islet function caused by NO formation. The iNOS-/- islets seemed to be less prone to increased proinsulin secretion. This could partially be explained by the lack of suppression of PC1 mRNA expression at 24 h of IL-1β exposure.

We also examined the effect of cytokine exposure on the expression of PDX-1 mRNA. PDX-1 is an important regulator of the function of the β-cell (119). The results from previous studies have been conflicting regarding the influence of NO on PDX-1 expression after cytokine exposure (120,121). By using the iNOS-/- islets we could see that PDX-1 expression is clearly decreased irrespective of NO formation.

The data in paper I demonstrate that *in vitro* exposure of iNOS-/- islets to cytokines induces a prolonged early stimulatory phase of insulin secretion, which may involve the DAG/PLD/PKC pathway and partially NO-dependent effects on proinsulin over insulin secretion. The cytokine exposure of the wt and the iNOS-/- islets was shown to decrease mRNA levels of PDX-1.

Paper II

Survival of an islet allograft deficient in iNOS after implantation into diabetic NOD mice. *Cell Transplantation* 2006; 15: 769-775

Herein we tested the hypothesis that transplantation of iNOS-/- islets would permit increased graft survival. In experiments *in vitro* we found that glucose-stimulated insulin release from wt and iNOS -/- islets resulted in a sevenfold increase in insulin secretion. This demonstrates that the two types of donor islets were functional and comparable in this aspect.

Spontaneously diabetic NOD mice were transplanted with 600 islets beneath the left kidney capsule. Islet graft function was followed up by the daily monitoring of blood glucose levels. We found that on the first day after transplantation, all animals had become normoglycemic. Animals in both experimental groups began to revert to hyperglycemia from day 3 and onwards. Transplantation of the two types of islets could maintain the normoglycemia for on average one week posttransplantation. Monitoring blood glucose is a way to follow graft function and our results are in line with recent studies using the NOD mice to investigate destruction of islet transplants (122).

The renal subcapsular space is one of the most extensively used sites for islet transplantations in rodents. From this site, it is relatively easy to retrieve islet grafts for further studies. In this study the mononuclear cell infiltration and insulin positive cells were evaluated. It was found that both types of islet grafts had infiltration of immune cells around and within the grafts. Some remaining endocrine cells were detected in the two types of grafts. The heavy infiltration observed in most of the grafts is due to the fact that we waited to retrieve the grafts until animals had already returned to hyperglycemia. At this time-point most of the transplanted β-cells were already destroyed. It was not possible to see an altered magnitude of infiltration during rejection of the two types of grafts. Remaining insulin-positive cells could be observed and confirmed the presence of donor islets. No apparent difference in the remaining insulin-positive cells could be observed. However, it is likely that the situation with hyperglycemic animals has led to a strong stimulation of the insulin secretion from the transplanted islets. Therefore we cannot exclude that there actually was a difference in β-cell content of the grafts at the examination.

Furthermore, it was possible to extract mRNA from the retrieved grafts and by RT-PCR we could detect the relative mRNA levels of certain genes. The mRNA levels of insulin and proinsulin convertases represent islet cell genes and our findings indicate that iNOS-/- islet grafts were more resistant to rejection/destruction than grafts consisting of wt islets.

The analysis of mRNA levels of cytokines believed to be involved in islet destruction revealed no differences in IFN-γ or TNF-α. However, mRNA levels of IL-1β were higher in grafts consisting of islets isolated from iNOS-/- islets.

Taken together, the RT-PCR results may suggest that iNOS-/- islet grafts are more resistant to rejection compared to wt islet grafts. The elevated IL-1β mRNA levels in grafts of iNOS-/- islets could be due to a more active and ongoing rejection process depending on the remaining islet tissue.

In this study, allogeneic transplantations have been examined. Apart from an allogeneic rejection, it is also possible that an autoimmune attack has also taken place. It has been suggested that it would be easier to delay islet allograft rejection than circumvent destruction of the islets by the autoimmune "recurrence of disease" process (79). If this is true such a mechanism could have contributed to the failure to demonstrate a difference between the two groups post-transplantation. From the data in paper II we conclude that deficiency of iNOS in transplanted pancreatic islets does not lead to a delayed allograft rejection in the examined transplantation model.

Paper III

Altered proinsulin conversion in rat pancreatic islets exposed long-term to various glucose concentrations or interleukin-1β. *J Endocrinol* 2007; 192: 381-387

Most studies of glucose regulation of the proinsulin conversion in β-cells have been performed over relatively short time-periods. In addition, previous studies of proinsulin conversion have mostly been performed using isolated pancreatic β-cells or cell lines. It is of importance to perform studies of islets as well as β-cells since results often are divergent because paracrine interactions between cells are very important for regulation of β-cell function. Currently, we examined the effects of long-time glucose culture or IL-1β exposure on proinsulin conversion in isolated rat pancreatic islets.

Isolated rat pancreatic islets were cultured for 1 week at different glucose concentrations (5.6, 11, 28 and 56 mM). Previously, studies have been performed with the addition of L-glucose (5.6-56 mM) to the RPMI 1640 medium with 5 or 11 mM D-glucose and have excluded possible osmotic effects underlying the findings caused by high concentrations of D-glucose (123).

Levels of accumulated proinsulin and insulin in media and within islets were analysed by ELISA. The ELISA measurements revealed increased proinsulin accumulation in media from islets cultured at increased glucose

concentrations. The increased proinsulin secretion can be explained by a sustained state of β-cell activation.

Real-time PCR was used to study the relative levels of mRNA of the proinsulin converting enzymes PC1 and PC2. The mRNA expression of PC1 was increased after culture at 11 and 28 mM glucose. Our findings are in line with previous studies that indicate that PC1 is the rate limiting enzyme in proinsulin conversion (23).

Relative contents of newly synthesized proinsulin and insulin were determined by pulse-chase labelling and immunoprecipitation. After a 90-min chase period it was found that islets cultured at 56 mM glucose had an increased proportion of newly synthesized proinsulin when compared with islets cultured at 5.6 mM glucose. No difference was observed in islets cultured at 11 or 28 mM glucose.

Our data suggest that a lasting increased functional stimulation of islets causes an increase in proinsulin ratio as well as in converting enzymes. This may reflect an adaptive response of the β-cell to an enhanced turnover of a diminished insulin pool. This adaptation, however, appears insufficient after culture at 56 mM glucose, as shown by increased levels of proinsulin and no adaptation of the converting enzymes.

The ELISA measurements showed that islet exposure to IL-1β increased proinsulin accumulation in the culture media. Islets exposed to IL-1β for 48 h displayed a decreased mRNA level of PC1 as well as PC2, indicating a decreased proinsulin conversion. Our data also showed a markedly increased proportion of newly synthesized proinsulin after 48 h of IL-1β exposure. These data are in line with previous studies, indicating a decreased proinsulin conversion in rat islets after a 24 h exposure to IL-1β (124). Treatment for 48 h with IL-1β increased the proportion of proinsulin both after 45 and 90 min chase periods, compared to control islets.

Increased proinsulin levels have been observed during the period preceding the clinical manifestation of the disease (21,125,126). It is possible that a high local IL-1β concentration may contribute to an increased proinsulin release.

The data in paper III show that stimulation by glucose of islets is coupled to a decreased conversion of proinsulin. Data also show that islets treatment with IL-1β is also coupled to a decreased proinsulin conversion. It is possible that this can contribute to the elevated proinsulin levels found both at the onset of type 1 diabetes as well as in type 2 diabetes.

Paper IV

Prolactin regulation of the expression of TNF-α, IFN-γ and IL-10 by splenocytes in murine multiple low dose streptozotocin diabetes. *Immunol Lett* 2006; 102: 25-30

Previously, the hormone prolactin has been found to be protective against development of type 1 diabetes induced by multiple injections of STZ (127). Based on *in vitro* experiments using isolated pancreatic islets in our earlier study, we found that it was unlikely that PRL *per se* would interfere with the direct β-cell inhibitory action of STZ. In paper IV, we investigated whether PRL can regulate the production of cytokines by splenocytes in mice treated with MLDSTZ. Immune cells of the pancreatic islets are considered to be difficult to isolate in sufficient quantities for studying immune responses. In this study we instead chose to isolate spleen cells, which may reflect the general immune state of the animal.

C57BL/Ks mice were treated with MLDSTZ. On day 15, splenocytes were isolated from the mice. Spleen cell preparations were stained for cytoplasmic TNF-α, IFN-γ and IL-10. Flow cytometry was used for analysis of the stained cells. Isolated spleen cells were also cultured for 24 h and mRNA analysed with real-time PCR and cytokine secretion measured by ELISA.

Freshly isolated spleen cells from PRL and STZ+PRL treated animals seemed to have an increased frequency of IL-10 positive cells. In cultured spleen cells isolated from MLDSTZ treated mice, IFN-γ and IL-10 mRNA levels were upregulated. Treatment with PRL downregulated the mRNA expression of these cytokines and also TNF-α in splenocytes from mice treated with MLDSTZ. The accumulation of these cytokines in the cultures of the explanted splenocytes showed only minor differences between the experimental groups.

In type 1 diabetes, it has been observed that a β-cell destructive insulitis is connected to a Th1 cytokine profile, while a non-destructive insulitis and disease protection is associated with Th2 cytokine secretion (128). In NOD mice, a Th1 response is considered to be involved in disease progression and a Th2 response is considered to help to suppress diabetes development (129). Treatments that can shift the cytokine profile in MLDSTZ from Th1 to Th2 have reduced diabetes incidence and decreased immune cell infiltration of the pancreatic islets (130-132). The data in paper IV support the view that PRL enhances a Th2 response and this may contribute to the preventive effect of PRL against development of multiple low doses STZ in mice.

However, it cannot be excluded that PRL possesses other immunoregulatory actions that can contribute to a reduced autoimmunity.

Moreover, PRL may be a growth factor for β-cells (133) which could be beneficial in prevention of ongoing loss of these cells.

Paper V

Suppressor of cytokine signalling-3 expression inhibits cytokine-mediated destruction of primary mouse and rat pancreatic islets and delays allograft rejection. *Diabetologia* 2008; 51: 1873-1882

Previously, Suppressor of cytokine signalling (SOCS)-3 has been shown to inhibit both IL-1β and IFN-γ mediated destruction of INS-1 cells (80). In paper V, mice with β-cell specific SOCS-3 expression and a SOCS-3 encoding adenovirus construct were used to characterise the protective effect of SOCS-3 in mouse and rat islets exposed to cytokines. Moreover, SOCS-3 transgenic islets were transplanted into spontaneously diabetic NOD mice and into alloxan-induced diabetic Balb/c mice.

In present study SOCS-3 transgenic islets showed significant resistance to cytokine induced apoptosis and impaired insulin release. A mixture of cytokines (IL-1β + IFN-γ) reduced the viability by 35% in wt islets, but transgenic islets were unaffected by this treatment. Analysis of accumulated insulin levels in media containing 16.7 mmol/l glucose following cytokine treatment demonstrated an impairment of insulin release (65% decrease) in wt islets, an effect that was completely counteracted by SOCS-3 expression in transgenic islets. Moreover, a 13-fold induction of apoptosis was observed in wt islets after exposure to a cytokine mixture (IL-1β + IFN-γ) for 3 days, whereas only a fourfold increase in apoptosis was observed in transgenic islets.

To be potentially useful in islet transplantation, SOCS-3 expression should not interfere with the glucose sensitivity/insulin secretion of the β-cell. Our present study reveal that neither glucose-stimulated insulin release, insulin content nor glucose oxidation were affected by SOCS-3. Thus, the data suggest that SOCS-3 expression at the level obtained in these studies did not alter the islet secretory function. Moreover, RIP-SOCS-3 transgenic mice have identical fasting blood glucose levels to their non-transgenic littermates (106).

Genetic engineering of islets/β-cells before transplantation would require *ex vivo* manipulation to introduce the transgene. To mimic this situation and at the same time investigate the effect of SOCS-3 following one possible *ex vivo* expression approach, SOCS-3 was expressed by using a SOCS-3 encoding adenoviral construct. Rat islet cultures transduced with SOCS-3 adenovirus displayed reduced cytokine-induced nitric oxide and apoptosis

associated with inhibition of the IL-1β induced NF-kB and mitogen-activated protein kinase (MAPK) pathways.

Next, 600 SOCS-3 transgenic or wt islets were transplanted into spontaneously diabetic NOD mice. All mice became normoglycaemic within 2 days, but from day 4 and onwards up to day 20 posttransplantation, all animals reverted to hyperglycaemia. Moreover, 300 transgenic or wt islets were transplanted into alloxan-induced diabetic BALB/c mice. In this transplantation model, the mice transplanted with SOCS-3 transgenic islets remained normoglycaemic longer then mice transplanted with wt islets. Thus, SOCS-3 β-cell over-expression was able to prolong islet survival in diabetic mice, but when autoimmunity was also superimposed (NOD mice) the protective effect was not sufficient.

In study V it was also found that SOCS-3 is upregulated in rat and human pancreatic islets following cytokine stimulation. In rat islets IL-1β alone induced SOCS-3 expression following cytokine exposure after 4 h, but at earlier time points an effect of the combination of IL-1β and IFN-γ was evident. In a non-β-cell fraction of rat islets, a more rapid induction of SOCS-3 mRNA in response to IL-1β was observed. This could explain why β-cells were killed selectively by cytokines, since non-β-cells are able to upregulate expression of SOCS-3 rapidly, thus preventing prolonged cytokine action and induction of apoptosis.

In human islets an increase in SOCS-3 mRNA was observed after 2 h of stimulation with IL-1β alone. Human β-cells require exposure to IL-1β, IFN-γ and TNF-α in combination for induction of apoptosis. When human islets were exposed to this mixture of cytokines, SOCS-3 mRNA was induced six- to tenfold after 4 to 6 h, suggesting a relatively slow induction and further supporting the hypothesis of a delayed and insufficient negative feedback of cytokine signalling in β-cells.

To conclude, the *in vitro* studies showed that SOCS-3 inhibits IL-1β induced signalling through the nuclear factor-κB and MAPK pathways and apoptosis induced by cytokines in primary β-cells. The *in vivo* studies revealed that SOCS-3 transgenic islets are protected in an allogeneic transplantation model. The results in paper V indicate that SOCS-3 may be a target for pharmacological treatment or genetic engineering in islet transplantation for treatment of type 1 diabetes.

Conclusions

The main conclusions of this thesis are:

- Exposure of isolated iNOS-/- islets to cytokines induces a prolonged early stimulatory phase of glucose stimulated insulin secretion. The prolonged stimulatory effect may involve the DAG/PLD/PKC pathway. (Paper I)

- Deficiency of iNOS in transplanted pancreatic islets does not lead to delayed allograft rejection. Remaining endocrine cells could be observed one week post islet transplantation both in iNOS-/- and wt islet grafts and mRNA for insulin, PC1 and PC2 could be detected by RT-PCR. (Paper II)

- Sustained glucose stimulation of islets is coupled to a decreased conversion of proinsulin to insulin. Islet treatment with IL-1β is also coupled to a decreased proinsulin conversion. (Paper III)

- When prolactin was administered to mice in the multiple low dose STZ model, PRL enhanced a Th2 response. This may contribute to the protective action by PRL in this model of autoimmune type 1 diabetes. (Paper IV)

- SOCS-3 overexpression inhibits IL-1β induced signalling through the NF-κB and MAPK pathways and apoptosis induced by cytokines in primary β-cells. SOCS-3 transgenic islets are protected in an allogeneic transplantation model. (Paper V)

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