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Role of Inducible Nitric Oxide Synthase and Melatonin in Regulation of β-cell Sensitivity to Cytokines

BY

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Abstract

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The mechanisms of β-cell destruction leading to type 1 diabetes are complex and not yet fully understood, but infiltration of the islets of Langerhans by autoreactive immune cells is believed to be important. Activated macrophages and T-cells may then secrete cytokines and free radicals, which could selectively damage the β-cells. Among the cytokines, IL-1β, IFN-γ and TNF-α can induce expression of inducible nitric synthase (iNOS) and cyclooxygenase-2. Subsequent nitric oxide (NO) and prostaglandin E_2 (PGE_2) formation may impair islet function.

In the present study, the ability of melatonin (an antioxidative and immunoregulatory hormone) to protect against β-cell damage induced by streptozotocin (STZ; a diabetogenic and free radical generating substance) or IL-1β exposure was examined. *In vitro*, melatonin counteracted STZ- but not IL-1β-induced islet suppression, indicating that the protective effect of melatonin is related to interference with free radical generation and DNA damage, rather than NO synthesis. *In vivo*, non-immune mediated diabetes induced by a single dose of STZ was prevented by melatonin.

Furthermore, the effects of proinflammatory cytokines were examined in islets obtained from mice with a targeted deletion of the iNOS gene (iNOS -/- mice) and wild-type controls. The *in vitro* data obtained show that exposure to IL-1β or (IL-1β + IFN-γ) induce disturbances in the insulin secretory pathway, which were independent of NO or PGE_2 production and cell death. Initially after addition, in particular IL-1β seems to be stimulatory for the insulin secretory machinery of iNOS –/- islets, whereas IL-1β acts inhibitory after a prolonged period. Separate experiments suggest that the stimulatory effect of IL-1β involves an increased gene expression of phospholipase D1a/b. In addition, the formation of new insulin molecules appears to be affected, since IL-1β and (IL-1β + IFN-γ) suppressed mRNA expression of both insulin convertase enzymes and insulin itself.

Keywords: β-cell, Cyclooxygenase, Cytokine, Diabetes, IFN-γ, IL-1β, iNOS, Insulin, Melatonin, Nitric oxide, Pancreatic islets, Phospholipase D, Prostaglandin E2, Streptozotocin

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”Det finns inget slut på hur komplicerat allt kan bli, med avseende på att en sak alltid leder till en annan.”

E. B. White

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, A K., Sandler, S. (2001) Melatonin protects against streptozotocin, but not Interleukin-1β-induced damage of rodent pancreatic β-cells. *J. Pineal Res.*, 30(3): 157-165

II. Andersson, A K., Flodström, M., Sandler, S. (2001) Cytokine-induced inhibition of insulin release from mouse pancreatic β-cells deficient in inducible nitric oxide synthase. *Biochem. Biophys. Res. Commun.*, 281(2): 396-403

III. Andersson, A K., Thorvaldson, L., Carlsson, C., Sandler, S. Cytokine-induced prostaglandin E_2 formation is reduced from mouse pancreatic islets deficient in inducible nitric oxide synthase. (manuscript)

IV. Andersson, A K., Börjesson, A., Sandler, S. Role of phospholipase D, insulin and proinsulin convertase gene expression in altered insulin secretion from β-cells deficient in inducible nitric oxide synthase following IL-1β and IFN-γ exposure. (manuscript)

Contents

Abbreviations

ANOVA	analysis of variance
AP-1	activator protein-1
APC	antigen presenting cell
ATF-2	activating transcription factor-2
BSA	bovine serum albumin
CD	cluster of differentiation
COX	cyclooxygenase
DAG	diacylglycerol
EMSA	electromobility shift assay
eNOS	endothelial nitric oxide synthase
FACS	fluorescence activated cell sorter
FasL	Fas ligand
FCS	foetal calf serum
G6PDH	glucose-6-phosphate dehydrogenase
GAF	gamma interferon activation factor
GAS	interferon-gamma activated site
HLA	human leukocyte antigen
HO-1	heme oxygenase-1
HPLC	high pressure liquid chromatography
HRP	horseradish peroxidase
IFN-γ	interferon-gamma
IL-	interleukin-
IL-1R	interleukin-1 receptor
IL-1β	interleukin-1beta
iNOS	inducible nitric oxide synthase
IP-10	interferon-inducible protein 10
IRAP	interleukin receptor antagonist

IRF-	interferon regulatory factor-
JAK	Janus activated kinase
JNK	c-Jun N-terminal kinase
KRBH	Krebs-Ringer bicarbonate HEPES
LPS	lipopolysaccharide
MAPK	mitogen activated kinase
MCP-1	macrophage chemoattractant protein-1
MHC	major histocompatibility complex
MLDSTZ	multiple low dose streptozotocin
MnSOD	manganese superoxide dismutase
NAD	nicotinamide adenine dinucleotide
NAT	serotonin N-acetyltransferase
NF-IL6	nuclear factor-IL6
NF-κB	nuclear factor-kappa B
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NOD	non-obese diabetic
PAGE	polyacrylamide gel electrophoresis
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffered saline
PGE	prostaglandin E
PI	propidium iodide
PKC	protein kinase C
PLD	phospholipase D
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SOCS	suppressors of cytokine signaling
SPI-3	serine protease inhibitor-3
STAT	transducers and activators of transcription
TNF-α	tumor necrosis factor alpha
wt	wild-type

1. Introduction

1.1 Type 1 Diabetes Mellitus

Despite extensive research efforts, type 1 diabetes remains to be a complex disorder, resulting in a life-long requirement of insulin therapy. The disease appears to evolve during a long preclinical period, with the absence of clinical symptoms (1,2). As the disease develops, β-cell mass is progressively lost, often with a marked acceleration about six months before clinical presentation. At time of diagnosis, most islets are deficient in β-cells and severe insulin depletion results in hyperglycemia and other clinical symptoms. The incidence of type 1 diabetes is increasing worldwide, and by the year 2010 it will be around 30-50 per 100 000 a year in high incidence populations (3). Genetic predisposition such as HLA alleles (4-6) together with environmental factors may influence disease development. Viral infections (e.g. enteroviruses) (7), dietary proteins and stress have been suggested to initiate or trigger the process leading to β-cell destruction, but the mechanisms of disease onset remains to be clarified (8).

During the pre-diabetic period and in newly diagnosed patients, various circulating antibodies against islet cell antigens (including insulin) are found, suggesting an ongoing process of β-cell damage (9,10). Islet cell antibodies also occur in close relatives of type 1 diabetes patients, especially in high risk siblings who share one or both HLA haplotypes with the diabetic proband (11). Several lines of evidence support the view that type 1 diabetes is an autoimmune disease, i.e. the insulin-producing β-cell is attacked and killed by the patients own immune system. Different HLA genotypes may influence susceptibility to type 1 diabetes, by affecting the degree of immune responsiveness to a β-cell antigen (5), or by modulating the normal immunologic tolerance to self antigens (12). The function of the HLA is to associate with antigenic peptides, and T-lymphocytes recognizing the HLA peptide complex trigger activation and proliferation of T-lymphocytes. While certain HLA genotypes increase the susceptibility for type 1 diabetes, others have been shown to be protective (13,14).

1.2 Insulitis and β-cell destruction

When pancreata of newly diagnosed type 1 diabetes patients are histologically examined, islets heavily infiltrated with immune cells are found (commonly referred to as insulitis). The distribution of insulitis appears to be uneven, with some islets infiltrated, others that are not infiltrated but deficient in β-cells, and also islets that appear to be normal (15-17).

Studies from animal models of type 1 diabetes (NOD mice and BB rats), suggest that the major populations of cells that infiltrate islets during early stage of insulitis are macrophages and dendritic cells (reviewed in (18)). The presentation of β-cell specific autoantigens by macrophages to $CD4^+$ helper T-cells may be the initial step in the autoimmune process (Fig 1). Gradually, cytotoxic $CD8^+$ T-cells are activated and play an important role in the β-cell destruction. At onset of type 1 diabetes, T-cells are the most abundant islet-infiltrating cell in humans (16,19,20), as well as in the insulitis of NOD mice (21). Depletion of $CD4^+$, $CD8^+$, T-cells or macrophages in NOD mice results in protection from disease (22-24), and T-cell clones can transfer the disease to non-diabetic animals (25-27).

Proinflammatory cytokines and free radicals secreted by invading macrophages and T-cells are believed to be important in the process of the selective β-cell destruction (28-30) (Fig 1). Macrophage derived oxygen and nitrogen free radicals may damage islet cell membranes and induce DNA lesions (31,32). In comparison to other cell types, β-cells have a low activity of specific free radical scavenging enzymes, making them particularly sensitive to oxidative stress (33-38). Cytokines bind to specific receptors on the β-cells, inducing signal transduction and gene transcription. Among the cytokines, the actions of interleukin-1β (IL-1β), interferon-γ (IFN-γ) and tumor necrosis factor α (TNF-α) have been the most studied. Pathways suggested to be activated by IL-β, IFN-γ and TNF-α (as single mediators or in combination) include induction of the enzyme inducible nitric oxide synthase (iNOS) and subsequent nitric oxide (NO) formation (39-41), activation of arachidonic acid metabolites (42), and expression of pro-apoptotic genes (36,43,44).

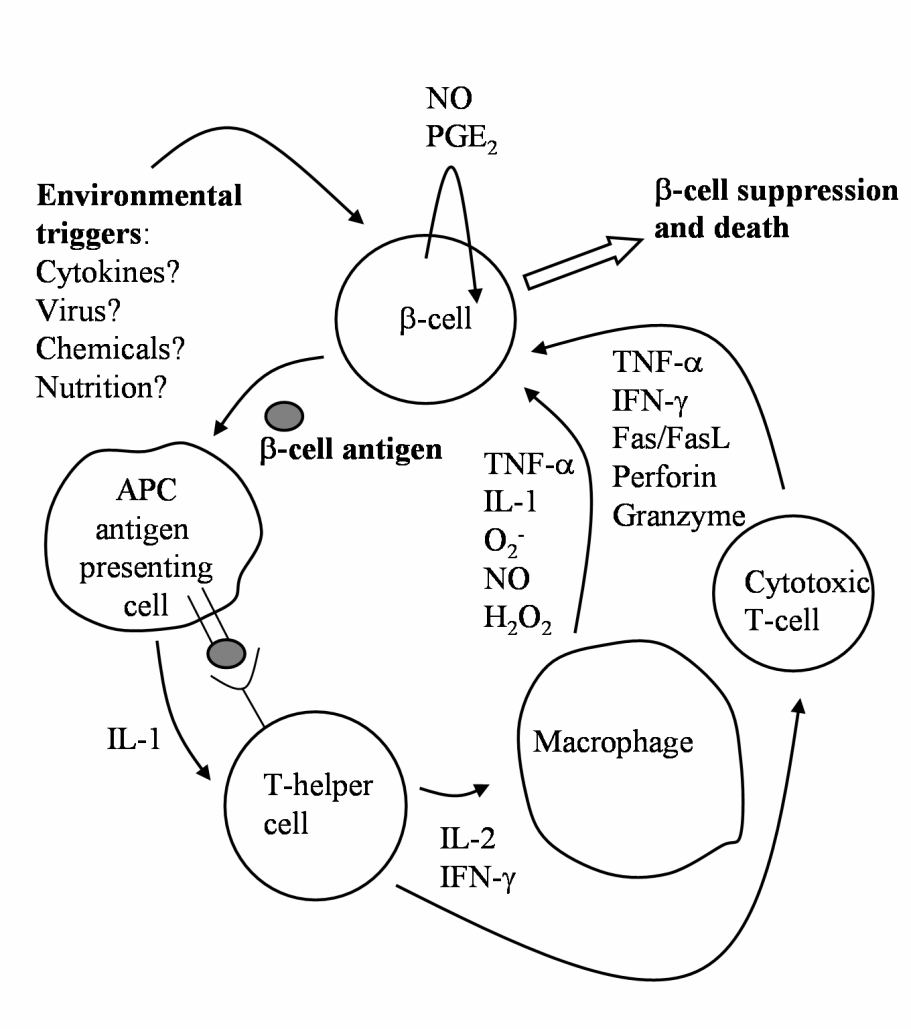

Figure 1. Proposed mechanisms of immune mediated β-cell destruction (modified from (29,30,45)). After initial damage, β-cell specific proteins are processed by APCs (macrophages and dendritic cells) and act as autoantigens. APCs present the antigen with MHC class II molecules to $CD4^+$ T-helper cells that become activated. $CD4^+$ T-cells then trigger macrophages and cytotoxic T-cells, that may secrete damaging cytokines and harmful molecules such as NO and other free radicals. Cytokines may induce inracellular production of NO and prostaglandin E_2, which could impair β-cell function. Antigen specific T-cells that bind to antigen-MHC class I complexes directly on the β-cell may also induce damage and cell death via interaction between FasL, and cytokine-induced Fas expression on the β-cells. Cytotoxic T-cells could also secrete harmful enzymes such as perforin and granzyme.

1.3 Proinflammatory cytokines and their effects in β-cells

1.3.1 IL-1β

IL-1β is an important regulator in immune and inflammatory responses (46). Mandrup-Poulsen and colleagues (47) first described IL-1 as a central mediator of β-cell destruction (47,48), and later it was found that primary β-cells express the type 1 IL-1 receptor (IL-1R1) (49). The natural IL-1β antagonist (IRAP), can be used *in vitro* to inhibit IL-1β-induced suppression of islets function (50). The major signalling pathways described from IL-1R1 in β-cells involve mitogen activated protein kinases (MAPKs), stress-activated protein kinases (JNKs) and tyrosine kinase activation, inducing various transcription factors including nuclear factor kappa B (NF-κB), activator protein-1 (AP-1), activating transcription factor-2 (ATF-2), nuclear factor-IL6 (NF-IL6) and interferon regulatory factor-1 (IRF-1) reviewed in (29,46,51). Activation of NF-κB is critical for IL-1β-mediated iNOS expression in β-cells (52,53), and NF-κB may also be involved in IL-1β-induced expression of cyclooxygenase-2 (COX-2) and subsequent prostaglandin E_2 (PGE_2) formation (54). Other factors suggested to be induced by IL-1β include ceramide, released from membranes by sphingomyelinase (55,56) and protein kinase C (PKC) activation via phospholipase (PLD) induction and diacylglycerol (DAG) formation (57,58).

In rat islets, IL-1β inhibits insulin secretion, insulin biosynthesis and oxidative metabolism (59). Moreover, IL-1β induces DNA damage and reduces cell viability of rat islets (60,61). In mouse islets, insulin secretion is impaired, and glucose oxidation can be affected, although usually not to the same extent as in rat islets (62,63). Human islets appear to be fairly resistant to exposure to IL-1β alone, whereas combinations of IL-1β, IFN-γ and TNF-α are suppressive and cytotoxic to human islet cells (64,65). Expression of iNOS and subsequent NO formation may mediate some of the inhibitory effects of IL-1β on β-cells, since inhibition of iNOS confers partial protection against IL-1β-induced impairment (39,66-69).

Another suggested mediator of IL-1β-induced inhibition of islet function is PGE_2, induced by COX-2 expression (42,70). COX-2 activity may be regulated by the same signal transduction pathways as iNOS (54,71), and NO may affect the level of PGE_2 formation (72,73). Selective inhibition of COX-2 was reported to counteract IL-1β-induced β-cell impairment (74,75) and streptozotocin-induced diabetes (76). IL-1β-induced ceramide formation

may also affect β-cell function, by inhibition of insulin secretion (55) and induction of stress response proteins (56). However, no increase in ceramide content was found in a β-cell line exposed to cytokines (77).

Along with the activation of various deleterious proteins and enzymatic products, IL-1β also induces expression of proteins involved in free radical defence and cellular repair, including heat-shock proteins, manganese superoxide dismutase (MnSOD), heme oxygenase-1 (HO-1) and serine protease inhibitor-3 (SPI-3) (4,32,62,78,79). IL-1β may also induce apoptosis in β-cells, by NO formation (discussed below) and/or up-regulation of the cell-surface receptor Fas. Interaction between Fas and its ligand (FasL), expressed on cytotoxic T lymphocytes, has been suggested to mediate islet cell apoptosis during insulitis (43,44).

1.3.2 IFN-γ

IFN-γ is known as a promoter of inflammatory reactions (reviewed in (80)). Signalling pathways of IFN-γ are mediated via Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) (81). Activation of STAT1α leads to formation of gamma interferon activation factor (GAF). GAF binds to gamma activated sites (GAS) in primary response genes of IFN-γ, inducing the transcription of different transcription factors, such as IRF-1 and IRF-2. IRF-1 is essential for IFN-γ-mediated induction of iNOS in macrophages (82), and is also believed to be involved in macrophage derived COX-2 expression (83). In islet cells, IFN-γ upregulates major histocompatibility complex class I (MHC class I) expression and induces IRF-1 expression (84,85). The reported effects of IFN-γ exposure to β-cells have been conflicting: in some studies IFN-γ was suggested to be inhibitory, while in others, no suppressive effects has been shown.

In rat and murine cell lines, IFN-γ was either shown to inhibit insulin production and cell replication (37,38,86,87), or not to affect β-cell function (88). It appears to be important (compared to other cytokines) to consider species specificity when studying the effect of IFN-γ added to β-cell cultures, since rat but not human IFN-γ showed some inhibitory effects on isolated rat islets (59,89). When murine IFN-γ was added to mouse islets, an impairment of insulin secretion was reported (90). On the other hand, an IFN-γ-induced reduction of the islet DNA content and glucose oxidation rate, without any effect on insulin secretion, has also been observed (91). In human islets, IFN-γ induced minor toxic effects and suppressed insulin secretion (64,92,93), although in one study, no inhibitory action was reported (65). It is possible that the inconsistent results regarding the effect

of IFN-γ on β-cells reflects differences in islet preparations and in *in vitro* culture methods, as especially in studies with human islets, the composition and quality of islet preparations may vary (94,95).

When IFN-γ is combined with IL-1β and TNF-α, IFN-γ acts synergistically to inhibit β-cell function and reduce β-cell viability; reviewed in (30). The synergistic effect of IFN-γ in cytokine-mediated β-cell damage may depend on enhancement of IL-1β-induced gene expression, as reported for iNOS induction, and/or via activation of non-endocrine cells within or close to the islets (e.g. macrophages and ductal cells) (85,96,97).

1.4 iNOS and NO formation

NO is a small molecule with a broad range of biological effects, including involvement in neurotransmission, vascular homeostasis and defence against microorganisms and transformed cells. There are three isoforms of NO synthases (NOS), all catalysing NO biosynthesis via a reaction involving conversion of L-arginine to L-citrulline (98). Two of them, endothelial NOS (eNOS) and neuronal NOS (nNOS), are calcium-dependent and constitutively expressed in many cell types (99,100). The third isoform, inducible NOS (iNOS), is calcium-independent and expressed at high levels after induction by cytokines and free radicals (101). NO derived from iNOS is suggested to be important in the defence against invading microorganisms and in the elimination of transformed cells (101,102).

During the process of insulitis, it is likely that infiltrating immune cells secrete cytokines that may induce iNOS expression and NO formation in β-cells, as well as in endothelial cells and activated intra-islet macrophages (103-106). *In vitro*, cytokine-induced expression of iNOS in islet cells is species specific. In rodent islets, exposure to IL-1β alone or in combination with IFN-γ or TNF-α or both, induces iNOS expression and NO formation, while in human islets a combination of (IL-1β + IFN-γ) or (IL-1β + IFN-γ + TNF-α) is required (93). The differences in regulation of iNOS expression in β-cells could be due to variation in the requirement of transcription factors between species (107).

Once produced, NO may react with superoxide to form peroxynitrite (108). NO itself or NO-derived peroxynitrite may inhibit the activity of [FeS] containing enzymes involved in β-cell metabolism, such as the Krebs cycle enzyme aconitase and proteins in the mitochondrial respiratory chain (40,109,110). Other heme-containing enzymes like dioxygenases and cyclooxygenases may be stimulated or inactivated by NO (111,112),

implicating a role for NO in prostaglandin synthesis. High levels of NO or peroxynitrite may lead to DNA strand breaks and β-cell death (113-116). Moreover, NO has been suggested to be involved in cytokine-mediated β-cell apoptosis (117-120). However, recent studies of β-cells from mice with a targeted deletion of iNOS (iNOS -/- mice), suggest that NO does not take an essential part in the process of cytokine-induced programmed cell death (121,122).

The importance of NO in cytokine-induced impairment of β-cell function and diabetes-induction have been illustrated by the use of different NOS inhibitors, some of which have been shown to be protective *in vivo* (123-125), while others only *in vitro* (67-69,126,127). iNOS -/- mice were partially protected against diabetes induced by multiple low-dose streptozotocin treatment (128), and islets from iNOS -/- mice were shown to be relatively resistant to cytokine-induced impairment of β-cell function (121,122,128). When transgenic mice expressing iNOS cDNA under control of the insulin promoter were generated, the mice developed diabetes without insulitis (129).

2 Specific aims of the thesis

- To examine whether melatonin could protect rodent pancreatic islets against STZ-induced damage, both *in vitro* and *in vivo*
- To investigate if melatonin could protect isolated rat islets against IL-1β-induced impairment *in vitro*
- To evaluate the function of iNOS -/- islets after a prolonged (48 h) exposure to IL-1β and (IL-1β + IFN-γ)
- To test whether IL-1β induces COX-2 activity and PGE_2 formation in iNOS -/- islets to a similar extent as in wt islets
- To study if IFN-γ affects IL-1β-induced COX-2 and PGE_2 formation in iNOS -/- islets
- To investigate the effect of IL-1β and (IL-1β + IFN-γ) exposure to iNOS -/- and wt mouse islets on early kinetics of glucose-stimulated insulin secretion
- To evaluate the gene expression of insulin, proinsulin-converting enzymes and the possible involvement of PKC activation after cytokine exposure to iNOS -/- and wt islets.

3 Methods

3.1 Streptozotocin and induction of β-cell damage and diabetes

Streptozotocin (STZ) is a naturally occurring antibiotic, originally isolated from *Streptomyces achromogenes* (130). Besides being a potent agent against microorganisms, it was found to suppress tumours (131). During investigations of the cytostatic effect, it was discovered that STZ could cause hyperglycaemia in rats and dogs (132), and later on also in various other animals, including mice (133).

STZ consists of a glucose molecule and an *N*-nitrosomethylurea side chain linked to position C2 of the glucose residue (Fig 2). Since STZ is structurally similar to glucose, it is believed to be taken up by β-cells and hepatocytes through the GLUT2 glucose transporter (134). STZ decomposes shortly (in 5-15 min) after entering the cell, producing free radicals and reactive methyl cations that may induce DNA alkylations and cross-link the DNA strands (135,136). DNA alkylations and other DNA lesions activate nuclear excision repair enzymes, which create DNA strand breaks (137). Single-strand breaks and endonucleolytic activity during excision repair stimulates the synthesis of poly(ADP-ribose) polymers by the enzyme poly(ADP-ribose) polymerase (PARP) (138). Activity of PARP is thought to be important in the process of excision repair, by mediating unfolding of the DNA and affecting the access for DNA repair enzymes (139,140). Nicotinamide adenine dinucleotide (NAD) is used as a substrate during the PARP-mediated synthesis of poly(ADP-ribose) polymers, reducing the cellular NAD content of STZ-treated islets (141) and liver cells (142). NAD is an important co-factor for oxidative phosphorylation, and its depletion reduces the cellular ATP production, leading to impairment of glucose oxidation and protein synthesis (143). The diabetogenic effect of STZ is reduced by different free radical scavengers (144-147), inhibition of PARP by nicotinamide (148), or a disruption of the gene coding for the PARP enzyme (149-151).

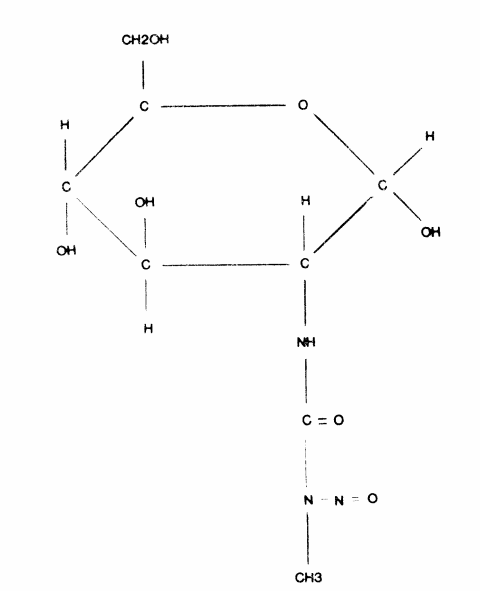

Figure 2. Structure of STZ

When STZ is given to mice as a single high injection (140-160 mg/kg body weight) it produces an acute β-cell degeneration and hyperglycaemia in 2-3 days (152), without any signs of islet inflammation. However, in 1976, Like and Rossini (153) discovered that multiple low dose injections (40 mg/kg body weight) of STZ to certain mice strains produced pancreatic insulitis and hyperglycaemia approximately 7 days after the first injection. It is believed that multiple low dose injections of STZ induce substantial islet damage, and that infiltrating lymphocytes and macrophages are responsible for the progressive β-cell destruction seen in the multiple low-dose STZ model (154,155).

3.2 The use of melatonin as a possible intervention against β-cell destruction

In 1958, Lerner and colleagues isolated melatonin, and identified it as the active substance from the pineal gland (156). Melatonin (Fig 3) is synthesised from the amino acid tryptophan with serotonin as an intermediate. One of the enzymes taking part in the melatonin synthesis,

serotonin N-acetyltransferase (NAT), displays a rhythmic pattern of activity, and variation in NAT activity has been suggested to mediate peaks of melatonin secretion (157). In humans and other mammals including rodents, melatonin secretion peaks at night, independently of species differences in night or day activity. Melatonin is an important messenger of photoperiodic information to the organism, and provides information concerning time of the day and time of the year to organs in the body; reviewed in (158). Besides involvement in timing of circadian rhythms and regulation of reproduction, proposed therapeutic effects of melatonin include sleep induction (159), alleviation of jet lag (160,161) and reduction of tumour growth (162).

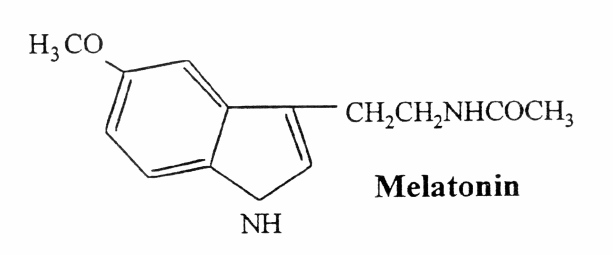

Figure 3. Structure of melatonin

Recently, it was found that in addition to melatonin's receptor-mediated effects (see above), it is also a scavenger of free radicals (163-165). Receptor-mediated effects of melatonin action may be G-protein coupled and result in inhibition of cAMP levels (166), while scavenging of free radicals does not seem to require receptor binding. Due to the combined lipophilic and hydrophilic properties of melatonin, it may easily pass through biological membranes and rapidly enter different cellular compartments, including the nucleus (167). The antioxidant effect of melatonin in experimental conditions has mostly been observed at non-physiological concentrations (μM-mM), compared to the level of endogenous melatonin found in serum (around 0.3 nM) (168).

In addition to a direct scavenging effect, melatonin may upregulate various antioxidative enzymes such as glutathione peroxidase and superoxide dismutases (169-171). Melatonin has been found to be protective in different animal models of inflammation and oxidative stress, such as kainate-induced brain damage (172), LPS-induced multiple organ dysfunction (173) and zymosan- and carrageenan-induced inflammation (174-177). Furthermore, melatonin was shown to counteract hyperglycaemia in STZ-treated rats (178), and in STZ or alloxan-injected mice (179,180).

Besides the protective effect against chemically induced diabetes, melatonin was shown to protect against development of spontaneous autoimmune diabetes in NOD mice (181). The protective effect seen in models of inflammation and free radical-induced tissue destruction has mostly been explained by the antioxidant properties of melatonin, although a role in regulation of immune responses also has been suggested (182).

In the present investigation, melatonin treatment was tested as a possible intervention against STZ and IL-1β-induced β-cell impairment (I).

3.3 STZ treatment *in vivo* (II)

To study the effect of melatonin against STZ induced hyperglycaemia, inbred male C57BL/Ks mice were used (bred in a local colony at The Animal Department, Biomedical Centre, Uppsala, Sweden). The mice were divided into four different groups of treatment: (vehicle + citrate buffer, melatonin + citrate buffer, vehicle + STZ, melatonin + STZ). The animals first received either an intraperitoneal injection of vehicle (30% ethanol/PBS, 0.2 ml) or of melatonin (100 mg/kg body-weight, 0.2 ml), and 30 min later the mice were given a second intravenous injection in a tail vein of either citrate buffer or STZ (140 mg/kg body weight, 0.2 ml). Blood glucose concentrations were determined before treatments was started (day 0), and once a day until animals were killed by cervical dislocation on day 7. Animals were considered hyperglycaemic when their blood glucose value was ≥ 11.1 mM.

3.4 The iNOS -/- mouse

Mice deficient in iNOS were generated by MacMicking and colleagues to test the hypothesis that iNOS defends the host against infectious agents and tumour cells at the risk of contributing to tissue damage and shock (183). A gene replacement vector (pINOS-RV1) was designed to delete the proximal 585 bases of the iNOS promoter, plus exons 1-4, including an ATG translational start site in exon 2. The lack of iNOS expression from homozygotes was evaluated by northern and western blots. The absence of iNOS expression in islets from iNOS -/- mice was confirmed in paper II , where we did not find any iNOS mRNA expression. Furthermore, we observed no elevation in NO formation after cytokine exposure to islets from

iNOS -/- mice. Under specific pathogen free conditions, iNOS -/- mice survive weaning, gain weight and mature at the same rate as wt mice They also produce litters at normal frequency and size (183).

MacMicking *et al* found that most examined tissues appeared to be histologically normal, and also lymphocyte expression of immunoglobulin, CD3, CD4, CD8 and MHC class II antigens was not affected. In addition, iNOS -/- mice showed normal inflammatory responses regarding accumulation of leukocytes upon chemically induced peritonitis, formation of granulomas after *Listeria* infection, migration of neutrophils in LPS treated lung and production of inflammatory cytokines in endotoxin-treated mice (183). The absence of iNOS expression seems mostly to affect responses to certain bacterial infections, susceptibility to septic shock and defence against growth of tumours (183-185).

In the present study, isolated islets from iNOS -/- mice were used to examine NO-dependent versus NO-independent effects of proinflammatory cytokines on β-cell function (II-IV).

3.5 Islet isolation

Male Sprague-Dawley (SD) rats bred in a local colony (Animal Department, Biomedical Centre, Uppsala, Sweden) were used as donors of rat islets (I).

Male mice deficient in iNOS (iNOS -/- mice, background C57BL/6x129SvEv, H-2^b) and male wild-type controls (wt, C57BL/6x129SvEv, H-2^b) were used as donors of mouse islets (II-IV). The iNOS -/- mice were generated from embryonic stem cells with a targeted disruption of the iNOS gene (183), and breeding pairs of iNOS -/- mice were kindly provided by J.S. Mudgett (Merck Research laboratories, Rahway, NJ, USA) and J.D. MacMicking and C. Nathan (Cornell University Medical College, New York, NY, USA). Homozygous iNOS mutants were maintained by interbreeding the F2 generation. The mice have subsequently been bred in the Animal Department, Biomedical Centre, Uppsala, Sweden. Wild-type controls were bought from Taconic, Germantown, NY, USA.

Animals were killed by cervical dislocation, and islets were isolated by a collagenase digestion procedure, and subsequent hand-picking (59). Groups of 150 islets were precultured free-floating in 5 ml RPMI 1640 medium containing 11.1 mM glucose and supplemented with 10% (vol/vol) foetal calf serum (FCS), benzylpenicillin (100 U/ml) and streptomycin (0.1 mg/ml)

in an atmosphere of humidified air and 5% CO_2. Islets were cultured 6-8 days before experiments, and medium was changed every second day.

3.6 STZ, melatonin and cytokine exposure *in vitro*

In paper I, STZ (0.5 mM) was dissolved in Krebs-Ringer bicarbonate buffer (KRBH buffer) supplemented with 5.6 mM glucose and 2 mg/ml bovine serum album, and melatonin (100 μM – 1mM) was dissolved in ethanol (final concentration 1%)

For *in vitro* experiments with cytokines (I-IV), human IL-1β (25 U/ml) and murine IFN-γ (1000 U/ml) were used. Murine IFN-γ was used because species specificity appears to be important for the biological activity of IFN-γ (while less important for the activity of IL-1β) (59,89).

3.7 Assessment of islet function and viability

3.7.1 Evaluation of mitochondrial function by glucose oxidation

To examine mitochondrial function, islets were incubated with radioactively labelled glucose. Liberated CO_2 was trapped, and radioactivity was measured (186). This method only determines the fraction of glucose metabolised in the mitochondria, and not the islet glucose utilisation. Duplicate (iNOS -/- islets) or triplicate (SD islets) groups of islets were transferred to sealed glass vials containing KRBH, supplemented with D-[U-^{14}C]glucose and nonradioactive glucose to a final specific activity of 0.5 mCi/mM and glucose concentration of 1.7 or 16.7 mM. After a 90 min incubation at 37° C, islet metabolism was arrested by addition of antimycin A. During a further 120 min incubation, CO_2 produced was trapped in hyamine hydroxide after liberation from the KRBH by addition of NaH_2PO_4 (pH 6.0). Radioactivity was measured by liquid scintillation.

3.7.2 Islet insulin release and insulin content

Duplicate or triplicate groups of islets were transferred to flat bottom multi-well plates containing KRBH supplemented with 2 mg/ml bovine serum

albumin and 1.7 or 16.7 mM glucose (I-II), or RPMI 1640 supplemented with 16.7 mM glucose (IV). Islets were then incubated for various time-periods at 37°C in an atmosphere of 95% air and 5% CO_2. After incubation, islets were pooled and ultrasonically disrupted in 0.1 ml redistilled water (I-II). A fraction of the aqueous homogenate was mixed with acidic ethanol (0.18 M HCl in 96% (vol/vol) ethanol) and the insulin was extracted overnight at 4°C. The insulin released to the media and in the extracts was measured by RIA (I-II) or ELISA (IV). The measured level of insulin obtained using the two different techniques was comparable.
For the RIA, samples were diluted in phosphate buffer, and mixed with anti-insulin serum (guinea pig). Samples were allowed to react for 20 h, and ^{125}I-insulin was added. After incubation at 4°C, free and antibody-bound insulin was separated by addition of ethanol, and free ^{125}I-insulin radioactivity was counted (187).

In paper IV, an insulin ELISA was used. This specific rat insulin ELISA is based on a direct sandwich technique, in which two monoclonal antibodies are directed against separate antigenic determinants of the insulin molecule. During incubation, insulin in the sample reacted with peroxidase-conjugated anti-insulin antibodies and anti-insulin antibodies bound to microtiration wells. Bound conjugate was detected by reaction with 3,3´,5,5´-tetramethylbenzidine. The reaction was stopped by addition of acid, giving a colometric endpoint that was read spectrophotometrically. The rat insulin ELISA has an 81% cross reactivity for mouse insulin, and 71% cross reactivity for human proinsulin.

3.7.3 Total protein and (pro)insulin biosynthesis

To determine total protein and (pro)insulin biosynthesis, islets were incubated at 1.7 mM and 16.7 mM glucose for either 90 min or 2 h at 37°C, with the addition of radioactively labeled L-leucine (50 µCi/ml L-[4,5-3H]leucine). The amount of incorporated radioactive L-leucine into insulin was estimated by precipitating insulin molecules with anti–insulin antibodies, and then radioactivity was counted and compared with the radioactivity of whole protein extracts (188). In the immunoprecipitation procedure, polyclonal anti-insulin serum was used (guinea pig), which is likely to bind both proinsulin and insulin molecules.

3.7.4 Measurement of nitrite formation

Nitrite is a stable product of NO and molecular oxygen, used as an indicator of NO formation in biological fluids (189). Nitrites react with primary aromatic amines in acidic solutions, with the formation of diazonium salts, which will couple with certain compounds to form intensely colored azo dyes (originally discovered by Griess in 1879 (190)) Aliquots of incubation media were added to a mixture of 0.5 % naphtylenediamine dihydrochloride, 5% sulphanilamide and 25% concentrated H_3PO_4 and incubated at 20ºC for 5 min. The absorbance was measured spectrophotometrically at 546 nm against a standard curve of sodium nitrite.

3.7.5 Quantification of PGE_2 formation

PGE_2 content in culture medium was determined with a PGE_2 competitive enzyme immunoassay system from Amersham Pharmacia Biotech. The assay is based on competition between unlabelled PGE_2 and a fixed quantity of peroxidase-labelled PGE_2 for a limited number of binding sites on a PGE_2 specific antibody. The reaction was stopped by addition of acid, giving a colometric endpoint that was read spectrophotometrically.

3.7.6 Determination of islet NAD content

For determination of NAD content, high pressure liquid chromatography (HPLC) was used. In HPLC, a liquid mixture of components is separated by high pressure force through a column, which retains the component of interest. NAD from groups of islets was extracted with 0.5 M $HClO_4$. After neutralisation with N,N-dioctylmethylamine, samples were separated on a 250 x 4.6 mm partisil 10 SAX column and eluted in a 20 mM $NH_4H_2PO_4$, 20 mM sodium acetate buffer; pH 4.15. Peaks were identified by UV-absorption (191).

3.7.7 Assessment of cell viability by flow cytometry

Propidium iodide (PI) is a polar dye, which stains both apoptotic and necrotic cells, the latter with high intensity (192). To examine cell viability, islets were incubated with 20 µg/ml PI for 15 min, and were then dissociated with trypsin. Changes in light scattering and fluorescence emission were

determined by flow cytometry with a Becton Dickinson FACSCalibur equipped with CellQuest software. Five to ten thousand cells per sample were analysed by exciting the cells at 488 nm and examining fluorescence at 650 nm. As a cell shrinks or loses volume, forward-scattered light decreases. Untreated cells were gated on a forward scatter versus PI fluorescence dot plot, and the same gates were then applied to cytokine treated cells for calculation of the fractions of apoptotic and/or necrotic cells.

3.8 Analysis of transcription factor activation, mRNA and protein expression

3.8.1 Extraction of nuclear proteins and electromobility shift assay (EMSA)

EMSA is a method that allows detection of binding between proteins and RNA or DNA oligonucleotides. To analyse the presence of the active form of the transcription factor NF-κB, extraction of islet nuclear proteins was performed with a method modified from Dignam (193). For the EMSA, a double stranded 26 mer oligonucleotide containing the kappa B binding site (5´-AGCTTCAGAGGGGACTTTCCGAGAGG-3´) was labeled with [32-P] dCTP. Nuclear protein extracts were denatured with formamide and then incubated with the radiolabelled nucleotide. A 100-fold excess of non-labeled oligonucleotide was used as negative control. The samples were then separated on a non-denaturing polyacrylamide gel, gel dried and exposed to X-ray film.

3.8.2 RNA isolation and cDNA synthesis

Total RNA from islets exposed to different conditions was isolated with an RNeasy Mini kit, where RNA molecules longer than 200 nucleotides are bound to a silica-gel-based membrane. Any contaminating DNA was digested with RNase-free DNAse, and RNA was eluted in water. To obtain a higher concentration of RNA than provided after using the RNeasy Mini kit, RNA was concentrated by sodium acetate/ethanol precipitation. Synthesis of cDNA was performed with a Reverse Transcription System using 9/10 of total RNA per 20 µl cDNA synthesis reaction.

3.8.3 Real Time PCR and calculation of relative mRNA expression values

A LightCycler Instrument combined with sequence-independent detection with SYBR Green I was used to amplify and analyse generated cDNA. For each PCR reaction, cDNA was mixed with nucleotides, polymerase and primers (one forward and one reverse) specific for the genes of interest (Table 1). During the amplification process, DNA is denatured at high temperature, and then the temperature is lowered to allow annealing of primers. After annealing, temperature is raised to 72 °C, the optimal temperature for the polymerase, which produces the PCR products. SYBR Green I is a dye that binds specifically to double-stranded DNA. During PCR, SYBR Green I binds to DNA products as they are synthesised. Thus, the increase in fluorescence, when measured at the end of each elongation cycle, indicates the amount of PCR product formed during that cycle. Cycle threshold (Ct) values were obtained for individual samples with the second derivative maximum method, which uses a software algorithm that identifies the first turning point of the fluorescence curve, i.e. the estimated amplification cycle number when the fluorescence exceeds a specified threshold value (194,195). To control the efficacy of the PCR, cDNA from cytokine treated islets was diluted (4x, 16x, 64x and 256x) and amplified. Ct values were plotted against the dilution of cDNA, and a two-fold amplification was obtained for each primer pair for each dilution. Negative controls without template or with cDNA samples run without reverse transcriptase were added to the PCR to screen for contamination or genomic amplification.

To obtain relative mRNA expression values, one PCR reaction was performed with G6PDH primers and one with specific primers of genes of interest (Table 1), for each experimental group. The Ct values were used to calculate the amount of PCR product in comparison to G6PDH as a “house-keeping gene”, by subtracting Ct values for specific primers from the Ct value for G6PDH (ΔCt). Relative mRNA expression was calculated as $2^{-\Delta Ct}$.

Table 1. Primers used

Gene	Accession number GenBank	primer sequence (5´-3´) F=forward R=reverse
G6PDH	Z11911	F ATTGACCACTACCTGGGCAA
		R GAGATACACTTCAACACTTTGACCT
iNOS	M87039	F CAGCTGGGCTGTACAAACCTT
		R CATTGGAAGTGAAGCGTTTCG
COX-2	NM011198	F GAGTCATTCACCAGACAGATTGC
		R AGTATTGAGGAGAACAGATGGGAT
PC1	NM013628	F TGAATGTTGTGGAGAAGCGG
		R GCACTTGGAGACTTCTTTGGTG
PC2	NM008792	F CTGAGGCTGGTGTGGCTAC
		R AGCTGGCGTGTTTGCATTA
PLD1a*	U87868	F GGGGCTCACTAGGCACAG
		R ATTTTGGCTTCATAATCTTCGTG
PLD1b*	AH007049	F CGACAGCACCTCCAACACT
		R The same as reverse primer for PLD1a
Insulin#	X04725	F CCATCAGCAAGCAGGTTAT
		R GGGTGTGTAGAAGAAGCCA

***** The reverse primer for PLD1a and PLD1b was designed to be the same, since PLD1a and PLD1b are two isoforms of an enzyme formed by different splice-variants of the same mRNA**.** # Primers for insulin were designed to amplify both mouse preproinsulin 1 and preproinsulin 2 mRNA similtaneously.

3.8.4 Western blot

To examine COX-2 protein expression, SDS-polyacrylamide gel electrophoresis was used to separate proteins from lysed islets by size. The separated proteins were then transferred onto Hybond-P polyvinylidene difluoride membranes, and incubated with a specific primary antibody for COX-2. After this, a secondary antibody conjugated to horseradish peroxidase (HRP) was added and blots were developed using ECL+ Western Blotting Detection System, where peroxidase in conjunction with a chemiluminiscent substrate generates a signal that can be captured on film. To correlate the amount of loaded protein from each experimental group, bound COX-2 antibodies were washed ("stripped") with SDS/β-mercaptoethanol and the membranes were incubated with actin antibodies and blotted as described above.

3.9 Statistical analysis

Values are expressed as means ± SEM. When experiments were performed in duplicates or triplicates, a mean was calculated for each experimental group, and this was considered as one separate observation. Single comparisons were performed by unpaired or paired t-test. Multiple comparisons were analysed by repeated measures ANOVA followed by Bonferroni t-test. To test for relative differences rather than absolute differences, the logarithm of relative mRNA expression values was calculated (III-IV) before statistical analysis. For *in vivo* experiments (II), blood glucose values and changes in weight were pooled from two sets of experiments performed at two different occations. Each time five to six mice were used in each experimental group. To compare the proportion of hyperglycaemic mice between each treatment group, Fisher's exact test was used.

4. Results and discussion

4.1 Melatonin and protection against β-cell impairment (I)

We found that melatonin (at 1 mM) counteracted STZ-induced (0.5 mM) inhibition of glucose oxidation and insulin release in cultured rat islets after 18 h of recovery. However, acutely after STZ-induced injury there were no significant differences between the STZ and STZ + melatonin treated groups regarding inhibition of glucose oxidation. Moreover, the NAD content was about 50 % higher in STZ + melatonin treated islets compared to STZ alone after 18 h of recovery, while the NAD content of both treatment groups were equally suppressed acutely after STZ exposure. Furthermore, diabetes induced by single, high dose injection of STZ (140 mg/kg body weight, i.v.) in C57BL/Ks mice was effectively prevented by administration of melatonin (100 mg/kg body weight, i.p.) 30 min before STZ injection.

When STZ is taken up by the β-cell, it rapidly decomposes forming free radicals and reactive methyl cations that may induce DNA alkylations and cross-link the DNA strands (135,136). Protection by addition of the hydroxyl radical scavenger dimethyl urea (146), superoxide dismutase (144,196,197) and vitamin E (198) support a role of free radicals in STZ-mediated β-cell suppression, although some investigators found DNA alkylation to be the major pathway of STZ action (136,145). Melatonin has been suggested to directly scavenge highly reactive molecules such as hydroxyl radicals ($^{\cdot}OH$) (163,199), singlet oxygen ($^1O_2$) (200), peroxynitrite ($ONOO^-$) (201,202) and NO (203). It is possible that the protection seen by melatonin against STZ-induced suppression of β-cell function may be a result of a neutralisation of STZ-derived radicals, resulting in reduced PARP activation and NAD depletion. A similar mechanism of protection has been suggested in previous studies of melatonin in relation to free radical-induced DNA damage (175,201). Moreover, it was shown that melatonin protected against alloxan-induced β-cell impairment in an *in vitro* islet perifusion model (204). Alloxan is a diabetogenic substance, which (in similarity to STZ) probably enter the cells via glucose transporters, and generates hydroxyl radicals and

other reactive substances inside the cell (205). The postulated mechanism of a direct neutralisation of free radicals by melatonin suggests that melatonin acts as an electron donor, and thereby becomes a low-reactive radical itself, the melatonyl cation radical (206). The latter molecule is believed to scavenge $O_2^{\cdot -}$ to generate a kynuramine, which *in vivo*, can be secreted in the urine.

In addition to direct scavenging of free radicals, melatonin has been shown to upregulate the activity of the antioxidative enzyme glutathione peroxidase (169-171), and increase the expression of glutathione peroxidase and manganese (Mn-SOD) and copper-zinc (Cu.Zn-SOD) superoxide dismutases (171,207). Upregulation of antioxidant enzymes may increase β-cell capacity to repair after injury, since β-cell damage was partially reduced by increased SOD activity in STZ-treated animals (144,147). We observed no increase in Mn-SOD mRNA after 6 h exposure of 1 mM melatonin to the rat pancreatic β-cell line RINm5F (AK Andersson, DL Eizirik and S Sandler, unpublished results). However, this result does not exclude an effect of melatonin on Mn-SOD activity in primary β-cells or pancreatic islets *in vivo*.

Recently it was shown that melatonin may affect rat liver and brain mitochondrial function by enhancement of the activity of the respiratory complexes I and IV (208,209). If islet mitochondria are affected in a similar way by melatonin treatment, this may facilitate β-cell ATP production and increase the ATP available for energy-dependent processes of cellular repair.

Moreover, when isolated rat islets were exposed to melatonin in an *in vitro* perifusion system, this caused a reversible inhibition of glucose-stimulated insulin release (210). A reversible melatonin-mediated reduction in insulin production during STZ-treatment in the present study could contribute to an increase in the ATP available for cellular repair mechanisms, by a reduction of energy demand due to the lowered insulin secretion. It has been proposed that an excessive supply of nutrients could increase the capability of β-cell repair after injury, since isolated rat and mouse islets were protected from STZ-induced cell death and IL-1β-induced impairment by culture in high glucose concentration (211-214).

It is likely that the mechanism of protection of melatonin operating *in vitro* against STZ-induced β-cell damage also function *in vivo*, as indicated by the well preserved metabolic control of STZ + melatonin exposed mice in the present study. Nevertheless, the reduction in pancreatic insulin content and weight of (melatonin + STZ) treated animals compared to controls indicate that there was a loss of β-cells in these mice, despite normal blood glucose levels. In line with our findings, a number of studies dealing with melatonin administration during STZ treatment of mice and rats showed decreased hyperglycemia and diabetes related complications such as lipid

peroxidation (178,179,215-217). These results, together with our observations in the present study, further support the hypothesis that melatonin may cause a reduction of STZ-induced oxidative stress and β-cell damage *in vivo*.

When we exposed cultured rat islets to melatonin and IL-1β, we observed no protection against NO formation or suppression of glucose oxidation, indicating that melatonin did not prevent IL-1β-induced NFκB activation or iNOS induction. In previous studies, melatonin inhibited LPS induced iNOS expression in macrophages and hepatocytes (173,218), and it was suggested that melatonin suppressed the activation of NFκB. Differences in activation of proteins in the NFκB family by LPS and IL-1β, affecting NFκB binding sites differently in the iNOS promoter, may explain the discrepancies of the effect of melatonin on iNOS expression.

The finding that melatonin protected against development of autoimmune diabetes in the NOD mouse (181) led to the speculation that melatonin could affect the influence of cytokines and/or affect T helper cells during the process of islet inflammation. Our observations that melatonin did not counteract IL-1β-induced damage in isolated rat islets *in vitro* suggest minor beneficial effects of melatonin against this particular cytokine. Moreover, we found no protective effect of melatonin against development of hyperglycaemia after multiple low-dose injections of STZ to C57BL/Ks mice (melatonin 4 mg/kg given 30 min prior to 40 mg/kg STZ, for 5 consecutive days, mean blood glucose (mM) at 14 days after first injection: control 7.9 ± 0.9; STZ 15.1 ± 1.0; melatonin + STZ 18.7 ± 0.8; AK Andersson and S Sandler, unpublished results).

In contrast to the beneficial effects of melatonin in the NOD mouse, melatonin caused a significant worsening of the disease in a mouse model of collagen-induced arthritis (219). Furthermore, melatonin has been reported to enhance immunologic responses in various conditions (220,221). The fact that melatonin may potentiate various immune responses indicate that receptor-related effects of melatonin have to be carefully elucidated, before therapeutic attempts with melatonin against immune-mediated β-cell destruction are undertaken.

4.2 Inducible nitric oxide synthase and regulation of β-cell sensitivity to cytokines (II-IV)

4.2.1 Prolonged (48 h) exposure to IL-1β impairs glucose-stimulated insulin secretion from iNOS -/- islets (II)

To investigate the effect of treatment with combined cytokines on iNOS -/- islet function, we exposed wt and iNOS -/- islets to IL-1β alone or IL-1β in combination with IFN-γ for 48 h. IL-1β induced NO formation from wt islets, which was further potentiated by IFN-γ. Neither IL-1β nor (IL-1β + IFN-γ) induced NO formation from iNOS -/- islets, confirming that these islets indeed were iNOS deficient.

In agreement with previous observations (62,63,91), we found that IL-1β and (IL-1β + IFN-γ) suppressed glucose-stimulated insulin release from wt islets, and that (IL-1β + IFN-γ) reduced (pro)insulin biosynthesis and glucose oxidation after 48 h (63,91). When iNOS -/- islets were exposed to IL-1β, glucose-stimulated insulin release was impaired, and in parallel, there was an increased insulin accumulation to the media a reduced insulin content of the islets. Exposure to (IL-1β + IFN-γ) did not affect glucose oxidation rates, glucose-stimulated insulin release or insulin accumulation, but lowered the insulin content of iNOS -/- islets. When we measured cell death by flow cytometry analysis of dispersed iNOS -/- islets, there was no increase in cell death in the IL-1β or (IL-1β + IFN-γ) treated groups compared to the control. Thus, the IL-1β-induced impairment of insulin secretion and reduction in insulin content of iNOS -/- islets seem to be independent of cell death. Altogether, when iNOS -/- islets are subjected to a prolonged (48 h) exposure to IL-1β, this induces exhaustion of the insulin secretory machinery, which results in a reduced response in insulin secretion to a glucose challenge. The ability of IFN-γ to counteract the IL-β-induced changes in insulin secretion may be due to effects of PLD1 or SOCS-3 (see section 4.2.3).

The effect of IL-1β in iNOS -/- islets is similar to what has been observed in other experimental conditions, where IL-1β exposure did not result in NO formation. Exposure of IL-1β alone to human islets does not induce iNOS expression. Instead, a combination of (IL-1β + IFN-γ) or (IL-1β + IFN-γ +TNF-α) is required (93). When human islets were exposed to IL-1β for 48 h, this resulted in an increase in insulin accumulation and glucose-stimulated insulin release, and a reduction of the insulin content, without any NO formation (222). A comparable effect of IL-1β was observed in rat and human islets cultured in absence of L-arginine (the only physiological substrate of NO synthesis by iNOS). In one of these studies, the insulin

accumulation from rat islets was higher in the IL-1β exposed group after 6 h compared to control islets, but after 6-24 h of IL-1β exposure, the cytokine exposed islets showed a significantly lower insulin accumulation during the 6-24 h time period (223). A similar result was obtained in another study with rat islets, where glucose-induced insulin release was impaired by IL-1β after 24 h exposure in arginine free medium, without any reduction in (pro)insulin biosynthesis or glucose oxidation rates (224). Human islets exposed to (IL-1β + IFN-γ + TNF-α) in arginine-depleted medium showed a reduction in insulin content, but no increase in insulin accumulation after 24 h of cytokine exposure (224).

The mechanism of NO-independent, IL-1β-derived changes in insulin secretion remains to be clarified. Suggested pathways include impairment of insulin secretion by oxygen radicals generated by iNOS (225), effects of IL-1β-induced ceramide (55,56) or PGE_2 (42), an early activation of PKC and a subsequent increase in insulin release (57), and changes in the activity of (pro)insulin convertases (226). Free radicals derived from iNOS activity are not likely to be involved in the present study, since the complete lack of iNOS expression has been confirmed in iNOS -/- islets (128), paper III.

It should be noted that the effect of IL-1β and (IL-1β + IFN-γ) on iNOS -/- islets does not necessarily need to be a β-cell specific phenomenon. In addition to β-cells, islets contain other endocrine and non-endocrine cells that may secrete factors that could affect β-cell function. Among the non-β-cells, macrophages, endothelial and ductual cells are all likely to be affected by cytokines (97,227,228).

4.2.2 Islets deficient in iNOS show a reduced ability to increase their PGE_2 formation upon cytokine treatment (III).

In paper III, the possible involvement of PGE_2 formation in the IL-1β-induced impairment of insulin secretion from iNOS -/- islets was investigated. IL-1β and (IL-1β + IFN-γ) exposed iNOS -/- islets responded with an increase in PGE_2 formation compared to non-cytokine exposed iNOS -/- islets, but the amount of PGE_2 formed was only about 20 % of that from cytokine exposed wt islets. Examination of the expression of the enzyme responsible for cytokine-induced PGE_2 formation in islets (COX-2), revealed that both COX-2 mRNA and protein was induced to similar levels in wt and iNOS -/- islets after 6 h of IL-1β or (IL-1β + IFN-γ) exposure. In addition, the activation of the transcription factor NF-κB, suggested to be important in iNOS and COX-2 transcriptional activation, was induced to the

same extent in islets of both genotypes. Furthermore, when iNOS -/- islets was incubated with IL-1β in the presence of the NO donor DETA NONOate, this increased the PGE_2 formation about 50%, compared to islets exposed to IL-1β alone. Taken together, the findings in paper III indicate that an absence of NO formation after cytokine exposure impairs the efficacy of the COX-2 enzyme activity. The decreased ability of PGE_2 formation from cytokine-treated iNOS -/- islets suggests that PGE_2 formation is probably not part of the IL-1β-induced suppression of insulin secretion observed in paper I. Also in other studies with islets, where iNOS was chemically inhibited, a reduction in PGE_2 formation after cytokine exposure was observed (71,72).

The presence of NO during COX-2 activity seems to directly stimulate the COX enzyme by scavengeing oxygen radicals formed (229). Peroxynitrite formed after interaction of NO with oxygen radicals may also activate COX enzymes (230). Another mechanism suggested to operate in an NO-dependent enhancement of prostaglandin H synthesis is S-nitrosation of cysteine residues in the catalytic domain, which enhances prostaglandin synthesis (231).

Interestingly, some studies suggest that inhibition of PGE_2 formation in turn could decrease iNOS-induced NO formation. For example, inhibition of COX activity by indomethacin resulted in a reduction in nitrite accumulation from rat Kupffer cells (232), as well as from rat alveolar macrophages (233). In a model of inflammation induced by zymosan injection into the mouse air pouch, treatment of animals with the selective COX-2 inhibitor NS-398 lowered iNOS activity and nitrite present in air pouch exudats (234). The relation between COX inhibition and NO formation in islets cells remains to be clarified. The use of specific COX-2 inhibitors such as NS-398 has been shown to act protectively against cytokine-induced suppression *in vitro* (74), and against STZ-induced diabetes *in vivo* (76). PGE_2 formation may be involved in recruitment of immune cells during an ongoing inflammation (so-called homing), and it is possible that an inhibition of PGE_2 formation *in vivo* could decrease an inflammatory infiltrate during the process of insulitis. When iNOS -/- mice were subjected to diabetes-induction by MLDSTZ, hyperglycaemia was partially counteracted and there was less inflammation around iNOS -/- islets compared to islets from MLDSTZ treated control mice (128). It is possible that the reduced ability to produce PGE_2 upon cytokine stimulation *in vitro* also operates *in vivo*, and may participate in the observed protection of iNOS -/- mice against diabetes development. Conversely, PGE_2 has been suggested to act as an inhibitor for $CD4^+$ and $CD8^+$ T-cell effector functions which thereby counteracts autoimmunity (235).

Recently it was suggested that COX-2 is involved in cytokine-mediated generation of free radicals from β-cells *in vivo* (236), indicating that other

COX-2 activities than PGE_2 formation may harm the β-cells. It is currently not known if the presence or absence of NO can influence the COX-2 mediated generation of free radicals in a similar way as seem to be the case with PGE_2 formation. The possible effects of COX-2 activity and PGE_2 production during diabetes development remain unclear, implicating a complex role for this pathway during inflammation.

4.2.3 Altered kinetics of early cytokine-stimulated insulin secretion from iNOS -/- islets – role of PLD1, proinsulin convertase and insulin mRNA expression (IV)

In paper I, we concluded that when iNOS -/- islets are subjected to a prolonged (48 h) exposure to IL-1β, this induces exhaustion of the insulin secretory machinery, which results in a reduced response in insulin secretion to a glucose challenge. To examine if the effects observed after a long term exposure to IL-1β could be a result of early events after IL-1β treatment, the insulin secretion from iNOS -/- islets after 2-20 h of cytokine exposure was evaluated in paper IV. In this study, we found that exposure of iNOS -/- islets to IL-1β at high glucose induces a delayed and prolonged early stimulatory phase (4-6 h) of insulin secretion compared to wt islets. Furthermore, the stimulatory effect of IL-β exposure was counteracted by the addition of IFN-γ.

Previous studies with mouse and rat islets have suggested that PLD1 induction, DAG formation and PKC activation may have a role in the early stimulatory phase of insulin secretion after cytokine exposure (57,58). In order to investigate if the PLD/DAG/PKC pathway was involved in the observed effects on insulin secretion from iNOS -/- islets after cytokine exposure, we examined PLD1 mRNA expression. There was, indeed, a more pronounced induction of PLD1 expression from iNOS -/- islets compared to the corresponding cytokine treated wt islets, especially regarding PLD1a expression. It is possible that the increase in PLD1 expression observed from iNOS -/- islets may reflect an increase in PKC activity, which in turn could mediate an increase in insulin secretion from these islets. The fact that the IL-1β-induced PLD1b expression from iNOS -/- islets was counteracted by the addition of IFN-γ may partially explain the observed effects of IFN-γ, suppressing the IL-1β-induced increase in insulin secretion. However, other mechanisms could operate as well.

Recently, a new family of intracellular proteins controlling the magnitude and/or duration of cytokine signaling (suppressors of cytokine signaling, SOCS) was described (reviewed in (237)). The expression of SOCS proteins represent an intracellular negative feedback loop in cytokine signaling, as

they inhibit the cytokine signalling from cytokines that induce their own expression. IFN-γ signaling has been shown to be inhibited by SOCS-1 and SOCS-3, via interference with JAK signaling and subsequent STAT dimerisation, necessary for transcriptional activation of target genes. In a study by Karlsen and coworkers (238), SOCS-3 overexpression was shown to counteract cytokine-induced apoptosis, and to reduce IL-1β-induced iNOS promoter activity and nitrite formation. The time course of SOCS-3 induction in normal rat islets appears to be different after IL-1β or IFN-γ exposure, with IFN-γ inducing a small increase in SOCS-3 mRNA after 1 h of exposure and IL-β inducing SOCS-3 expression only after a longer time period (24 h) (238). If IFN-γ treatment of iNOS-/- islets induces an early increase in SOCS-3 expression, SOCS-3 might contribute to the inhibitory action of IFN-γ against IL-β induced signals in these islets.

When rat and human islets were exposed to IL-1β or IL-1β in combination with IFN-γ, cytokine treatment was shown to reduce the expression of proinsulin convertases, with an increase in proinsulin over insulin secretion as a result (226,239). In rat islets, the inhibition of PC expression was shown to be NO-dependent, which was not the case in human islets. In iNOS -/- islets, we found that suppression of PC1 mRNA expression was partly NO-dependent, while IL-1β-induced impairment of PC2 expression was not. A reduced expression of PC enzymes may increase the proinsulin over insulin secretion from iNOS -/- islets. However, it was not possible to discriminate between insulin and proinsulin in the present investigation, due to limitations of the ELISA used for insulin determination (see methods). Elevated proinsulin levels has been associated with the prediabetic stage of type 1 diabetes in humans (240), and proinsulin is also elevated in first-degree relatives of type 1 diabetic patients positive for islet cell antibodies (241). The present *in vitro* data, together with results from other studies, indicate that cytokines participating in a process of insulitis may affect proinsulin conversion. It remains to be clarified if there is a relation between the *in vitro* findings of a disturbed insulin processing and the elevated proinsulin levels found in serum. Nevertheless, it is an interesting possibility that changes in serum proinsulin levels might serve as an early, peripheral marker of β-cells facing active immune cells.

Cytokine-induced suppression of insulin mRNA expression appears to be NO-independent, as cytokine treatment of wt and iNOS -/- islets suppressed insulin mRNA expression to a similar extent after 24 h. Moreover, the reduction in insulin mRNA levels did not seem to affect iNOS -/- islets ability to release insulin at this time-point. On the other hand, long term inhibition of insulin mRNA expression could affect the capacity to produce insulin, as indicated by the (pro)insulin synthesis data from iNOS -/- islets in paper I. In a study of NF-κB-dependent gene expression from cytokine

exposed rat islets, the mRNA expression of IP-10, IL-15, MCP-1, c-myc, MnSOD and iNOS was shown to be NF-κB-dependent, but not blocked by iNOS inhibition (242). Of these genes, the transcription factor c-myc has been suggested to be involved in suppression of insulin transcription and inhibition of insulin secretion (243,244), making c-myc an interesting target for further studies of mechanisms of cytokine-induced, NO-independent impairment of β-cell function.

4.2.4 Concluding remarks about NO-dependent versus NO-independent effects of IL-1β and IFN-γ exposure to mouse pancreatic islets

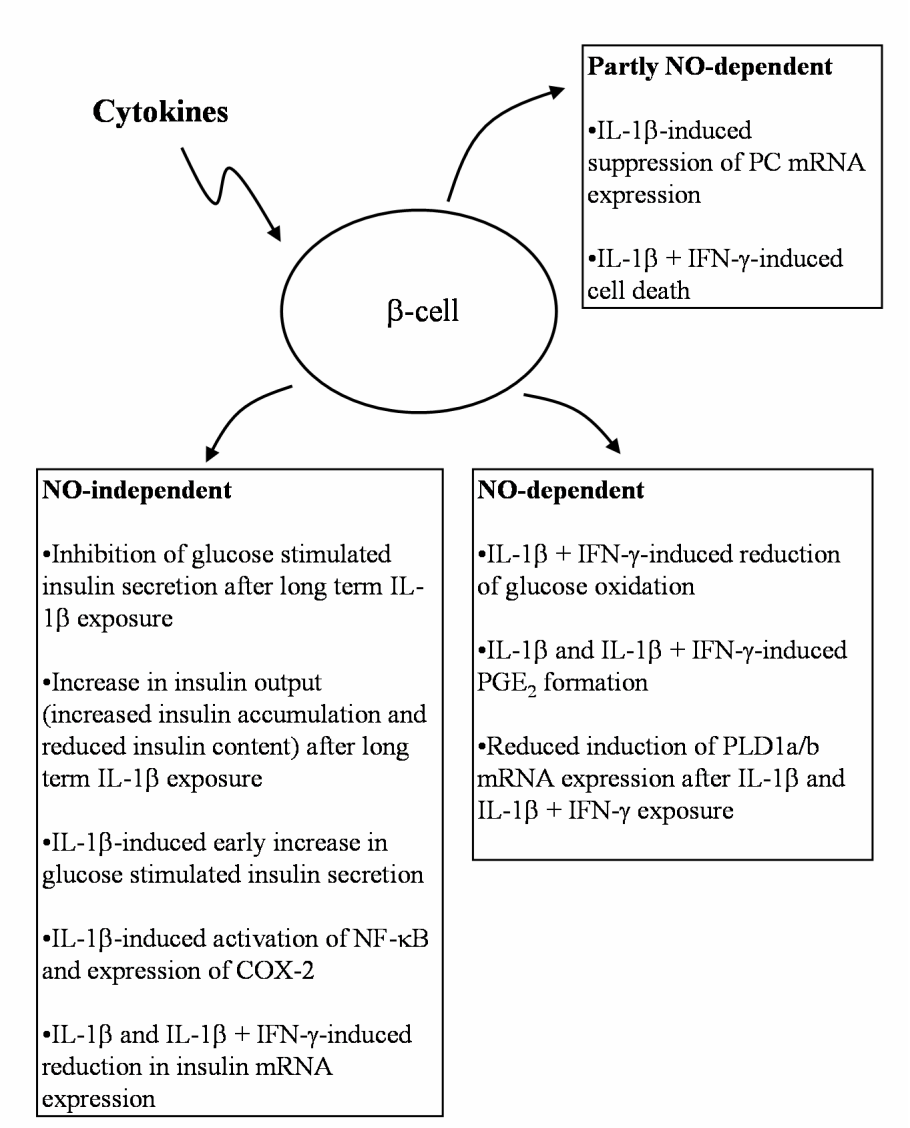

Figure 4.Outline of NO-dependent versus NO-independent effects of cytokine exposure to mouse β-cells.

The findings in papers II, III and IV, together with results from other studies, clearly show that cytokines damage islets by non-NO dependent mechanisms. The main function of the β-cell, glucose stimulated insulin secretion, appears to be disturbed at several different steps by cytokines, in the absence of NO formation. Initially, in particular IL-1β exposure seems to be stimulatory for the insulin secretion machinery, while after a longer time-period of exposure, IL-1β act inhibitory. Also the formation of new insulin molecules appears to be affected, since IL-1β and IL-β + IFN-γ suppress mRNA expression of both insulin convertase enzymes and insulin itself. Several of the observed effects of cytokine exposure to iNOS -/- islets resembles the effects obtained after cytokine treatment of human islets, making the iNOS -/- mouse islets an interesting model system to use to further characterise the mechanisms of cytokine-induced islet damage in type 1 diabetes.

5. Conclusions

- Melatonin may prevent against STZ-induced β-cell damage *in vitro*. *In vivo*, melatonin counteracts hyperglycaemia, induced in mice by a single high dose injection of STZ. The protective actions of melatonin in the present study may be related to interference with DNA damage and PARP activation, rather than through effects on NO generation pathways, since IL-1β-induced impairment of isolated islets could not be counteracted by melatonin treatment.

- Prolonged exposure of IL-1β or (IL-1β + IFN-γ) to iNOS -/- islets induces changes in the insulin secretory machinery, with an IL-1β-induced exhaustion which results in a reduced response in insulin secretion to a glucose challenge. This is independent of NO formation pathways and cell death.

- Exposure of iNOS -/- islets to IL-1β at a high glucose concentration induces a delayed and prolonged stimulatory phase of insulin secretion compared to wt islets. PKC activation, via increased phospholipase D expression and effects on proinsulin to insulin conversion, but probably not changes in insulin mRNA expression, may be involved in early NO-independent regulation of cytokine-stimulated insulin secretion from β-cells.

- The prolonged stimulatory phase of insulin secretion from iNOS -/- islets early after IL-1β exposure could contribute to the inhibitory effect on glucose-stimulated insulin secretion from these islets after long term exposure to IL-1β.

- iNOS -/- islets have a reduced ability to respond with PGE_2 formation after cytokine exposure. This is due to posttranslational inhibition of the COX-2 enzyme activity in the absence of NO formation, rather than on expression of COX-2.

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7. References

1. Gorsuch, AN, Spencer, KM, Lister, J, McNally, JM, Dean, BM, Bottazzo, GF, Cudworth, AG: Evidence for a long prediabetic period in type I (insulin-dependent) diabetes mellitus. *Lancet* 2:1363-1365, 1981

2. Eisenbarth, GS, Connelly, J, Soeldner, JS: The "natural" history of type I diabetes. *Diabetes Metab. Rev.* 3:873-891, 1987

3. Onkamo, P, Väänänen, S, Karvonen, M, Tuomilehto, J: Worldwide increase in incidence of Type I diabetes--the analysis of the data on published incidence trends. *Diabetologia* 42:1395-1403, 1999

4. Andersen, HU, Larsen, PM, Fey, SJ, Karlsen, AE, Mandrup-Poulsen, T, Nerup, J: Two-dimensional gel electrophoresis of rat islet proteins. Interleukin 1 β-induced changes in protein expression are reduced by L-arginine depletion and nicotinamide. *Diabetes* 44:400-407, 1995

5. Nepom, GT: HLA and type I diabetes. *Immunol.Today* 11:314-315, 1990

6. Pociot, F, Rønningen, KS, Bergholdt, R, Lorenzen, T, Johannesen, J, Ye, K, Dinarello, CA, Nerup, J: Genetic susceptibility markers in Danish patients with type 1 (insulin- dependent) diabetes--evidence for polygenicity in man. Danish Study Group of Diabetes in Childhood. *Autoimmunity* 19:169-178, 1994

7. Hyöty, H, Taylor, KW: The role of viruses in human diabetes. *Diabetologia* 45:1353-1361, 2002

8. Dahlquist, G: Non-genetic risk determinants of type 1 diabetes. *Diabete Metab.* 20:251-257, 1994

9. Bottazzo, GF, Florin-Christensen, A, Doniach, D: Islet-cell antibodies in diabetes mellitus with autoimmune polyendocrine deficiencies. *Lancet* ii:1279-1283, 1974

10. Palmer, JP, Asplin, CM, Clemons, P, Lyen, K, Tatpati, O, Raghu, PK, Paquette, TL: Insulin antibodies in insulin-dependent diabetics before insulin treatment. *Science* 222:1337-1339, 1983

11. Vahasalo, P, Knip, M, Karjalainen, J, Tuomilehto-Wolf, E, Lounamaa, R, Åkerblom, HK: Islet cell-specific autoantibodies in children with insulin-dependent diabetes mellitus and their siblings at clinical manifestation of the disease. Childhood Diabetes in Finland Study Group. *Eur.J.Endocrinol.* 135:689-695, 1996

12. Sheehy, MJ: HLA and insulin-dependent diabetes. A protective perspective. *Diabetes* 41:123-129, 1992

13. Sterkers, G, Zeliszewski, D, Chaussee, AM, Deschamps, I, Font, MP, Freidel, C, Hors, J, Betuel, H, Dausset, J, Levy, JP: HLA-DQ rather than HLA-DR region might be involved in dominant nonsusceptibility to diabetes. *Proc.Natl.Acad.Sci.U.S.A.* 85:6473-6477, 1988

14. Baisch, JM, Weeks, T, Giles, R, Hoover, M, Stastny, P, Capra, JD: Analysis of HLA-DQ genotypes and susceptibility in insulin-dependent diabetes mellitus. *N.Engl.J.Med.* 322:1836-1841, 1990

15. Gepts, W: Pathologic anatomy of the pancreas in juvenile diabetes mellitus. *Diabetes* 14:619-633, 1965

16. Foulis, AK, Liddle, CN, Farquharson, MA, Richmond, JA, Weir, RS: The histopathology of the pancreas in type 1 (insulin-dependent) diabetes mellitus: a 25-year review of deaths in patients under 20 years of age in the United Kingdom. *Diabetologia* 29:267-274, 1986

17. Gepts, W, In't Veld, PA: Islet morphologic changes. *Diabetes Metab. Rev.* 3:859-872, 1987

18. Yoon, JW, Jun, HS, Santamaria, P: Cellular and molecular mechanisms for the initiation and progression of β cell destruction resulting from the collaboration between macrophages and T cells. *Autoimmunity* 27:109-122, 1998

19. Bottazzo, GF, Dean, BM, McNally, JM, MacKay, EH, Swift, PG, Gamble, DR: In situ characterization of autoimmune phenomena and expression of HLA molecules in the pancreas in diabetic insulitis. *N.Engl.J.Med.* 313:353-360, 1985

20. Hänninen, A, Jalkanen, S, Salmi, M, Toikkanen, S, Nikolakaros, G, Simell, O: Macrophages, T cell receptor usage, and endothelial cell

activation in the pancreas at the onset of insulin-dependent diabetes mellitus. *J.Clin.Invest.* 90:1901-1910, 1992

21. Miyazaki, A, Hanafusa, T, Yamada, K, Miyagawa, J, Fujino-Kurihara, H, Nakajima, H, Nonaka, K, Tarui, S: Predominance of T lymphocytes in pancreatic islets and spleen of pre-diabetic non-obese diabetic (NOD) mice: a longitudinal study. *Clin.Exp.Immunol.* 60:622-630, 1985

22. Koike, T, Itoh, Y, Ishii, T, Ito, I, Takabayashi, K, Maruyama, N, Tomioka, H, Yoshida, S: Preventive effect of monoclonal anti-L3T4 antibody on development of diabetes in NOD mice. *Diabetes* 36:539-541, 1987

23. Lee, KU, Amano, K, Yoon, JW: Evidence for initial involvement of macrophage in development of insulitis in NOD mice. *Diabetes* 37:989-991, 1988

24. Shizuru, JA, Taylor-Edwards, C, Banks, BA, Gregory, AK, Fathman, CG: Immunotherapy of the nonobese diabetic mouse: treatment with an antibody to T-helper lymphocytes. *Science* 240:659-662, 1988

25. Haskins, K, McDuffie, M: Acceleration of diabetes in young NOD mice with a CD4+ islet-specific T cell clone. *Science* 249:1433-1436, 1990

26. Healey, D, Ozegbe, P, Arden, S, Chandler, P, Hutton, J, Cooke, A: In vivo activity and in vitro specificity of CD4+ Th1 and Th2 cells derived from the spleens of diabetic NOD mice. *J.Clin.Invest.* 95:2979-2985, 1995

27. Wong, FS, Visintin, I, Wen, L, Flavell, RA, Janeway, CA, Jr.: CD8 T cell clones from young nonobese diabetic (NOD) islets can transfer rapid onset of diabetes in NOD mice in the absence of CD4 cells. *J.Exp.Med.* 183:67-76, 1996

28. Sandler, S, Eizirik, DL, Svensson, C, Strandell, E, Welsh, M, Welsh, N: Biochemical and molecular actions of interleukin-1 on pancreatic β- cells. *Autoimmunity* 10:241-253, 1991

29. Mandrup-Poulsen, T: The role of interleukin-1 in the pathogenesis of IDDM. *Diabetologia* 39:1005-1029, 1996

30. Rabinovitch, A, Suarez-Pinzon, WL: Cytokines and their roles in pancreatic islet β-cell destruction and insulin-dependent diabetes mellitus. *Biochem. Pharmacol.* 55:1139-1149, 1998

31. Heller, B, Burkle, A, Radons, J, Fengler, E, Jalowy, A, Muller, M, Burkart, V, Kolb, H: Analysis of oxygen radical toxicity in pancreatic islets at the single cell level. *Biol. Chem. Hoppe Seyler* 375:597-602, 1994

32. Chen, MC, Schuit, F, Pipeleers, DG, Eizirik, DL: IL-1β induces serine protease inhibitor 3 (SPI-3) gene expression in rat pancreatic β-cells. Detection by differential display of messenger RNA. *Cytokine* 11:856-862, 1999

33. Grankvist, K, Marklund, S, Sehlin, J, Täljedal, IB: Superoxide dismutase, catalase and scavengers of hydroxyl radical protect against the toxic action of alloxan on pancreatic islet cells in vitro. *Biochem. J.* 182:17-25, 1979

34. Malaisse, WJ, Malaisse-Lagae, F, Sener, A, Pipeleers, DG: Determinants of the selective toxicity of alloxan to the pancreatic B cell. *Proc.Natl.Acad.Sci.U.S.A* 79:927-930, 1982

35. Tiedge, M, Lortz, S, Drinkgern, J, Lenzen, S: Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. *Diabetes* 46:1733-1742, 1997

36. Amrani, A, Verdaguer, J, Thiessen, S, Bou, S, Santamaria, P: IL-1α, IL-1β, and IFN-γ mark beta cells for Fas-dependent destruction by diabetogenic CD4(+) T lymphocytes. *J Clin.Invest.* 105:459-468, 2000

37. Campbell, IL, Oxbrow, L, Harrison, LC: Interferon-γ pleiotropic effects on a rat pancreatic β cell line. *Mol.Cell. Endocrinol.* 52:161-167, 1987

38. Sjöholm, Å: Cytokines inhibit proliferation and insulin secretion by clonal rat insulinoma cells (RINm5F) non-synergistically and in a pertussis toxin- insensitive manner. *Immunol.Lett.* 30:81-86, 1991

39. Southern, C, Schulster, D, Green, IC: Inhibition of insulin secretion by interleukin-1 β and tumour necrosis factor-α via an L-arginine-dependent nitric oxide generating mechanism. *FEBS Lett.* 276:42-44, 1990

40. Welsh, N, Eizirik, DL, Bendtzen, K, Sandler, S: Interleukin-1 β-induced nitric oxide production in isolated rat pancreatic islets requires gene transcription and may lead to inhibition of the Krebs cycle enzyme aconitase. *Endocrinology* 129:3167-3173, 1991

41. Eizirik, DL, Cagliero, E, Björklund, A, Welsh, N: Interleukin-1 β induces the expression of an isoform of nitric oxide synthase in insulin-producing cells, which is similar to that observed in activated macrophages. *FEBS Lett.* 308:249-252, 1992

42. Hughes, JH, Easom, RA, Wolf, BA, Turk, J, McDaniel, ML: Interleukin 1-induced prostaglandin E_2 accumulation by isolated pancreatic islets. *Diabetes* 38:1251-1257, 1989

43. Yamada, K, Takane-Gyotoku, N, Yuan, X, Ichikawa, F, Inada, C, Nonaka, K: Mouse islet cell lysis mediated by interleukin-1-induced Fas. *Diabetologia* 39:1306-1312, 1996

44. Loweth, AC, Williams, GT, James, RF, Scarpello, JH, Morgan, NG: Human islets of Langerhans express Fas ligand and undergo apoptosis in response to interleukin-1β and Fas ligation. *Diabetes* 47:727-732, 1998

45. Nerup, J, Mandrup-Poulsen, T, Mølvig, J, Helqvist, S, Wogensen, L, Egeberg, J: Mechanisms of pancreatic β-cell destruction in type I diabetes. *Diabetes Care* 11 Suppl 1:16-23, 1988

46. O'Neill, LA, Greene, C: Signal transduction pathways activated by the IL-1 receptor family: ancient signaling machinery in mammals, insects, and plants. *J. Leukoc. Biol.* 63:650-657, 1998

47. Bendtzen, K, Mandrup-Poulsen, T, Nerup, J, Nielsen, JH, Dinarello, CA, Svenson, M: Cytotoxicity of human pI 7 interleukin-1 for pancreatic islets of Langerhans. *Science* 232:1545-1547, 1986

48. Mandrup-Poulsen, T, Bendtzen, K, Nerup, J, Dinarello, CA, Svenson, M, Nielsen, JH: Affinity-purified human interleukin I is cytotoxic to isolated islets of Langerhans. *Diabetologia* 29:63-67, 1986

49. Scarim, AL, Arnush, M, Hill, JR, Marshall, CA, Baldwin, A, McDaniel, ML, Corbett, JA: Evidence for the presence of type I IL-1 receptors on β-cells of islets of Langerhans. *Biochim.Biophys.Acta* 1361:313-320, 1997

50. Eizirik, DL, Tracey, DE, Bendtzen, K, Sandler, S: Role of receptor binding and gene transcription for both the stimulatory and inhibitory effects of interleukin-1 in pancreatic β- cells. *Autoimmunity* 12:127-133, 1992

51. Eizirik, DL, Flodström, M, Karlsen, AE, Welsh, N: The harmony of the spheres: inducible nitric oxide synthase and related genes in pancreatic β cells. *Diabetologia* 39:875-890, 1996

52. Corbett, JA, Sweetland, MA, Lancaster, JR, Jr., McDaniel, ML: A 1-hour pulse with IL-1 β induces formation of nitric oxide and inhibits insulin secretion by rat islets of Langerhans: evidence for a tyrosine kinase signaling mechanism. *FASEB J* 7:369-374, 1993

53. Welsh, N: A role for tyrosine kinase activation in interleukin-1 β induced nitric oxide production in the insulin producing cell line RINm-5F. *Biosci.Rep.* 14:43-50, 1994

54. Kwon, G, Corbett, JA, Hauser, S, Hill, JR, Turk, J, McDaniel, ML: Evidence for involvement of the proteasome complex (26S) and NFκB in IL-1β-induced nitric oxide and prostaglandin production by rat islets and RINm5F cells. *Diabetes* 47:583-591, 1998

55. Sjöholm, Å: Ceramide inhibits pancreatic β-cell insulin production and mitogenesis and mimics the actions of interleukin-1 beta. *FEBS Lett.* 367:283-286, 1995

56. Welsh, N: Interleukin-1 β-induced ceramide and diacylglycerol generation may lead to activation of the c-Jun NH2-terminal kinase and the transcription factor ATF2 in the insulin-producing cell line RINm5F. *J. Biol. Chem.* 271:8307-8312, 1996

57. Eizirik, DL, Sandler, S, Welsh, N, Juntti-Berggren, L, Berggren, PO: Interleukin-1 β-induced stimulation of insulin release in mouse pancreatic islets is related to diacylglycerol production and protein kinase C activation. *Mol.Cell. Endocrinol.* 111:159-165, 1995

58. Chen, MC, Paez-Espinosa, V, Welsh, N, Eizirik, DL: Interleukin-1β regulates phospholipase D-1 expression in rat pancreatic β-cells. *Endocrinology* 141:2822-2828, 2000

59. Sandler, S, Andersson, A, Hellerström, C: Inhibitory effects of interleukin 1 on insulin secretion, insulin biosynthesis, and oxidative metabolism of isolated rat pancreatic islets. *Endocrinology* 121:1424-1431, 1987

60. Delaney, CA, Pavlovic, D, Hoorens, A, Pipeleers, DG, Eizirik, DL: Cytokines induce deoxyribonucleic acid strand breaks and apoptosis in human pancreatic islet cells. *Endocrinology* 138:2610-2614, 1997

61. Bolaffi, JL, Rodd, GG, Wang, J, Grodsky, GM: Interrelationship of changes in islet nicotine adeninedinucleotide, insulin secretion, and cell viability induced by interleukin-1 β. *Endocrinology* 134:537-542, 1994

62. Eizirik, DL, Welsh, M, Strandell, E, Welsh, N, Sandler, S: Interleukin-1 β depletes insulin messenger ribonucleic acid and increases the heat shock protein hsp70 in mouse pancreatic islets without impairing the glucose metabolism. *Endocrinology* 127:2290-2297, 1990

63. Welsh, N, Sandler, S: Interleukin-1 β induces nitric oxide production and inhibits the activity of aconitase without decreasing glucose oxidation rates in isolated mouse pancreatic islets. *Biochem. Biophys.Res.Commun.* 182:333-340, 1992

64. Kawahara, DJ, Kenney, JS: Species differences in human and rat islet sensitivity to human cytokines. Monoclonal anti-interleukin-1 (IL-1) influences on direct and indirect IL-1-mediated islet effects. *Cytokine* 3:117-124, 1991

65. Soldevila, G, Buscema, M, Doshi, M, James, RF, Bottazzo, GF, Pujol-Borrell, R: Cytotoxic effect of IFN-γ plus TNF-α on human islet cells. *J. Autoimmun.* 4:291-306, 1991

66. Bergmann, L, Kröncke, KD, Suschek, C, Kolb, H, Kolb-Bachofern, V: Cytotoxic action of IL-1 β against pancreatic islets is mediated via nitric oxide formation and is inhibited by N^G-monomethyl-L-arginine. *FEBS Lett.* 299:103-106, 1992

67. Corbett, JA, Mikhael, A, Shimizu, J, Frederick, K, Misko, TP, McDaniel, ML, Kanagawa, O, Unanue, ER: Nitric oxide production in islets from nonobese diabetic mice: aminoguanidine-sensitive and -resistant stages in the immunological diabetic process. *Proc.Natl.Acad.Sci.U.S.A.* 90:8992-8995, 1993

68. Holstad, M, Sandler, S: Aminoguanidine, an inhibitor of nitric oxide formation, fails to protect against insulitis and hyperglycemia induced by multiple low dose streptozotocin injections in mice *Autoimmunity* 15:311-314, 1993

69. Sternesjö, J, Welsh, N, Sandler, S: S-methyl-L-thiocitrulline counteracts interleukin 1 β induced suppression of pancreatic islet function in vitro, but does not protect against multiple low-dose streptozotocin-induced diabetes in vivo. *Cytokine* 9:352-359, 1997

70. Sjöholm, Å: Prostaglandins inhibit pancreatic β-cell replication and long-term insulin secretion by pertussis toxin-insensitive mechanisms but do not mediate the actions of interleukin-1 β. *Biochim.Biophys.Acta* 1313:106-110, 1996

71. Corbett, JA, Kwon, G, Marino, MH, Rodi, CP, Sullivan, PM, Turk, J, McDaniel, ML: Tyrosine kinase inhibitors prevent cytokine-induced expression of iNOS and COX-2 by human islets. *Am.J.Physiol.* 270:C1581-C1587, 1996

72. Corbett, JA, Kwon, G, Turk, J, McDaniel, ML: IL-1 β induces the coexpression of both nitric oxide synthase and cyclooxygenase by islets of Langerhans: activation of cyclooxygenase by nitric oxide. *Biochemistry* 32:13767-13770, 1993

73. Marnett, LJ, Wright, TL, Crews, BC, Tannenbaum, SR, Morrow, JD: Regulation of prostaglandin biosynthesis by nitric oxide is revealed by targeted deletion of inducible nitric-oxide synthase. *J.Biol.Chem.* 275:13427-13430, 2000

74. Tran, PO, Gleason, CE, Poitout, V, Robertson, RP: Prostaglandin E_2 mediates inhibition of insulin secretion by interleukin-1β. *J.Biol.Chem.* 274:31245-31248, 1999

75. Tran, PO, Gleason, CE, Robertson, RP: Inhibition of interleukin-1β-induced COX-2 and EP3 gene expression by sodium salicylate enhances pancreatic islet β-cell function. *Diabetes* 51:1772-1778, 2002

76. Tabatabaie, T, Waldon, AM, Jacob, JM, Floyd, RA, Kotake, Y: COX-2 inhibition prevents insulin-dependent diabetes in low-dose streptozotocin-treated mice. *Biochem.Biophys.Res.Commun.* 273:699-704, 2000

77. Major, CD, Gao, ZY, Wolf, BA: Activation of the sphingomyelinase/ceramide signal transduction pathway in insulin-secreting β-cells: role in cytokine-induced β-cell death. *Diabetes* 48:1372-1380, 1999

78. Helqvist, S, Polla, BS, Johannesen, J, Nerup, J: Heat shock protein induction in rat pancreatic islets by recombinant human interleukin 1 beta. *Diabetologia* 34:150-156, 1991

79. Borg, LA, Cagliero, E, Sandler, S, Welsh, N, Eizirik, DL: Interleukin-1 β increases the activity of superoxide dismutase in rat pancreatic islets. *Endocrinology* 130:2851-2857, 1992

80. Boehm, U, Klamp, T, Groot, M, Howard, JC: Cellular responses to interferon-γ. *Annu.Rev.Immunol.* 15:749-795, 1997

81. Darnell, JE, Jr., Kerr, IM, Stark, GR: Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* 264:1415-1421, 1994

82. Kamijo, R, Harada, H, Matsuyama, T, Bosland, M, Gerecitano, J, Shapiro, D, Le, J, Koh, SI, Kimura, T, Green, SJ: Requirement for transcription factor IRF-1 in NO synthase induction in macrophages. *Science* 263:1612-1615, 1994

83. Blanco, JC, Contursi, C, Salkowski, CA, DeWitt, DL, Ozato, K, Vogel, SN: Interferon regulatory factor (IRF)-1 and IRF-2 regulate interferon gamma-dependent cyclooxygenase 2 expression. *J. Exp.Med.* 191:2131-2144, 2000

84. Pavlovic, D, van de Winkel, M, van der Auwera, B, Chen, MC, Schuit, F, Bouwens, L, Pipeleers, D: Effect of interferon-gamma and glucose on major histocompatibility complex class I and class II expression by pancreatic. *J Clin.Endocrinol.Metab.* 82:2329-2336, 1997

85. Flodström, M, Eizirik, DL: Interferon-γ-induced interferon regulatory factor-1 (IRF-1) expression in rodent and human islet cells precedes nitric oxide production. *Endocrinology* 138:2747-2753, 1997

86. Shimizu, F, Shimizu, M, Kamiyama, K: Inhibitory effect of interferon on the production of insulin. *Endocrinology* 117:2081-2084, 1985

87. Baldeon, ME, Chun, T, Gaskins, HR: Interferon-α and interferon-γ differentially affect pancreatic β-cell phenotype and function. *Am.J. Physiol.* 275:C25-C32, 1998

88. Janjic, D, Asfari, M: Effects of cytokines on rat insulinoma INS-1 cells. *J. Endocrinol.* 132:67-76, 1992

89. Mandrup-Poulsen, T, Bendtzen, K, Dinarello, CA, Nerup, J: Human tumor necrosis factor potentiates human interleukin 1-mediated rat pancreatic β-cell cytotoxicity. *J. Immunol.* 139:4077-4082, 1987

90. Campbell, IL, Iscaro, A, Harrison, LC: IFN-γ and tumor necrosis factor-α. Cytotoxicity to murine islets of Langerhans. *J. Immunol.* 141:2325-2329, 1988

91. Cetkovic-Cvrlje, M, Eizirik, DL: TNF-α and IFN-γ potentiate the deleterious effects of IL-1 β on mouse pancreatic islets mainly via generation of nitric oxide. *Cytokine* 6:399-406, 1994

92. Rabinovitch, A, Sumoski, W, Rajotte, RV, Warnock, GL: Cytotoxic effects of cytokines on human pancreatic islet cells in monolayer culture. *J. Clin.Endocrinol.Metab.* 71:152-156, 1990

93. Eizirik, DL, Sandler, S, Welsh, N, Cetkovic-Cvrlje, M, Nieman, A, Geller, DA, Pipeleers, DG, Bendtzen, K, Hellerström, C: Cytokines suppress human islet function irrespective of their effects on nitric oxide generation. *J.Clin.Invest.* 93:1968-1974, 1994

94. Palmer, JP, Helqvist, S, Spinas, GA, Mølvig, J, Mandrup-Poulsen, T, Andersen, HU, Nerup, J: Interaction of β-cell activity and IL-1 concentration and exposure time in isolated rat islets of Langerhans. *Diabetes* 38:1211-1216, 1989

95. London, NJ, Swift, SM, Clayton, HA: Isolation, culture and functional evaluation of islets of Langerhans. *Diabetes Metab.* 24:200-207, 1998

96. Heitmeier, MR, Scarim, AL, Corbett, JA: Prolonged STAT1 activation is associated with interferon-γ priming for interleukin-1-induced inducible nitric-oxide synthase expression by islets of Langerhans. *J. Biol. Chem.* 274:29266-29273, 1999

97. Pavlovic, D, Chen, MC, Bouwens, L, Eizirik, DL, Pipeleers, D: Contribution of ductal cells to cytokine responses by human pancreatic islets. *Diabetes* 48:29-33, 1999

98. Griffith, OW, Stuehr, DJ: Nitric oxide synthases: properties and catalytic mechanism. *Annu.Rev.Physiol.* 57:707-736, 1995

99. Moncada, S, Palmer, RM, Higgs, EA: Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol.Rev.* 43:109-142, 1991

100. Garthwaite, J: Glutamate, nitric oxide and cell-cell signalling in the nervous system. *Trends Neurosci.* 14:60-67, 1991

101. Xie, Q, Nathan, C: The high-output nitric oxide pathway: role and regulation. *J. Leukoc.Biol.* 56:576-582, 1994

102. Nathan, C: Natural resistance and nitric oxide. *Cell* 82:873-876, 1995

103. Kolb, H, Kolb-Bachofen, V: Nitric oxide: a pathogenetic factor in autoimmunity. *Immunol.Today* 13:157-160, 1992

104. Kleemann, R, Rothe, H, Kolb-Bachofen, V, Xie, QW, Nathan, C, Martin, S, Kolb, H: Transcription and translation of inducible nitric oxide synthase in the pancreas of prediabetic BB rats. *FEBS Lett.* 328:9-12, 1993

105. Rothe, H, Faust, A, Schade, U, Kleemann, R, Bosse, G, Hibino, T, Martin, S, Kolb, H: Cyclophosphamide treatment of female non-obese diabetic mice causes enhanced expression of inducible nitric oxide synthase and interferon-γ, but not of interleukin-4. *Diabetologia* 37:1154-1158, 1994

106. Rabinovitch, A, Suarez-Pinzon, WL, Sorensen, O, Bleackley, RC: Inducible nitric oxide synthase (iNOS) in pancreatic islets of nonobese diabetic mice: identification of. *Endocrinology* 137:2093-2099, 1996

107. Darville, MI, Eizirik, DL: Regulation by cytokines of the inducible nitric oxide synthase promoter in insulin-producing cells. *Diabetologia* 41:1101-1108, 1998

108. Pryor, WA, Squadrito, GL: The chemistry of peroxynitrite: a product from the reaction of nitric oxide with superoxide. *Am.J. Physiol.* 268:L699-L722, 1995

109. Drapier, JC, Hibbs, JB, Jr.: Murine cytotoxic activated macrophages inhibit aconitase in tumor cells. Inhibition involves the iron-sulfur prosthetic group and is reversible. *J. Clin.Invest.* 78:790-797, 1986

110. Brown, GC: Nitric oxide regulates mitochondrial respiration and cell functions by inhibiting cytochrome oxidase. *FEBS Lett.* 369:136-139, 1995

111. Tsai, AL, Wei, C, Kulmacz, RJ: Interaction between nitric oxide and prostaglandin H synthase. *Arch.Biochem. Biophys.* 313:367-372, 1994

112. Curtis, JF, Reddy, NG, Mason, RP, Kalyanaraman, B, Eling, TE: Nitric oxide: a prostaglandin H synthase 1 and 2 reducing cosubstrate that does not stimulate cyclooxygenase activity or prostaglandin H synthase expression in murine macrophages. *Arch.Biochem. Biophys.* 335:369-376, 1996

113. Delaney, CA, Green, MH, Lowe, JE, Green, IC: Endogenous nitric oxide induced by interleukin-1 β in rat islets of Langerhans and HIT-T15 cells causes significant DNA damage as measured by the 'comet' assay. *FEBS Lett.* 333:291-295, 1993

114. Fehsel, K, Jalowy, A, Qi, S, Burkart, V, Hartmann, B, Kolb, H: Islet cell DNA is a target of inflammatory attack by nitric oxide. *Diabetes* 42:496-500, 1993

115. Eizirik, DL, Delaney, CA, Green, MH, Cunningham, JM, Thorpe, JR, Pipeleers, DG, Hellerström, C, Green, IC: Nitric oxide donors decrease the function and survival of human pancreatic islets. *Mol.Cell. Endocrinol.* 118:71-83, 1996

116. Delaney, CA, Tyrberg, B, Bouwens, L, Vaghef, H, Hellman, B, Eizirik, DL: Sensitivity of human pancreatic islets to peroxynitrite-induced cell dysfunction and death. *FEBS Lett.* 394:300-306, 1996

117. Ankarcrona, M, Dypbukt, JM, Brune, B, Nicotera, P: Interleukin-1 β-induced nitric oxide production activates apoptosis in pancreatic RINm5F cells. *Exp.Cell Res.* 213:172-177, 1994

118. Kaneto, H, Fujii, J, Seo, HG, Suzuki, K, Matsuoka, T, Nakamura, M, Tatsumi, H, Yamasaki, Y, Kamada, T, Taniguchi, N: Apoptotic cell death triggered by nitric oxide in pancreatic β-cells. *Diabetes* 44:733-738, 1995

119. Stassi, G, De Maria, R, Trucco, G, Rudert, W, Testi, R, Galluzzo, A, Giordano, C, Trucco, M: Nitric oxide primes pancreatic β cells for Fas-mediated destruction in insulin-dependent diabetes mellitus. *J. Exp.Med.* 186:1193-1200, 1997

120. Saldeen, J: Cytokines induce both necrosis and apoptosis via a common Bcl-2- inhibitable pathway in rat insulin-producing cells. *Endocrinology* 141:2003-2010, 2000

121. Zumsteg, U, Frigerio, S, Hollander, GA: Nitric oxide production and Fas surface expression mediate two independent pathways of cytokine-induced murine beta-cell damage. *Diabetes* 49:39-47, 2000

122. Liu, D, Pavlovic, D, Chen, M, Flodström, M, Sandler, S, Eizirik, DL: Cytokines induce apoptosis in beta-cells isolated from mice lacking the inducible isoform of nitric oxide synthase (iNOS-/-). *Diabetes* 49:1116-1122, 2000

123. Lukic, ML, Stosic-Grujicic, S, Ostojic, N, Chan, WL, Liew, FY: Inhibition of nitric oxide generation affects the induction of diabetes by streptozocin in mice. *Biochem. Biophys.Res.Commun.* 178:913-920, 1991

124. Kolb, H, Kiesel, U, Kröncke, KD, Kolb-Bachofen, V: Suppression of low dose streptozotocin induced diabetes in mice by administration of a nitric oxide synthase inhibitor. *Life Sci.* 49:L213-L217, 1991

125. Lindsay, RM, Smith, W, Rossiter, SP, McIntyre, MA, Williams, BC, Baird, JD: N^{ω}-nitro-L-arginine methyl ester reduces the incidence of IDDM in BB/E rats. *Diabetes* 44:365-368, 1995

126. Papaccio, G, Esposito, V, Latronico, MV, Pisanti, FA: Administration of a nitric oxide synthase inhibitor does not suppress low-dose streptozotocin-induced diabetes in mice. *Int.J. Pancreatol.* 17:63-68, 1995

127. Wu, G: Nitric oxide synthesis and the effect of aminoguanidine and N^{G}-monomethyl-L-arginine on the onset of diabetes in the spontaneously diabetic BB rat. *Diabetes* 44:360-364, 1995

128. Flodström, M, Tyrberg, B, Eizirik, DL, Sandler, S: Reduced sensitivity of inducible nitric oxide synthase-deficient mice to multiple low-dose streptozotocin-induced diabetes. *Diabetes* 48:706-713, 1999

129. Takamura, T, Kato, I, Kimura, N, Nakazawa, T, Yonekura, H, Takasawa, S, Okamoto, H: Transgenic mice overexpressing type 2 nitric-oxide synthase in pancreatic beta cells develop insulin-dependent diabetes without insulitis. *J. Biol. Chem.* 273:2493-2496, 1998

130. Vavra, JJ, DeBoer, C, Dietz, A, Hanka, J, Soloski, WT: Streptozotocin, a new antibacterial antibiotic. *In:Antibiotic Annual*

*1959-1960.Ed.Marti-Ibanez,F.Antibiotica Inc.New York N.Y.*230-235, 1960

131. White, FR: Streptozotocin. *Cancer Chemother.* 30:49-53, 1963

132. Rakieten, N, Rakieten, ML, Nadkarni, MV: Studies on the diabetogenic action of streptzotocin. *Cancer Chemother.* 29:91-98, 1963

133. Brosky, G, Logothetopoulos, J: Streptozotocin diabetes in the mouse and guinea pig. *Diabetes* 18:606-611, 1969

134. Schnedl, WJ, Ferber, S, Johnson, JH, Newgard, CB: STZ transport and cytotoxicity. Specific enhancement in GLUT2- expressing cells. *Diabetes* 43:1326-1333, 1994

135. Lown, JW, McLaughlin, LW, Chang, Y: Mechanisms of action of 2-haloethyl-nitrosureas on DNA and its relation to their antileukemic properties. *Bioorg.Chem.* 7:97-110, 1978

136. Murata, M, Takahashi, A, Saito, I, Kawanishi, S: Site-specific DNA methylation and apoptosis: induction by diabetogenic streptozotocin. *Biochem.Pharmacol.* 57:881-887, 1999

137. McCullough, AK, Dodson, ML, Lloyd, RS: Initiation of base excision repair: glycosylase mechanisms and structures. *Annu.Rev.Biochem* 68:255-285, 1999

138. Durkacz, BW, Omidiji, O, Gray, DA, Shall, S: (ADP-ribose)n participates in DNA excision repair. *Nature* 283:593-596, 1980

139. Satoh, MS, Lindahl, T: Role of poly(ADP-ribose) formation in DNA repair. *Nature* 356:356-358, 1992

140. Althaus, FR: Poly ADP-ribosylation: a histone shuttle mechanism in DNA excision repair. *J. Cell. Sci.* 102:663-670, 1992

141. Yamamoto, H, Uchigata, Y, Okamoto, H: Streptozotocin and alloxan induce DNA strand breaks and poly(ADP- ribose) synthetase in pancreatic islets. *Nature* 294:284-286, 1981

142. Schein, PS, Loftus, S: Streptozotocin: depression of mouse liver pyridine nucleotides. *Cancer Res.* 28:1501-1506, 1968

143. Gunnarsson, R, Berne, C, Hellerström, C: Cytotoxic effects of streptozotocin and N-nitrosomethylurea on the pancreatic B cells

with special regard to the role of nicotinamide- adenine dinucleotide. *Biochem. J.* 140:487-494, 1974

144. Robbins, MJ, Sharp, RA, Slonim, AE, Burr, IM: Protection against streptozotocin-induced diabetes by superoxide dismutase. *Diabetologia* 18:55-58, 1980

145. Uchigata, Y, Yamamoto, H, Kawamura, A, Okamoto, H: Protection by superoxide dismutase, catalase, and poly(ADP-ribose) synthetase inhibitors against all. *J.Biol.Chem.* 257:6084-6088, 1982

146. Sandler, S, Andersson, A: The partial protective effect of the hydroxyl radical scavenger dimethyl urea on streptozotocin-induced diabetes in the mouse in vivo and in vitro. *Diabetologia* 23:374-378, 1982

147. Papaccio, G, Pisanti, FA, Frascatore, S: Acetyl-homocysteine-thiolactone-induced increase of superoxide dismutase counteracts the effect of subdiabetogenic doses of streptozocin. *Diabetes* 35:470-474, 1986

148. Schein, PS, Cooney, DA, Vernon, ML: The use of nicotinamide to modify the toxicity of streptozotocin diabetes without loss of antitumor activity. *Cancer Res.* 27:2324-2332, 1967

149. Burkart, V, Wang, ZQ, Radons, J, Heller, B, Herceg, Z, Stingl, L, Wagner, EF, Kolb, H: Mice lacking the poly(ADP-ribose) polymerase gene are resistant to pancreatic beta-cell destruction and diabetes development induced by streptozocin. *Nature Med.* 5:314-319, 1999

150. Pieper, AA, Brat, DJ, Krug, DK, Watkins, CC, Gupta, A, Blackshaw, S, Verma, A, Wang, ZQ, Snyder, SH: Poly(ADP-ribose) polymerase-deficient mice are protected from streptozotocin-induced diabetes. *Proc.Natl.Acad.Sci.U.S.A.* 96:3059-3064, 1999

151. Masutani, M, Suzuki, H, Kamada, N, Watanabe, M, Ueda, O, Nozaki, T, Jishage, K, Watanabe, T, Sugimoto, T, Nakagama, H, Ochiya, T, Sugimura, T: Poly(ADP-ribose) polymerase gene disruption conferred mice resistant to streptozotocin-induced diabetes. *Proc.Natl.Acad.Sci.U.S.A.* 96:2301-2304, 1999

152. Rerup, CC: Drugs producing diabetes through damage of the insulin secreting cells. *Pharmacol.Rev.* 22:485-518, 1970

153. Like, AA, Rossini, AA: Streptozotocin-induced pancreatic insulitis: new model of diabetes mellitus. *Science* 193:415-417, 1976

154. Bonnevie-Nielsen, V, Steffes, MW, Lernmark, Å: A major loss in islet mass and B-cell function precedes hyperglycemia in mice given multiple low doses of streptozotocin. *Diabetes* 30:424-429, 1981

155. Fiordaliso, F, Li, B, Latini, R, Sonnenblick, EH, Anversa, P, Leri, A, Kajstura, J: Myocyte death in streptozotocin-induced diabetes in rats in angiotensin II- dependent. *Lab. Invest.* 80:513-527, 2000

156. Lerner, AB, Case, JD, Takahashi, Y, Lee, TH, Mori, W: Isolation of melatonin, the pineal gland factor that lightens melanocytes. *J.Am.Chem.Soc.* 80:2587, 1958

157. Klein, DC, Weller, JL: Rapid light-induced decrease in pineal serotonin N-acetyltransferase activity. *Science* 177:532-533, 1972

158. Reiter, RJ: Pineal melatonin: cell biology of its synthesis and of its physiological interactions. *Endocr.Rev.* 12:151-180, 1991

159. Mendelson, WB, Gillin, JC, Dawson, SD, Lewy, AJ, Wyatt, RJ: Effects of melatonin and propranolol on sleep of the rat. *Brain Res.* 201:240-244, 1980

160. Arendt, J, Broadway, J: Light and melatonin as zeitgebers in man. *Chronobiol.Int.* 4:273-282, 1987

161. Brown, GM: Day-night rhythm disturbance, pineal function and human disease. *Horm.Res.* 37 Suppl 3:105-111, 1992

162. Blask, DE, Wilson, ST, Zalatan, F: Physiological melatonin inhibition of human breast cancer cell growth in vitro: evidence for a glutathione-mediated pathway. *Cancer Res.* 57:1909-1914, 1997

163. Tan, DX, Chen, LD, Poeggeler, B, Manchester, LC, Reiter, RJ: Melatonin: A potent, endogenous hydroxyl radical scavenger. *Endocr.J.* 1:57-60, 1993

164. Reiter, RJ, Melchiorri, D, Sewerynek, E, Poeggeler, B, Barlow-Walden, L, Chuang, J, Ortiz, GG, Acuna-Castroviejo, D: A review of the evidence supporting melatonin's role as an antioxidant. *J.Pineal Res.* 18:1-11, 1995

165. Marshall, KA, Reiter, RJ, Poeggeler, B, Aruoma, OI, Halliwell, B: Evaluation of the antioxidant activity of melatonin in vitro. *Free Radic.Biol.Med.* 21:307-315, 1996

166. Morgan, PJ, Barrett, P, Hazlerigg, D, Milligan, G, Lawson, W, MacLean, A, Davidson, G: Melatonin receptors couple through a cholera toxin-sensitive mechanism to inhibit cyclic AMP in the ovine pituitary. *J Neuroendocrinol.* 7:361-369, 1995

167. Menendez-Pelaez, A, Reiter, RJ: Distribution of melatonin in mammalian tissues: the relative importance of nuclear versus cytosolic localization. *J.Pineal Res.* 15:59-69, 1993

168. Reiter, RJ, Guerrero, JM, Garcia, JJ, Acuna-Castroviejo, D: Reactive oxygen intermediates, molecular damage, and aging. Relation to melatonin. *Ann.N.Y.Acad.Sci.* 854:410-424, 1998

169. Barlow-Walden, LR, Reiter, RJ, Abe, M, Pablos, M, Menendez-Pelaez, A, Chen, LD, Poeggeler, B: Melatonin stimulates brain glutathione peroxidase activity. *Neurochem.Int.* 26:497-502, 1995

170. Pablos, MI, Agapito, MT, Gutierrez, R, Recio, JM, Reiter, RJ, Barlow-Walden, L, Acuna-Castroviejo, D, Menendez-Pelaez, A: Melatonin stimulates the activity of the detoxifying enzyme glutathione peroxidase in several tissues of chicks. *J.Pineal Res.* 19:111-115, 1995

171. Kotler, M, Rodriguez, C, Sainz, RM, Antolin, I, Menendez-Pelaez, A: Melatonin increases gene expression for antioxidant enzymes in rat brain cortex. *J.Pineal Res.* 24:83-89, 1998

172. Giusti, P, Lipartiti, M, Gusella, M, Floreani, M, Manev, H: In vitro and in vivo protective effects of melatonin against glutamate oxidative stress and neurotoxicity. *Ann.N.Y.Acad.Sci.* 825:79-84, 1997

173. Crespo, E, Macias, M, Pozo, D, Escames, G, Martin, M, Vives, F, Guerrero, JM, Acuna-Castroviejo, D: Melatonin inhibits expression of the inducible NO synthase II in liver and lung and prevents endotoxemia in lipopolysaccharide-induced multiple organ dysfunction syndrome in rats. *FASEB J.* 13:1537-1546, 1999

174. Cuzzocrea, S, Zingarelli, B, Costantino, G, Caputi, AP: Protective effect of melatonin in a non-septic shock model induced by zymosan in the rat. *J. Pineal Res.* 25:24-33, 1998

175. Cuzzocrea, S, Caputi, AP: Protective effect of melatonin on zymosan-induced cellular damage. *Biol.Signals Recept.* 8:136-142, 1999

176. El Sokkary, GH, Reiter, RJ, Cuzzocrea, S, Caputi, AP, Hassanein, AF, Tan, DX: Role of melatonin in reduction of lipid peroxidation and peroxynitrite formation in non-septic shock induced by zymosan. *Shock* 12:402-408, 1999

177. Cuzzocrea, S, Tan, DX, Costantino, G, Mazzon, E, Caputi, AP, Reiter, RJ: The protective role of endogenous melatonin in carrageenan-induced pleurisy in the rat. *FASEB J.* 13:1930-1938, 1999

178. Montilla, PL, Vargas, JF, Tunez, IF, Munoz de Agueda, MC, Valdelvira, ME, Cabrera, ES: Oxidative stress in diabetic rats induced by streptozotocin: protective effects of melatonin. *J.Pineal Res.* 25:94-100, 1998

179. Abdel-Wahab, MH, Abd-Allah, AR: Possible protective effect of melatonin and/or desferrioxamine against streptozotocin-induced hyperglycaemia in mice. *Pharmacol.Res.* 41:533-537, 2000

180. Pierrefiche, G, Topall, G, Courboin, G, Henriet, I, Laborit, H: Antioxidant activity of melatonin in mice. *Res.Commun.Chem.Pathol.Pharmacol.* 80:211-223, 1993

181. Conti, A, Maestroni, GJ: Role of the pineal gland and melatonin in the development of autoimmune diabetes in non-obese diabetic mice. *J.Pineal Res.* 20:164-172, 1996

182. Conti, A, Maestroni, GJ: Melatonin rhythms in mice: role in autoimmune and lymphoproliferative diseases. *Ann.N.Y.Acad.Sci.* 840:395-410, 1998

183. MacMicking, JD, Nathan, C, Hom, G, Chartrain, N, Fletcher, DS, Trumbauer, M, Stevens, K, Xie, QW, Sokol, K, Hutchinson, N: Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase. *Cell* 81:641-650, 1995

184. Darrah, PA, Hondalus, MK, Chen, Q, Ischiropoulos, H, Mosser, DM: Cooperation between reactive oxygen and nitrogen intermediates in killing of Rhodococcus equi by activated macrophages. *Infect.Immun.* 68:3587-3593, 2000

185. Crosara-Alberto, DP, Darini, AL, Inoue, RY, Silva, JS, Ferreira, SH, Cunha, FQ: Involvement of NO in the failure of neutrophil migration in sepsis induced by Staphylococcus aureus. *Br.J.Pharmacol.* 136:645-658, 2002

186. Andersson, A, Sandler, S: Viability tests of cryopreserved endocrine pancreatic cells. *Cryobiology* 20:161-168, 1983

187. Heding, LG: Determination of total serum insulin (IRI) in insulin-treated diabetic patients. *Diabetologia* 8:260-266, 1972

188. Halban, PA, Wollheim, CB, Blondel, B, Renold, AE: Long-term exposure of isolated pancreatic islets to mannoheptulose: evidence for insulin degradation in the beta cell. *Biochem. Pharmacol.* 29:2625-2633, 1980

189. Green, LC, Wagner, DA, Glogowski, J, Skipper, PL, Wishnok, JS, Tannenbaum, SR: Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids. *Anal. Biochem.* 126:131-138, 1982

190. Greiss, P: Bemerkungen zu der Abhandlung der HH. Weselsky und Benedict "Ueber einige Azoverbindungen". *Ber.Deutsch.Chem.Ges.* 12:426-428, 1879

191. Ericson, Å, Niklasson, F, de Verdier, CH: A systematic study of nucleotide analysis of human erythrocytes using an anionic exchanger and HPLC. *Clin.Chim.Acta* 127:47-59, 1983

192. Crompton, T, Peitsch, MC, MacDonald, HR, Tschopp, J: Propidium iodide staining correlates with the extent of DNA degradation in isolated nuclei. *Biochem. Biophys.Res.Commun.* 183:532-537, 1992

193. Dignam, JD, Lebovitz, RM, Roeder, RG: Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11:1475-1489, 1983

194. Heid, CA, Stevens, J, Livak, KJ, Williams, PM: Real time quantitative PCR. *Genome Res.* 6:986-994, 1996

195. Gibson, UE, Heid, CA, Williams, PM: A novel method for real time quantitative RT-PCR. *Genome Res.* 6:995-1001, 1996

196. Gandy, SE, Buse, MG, Crouch, RK: Protective role of superoxide dismutase against diabetogenic drugs. *J Clin.Invest.* 70:650-658, 1982

197. Asplund, K, Grankvist, K, Marklund, S, Täljedal, IB: Partial protection against streptozotocin-induced hyperglycaemia by superoxide dismutase linked to polyethylene glycol. *Acta Endocrinol.(Copenh)* 107:390-394, 1984

198. Slonim, AE, Surber, ML, Page, DL, Sharp, RA, Burr, IM: Modification of chemically induced diabetes in rats by vitamin E. Supplementation minimizes and depletion enhances development of diabetes. *J Clin.Invest.* 71:1282-1288, 1983

199. Matuszak, Z, Reszka, K, Chignell, CF: Reaction of melatonin and related indoles with hydroxyl radicals: EPR and spin trapping investigations. *Free Radic.Biol. Med.* 23:367-372, 1997

200. Cagnoli, CM, Atabay, C, Kharlamova, E, Manev, H: Melatonin protects neurons from singlet oxygen-induced apoptosis. *J. Pineal Res.* 18:222-226, 1995

201. Gilad, E, Cuzzocrea, S, Zingarelli, B, Salzman, AL, Szabo, C: Melatonin is a scavenger of peroxynitrite. *Life Sci.* 60:L169-L174, 1997

202. Zhang, H, Squadrito, GL, Pryor, WA: The reaction of melatonin with peroxynitrite: formation of melatonin radical cation and absence of stable nitrated products. *Biochem. Biophys.Res.Commun.* 251:83-87, 1998

203. Noda, Y, Mori, A, Liburdy, R, Packer, L: Melatonin and its precursors scavenge nitric oxide. *J. Pineal Res.* 27:159-163, 1999

204. Ebelt, H, Peschke, D, Bromme, HJ, Morke, W, Blume, R, Peschke, E: Influence of melatonin on free radical-induced changes in rat pancreatic beta-cells in vitro. *J. Pineal Res.* 28:65-72, 2000

205. Malaisse, WJ: Alloxan toxicity to the pancreatic B-cell. A new hypothesis. *Biochem Pharmacol.* 31:3527-3534, 1982

206. Hardeland, R, Reiter, RJ, Poeggeler, B, Tan, DX: The significance of the metabolism of the neurohormone melatonin: antioxidative protection and formation of bioactive substances. *Neurosci.Biobehav.Rev.* 17:347-357, 1993

207. Antolin, I, Rodriguez, C, Sainz, RM, Mayo, JC, Uria, H, Kotler, ML, Rodriguez-Colunga, MJ, Tolivia, D, Menendez-Pelaez, A: Neurohormone melatonin prevents cell damage: effect on gene expression for antioxidant enzymes. *FASEB J.* 10:882-890, 1996

208. Martin, M, Macias, M, Escames, G, Reiter, RJ, Agapito, MT, Ortiz, GG, Acuna-Castroviejo, D: Melatonin-induced increased activity of the respiratory chain complexes I and IV can prevent mitochondrial damage induced by ruthenium red in vivo. *J. Pineal Res.* 28:242-248, 2000

209. Martin, M, Macias, M, Leon, J, Escames, G, Khaldy, H, Acuna-Castroviejo, D: Melatonin increases the activity of the oxidative phosphorylation enzymes and the production of ATP in rat brain and liver mitochondria. *Int.J.Biochem.Cell Biol.* 34:348-357, 2002

210. Peschke, E, Peschke, D, Hammer, T, Csernus, V: Influence of melatonin and serotonin on glucose-stimulated insulin release from perifused rat pancreatic islets in vitro. *J. Pineal Res.* 23:156-163, 1997

211. Pipeleers, D, van de Winkel, M: Pancreatic B cells possess defense mechanisms against cell-specific toxicity. *Proc.Natl.Acad.Sci.U.S.A.* 83:5267-5271, 1986

212. Eizirik, DL, Strandell, E, Sandler, S: Culture of mouse pancreatic islets in different glucose concentrations modifies B cell sensitivity to streptozotocin. *Diabetologia* 31:168-174, 1988

213. Sandler, S, Bendtzen, K, Eizirik, DL, Strandell, E, Welsh, M, Welsh, N: Metabolism and β-cell function of rat pancreatic islets exposed to human interleukin-1 beta in the presence of a high glucose concentration. *Immunol.Lett.* 26:245-251, 1990

214. Burkart, V, Brenner, HH, Hartmann, B, Kolb, H: Metabolic activation of islet cells improves resistance against oxygen radicals or streptozocin, but not nitric oxide. *J Clin.Endocrinol.Metab* 81:3966-3971, 1996

215. Baydas, G, Canatan, H, Turkoglu, A: Comparative analysis of the protective effects of melatonin and vitamin E on streptozocin-induced diabetes mellitus. *J.Pineal Res.* 32:225-230, 2002

216. Gorgun, FM, Ozturk, Z, Gumustas, MK, Kokogu, E: Melatonin administration affects plasma total sialic acid and lipid peroxidation levels in streptozotocin induced diabetic rats. *J.Toxicol.Environ.Health A* 65:695-700, 2002

217. Rao, VS, Santos, FA, Silva, RM, Teixiera, MG: Effects of nitric oxide synthase inhibitors and melatonin on the hyperglycemic

response to streptozotocin in rats. *Vasc.Pharmacol.* 38:127-130, 2002

218. Gilad, E, Wong, HR, Zingarelli, B, Virag, L, O'Connor, M, Salzman, AL, Szabo, C: Melatonin inhibits expression of the inducible isoform of nitric oxide synthase in murine macrophages: role of inhibition of NFkappaB activation. *FASEB J.* 12:685-693, 1998

219. Hansson, I, Holmdahl, R, Mattsson, R: The pineal hormone melatonin exaggerates development of collagen- induced arthritis in mice. *J. Neuroimmunol.* 39:23-30, 1992

220. Mocchegiani, E, Bulian, D, Santarelli, L, Tibaldi, A, Muzzioli, M, Pierpaoli, W, Fabris, N: The immuno-reconstituting effect of melatonin or pineal grafting and its relation to zinc pool in aging mice. *J. Neuroimmunol.* 53:189-201, 1994

221. Currier, NL, Sun, LZ, Miller, SC: Exogenous melatonin: quantitative enhancement in vivo of cells mediating non-specific immunity. *J. Neuroimmunol.* 104:101-108, 2000

222. Eizirik, DL, Welsh, N, Hellerström, C: Predominance of stimulatory effects of interleukin-1 beta on isolated human pancreatic islets. *J Clin.Endocrinol.Metab.* 76:399-403, 1993

223. Andersen, HU, Jorgensen, KH, Egeberg, J, Mandrup-Poulsen, T, Nerup, J: Nicotinamide prevents interleukin-1 effects on accumulated insulin release and nitric oxide production in rat islets of Langerhans. *Diabetes* 43:770-777, 1994

224. Flodström, M, Morris, SM, Jr., Eizirik, DL: Role of the citrulline-nitric oxide cycle in the functional response of adult human and rodent pancreatic islets to cytokines. *Cytokine* 8:642-650, 1996

225. Mandrup-Poulsen, T, Helqvist, S, Wogensen, LD, Mølvig, J, Pociot, F, Johannesen, J, Nerup, J: Cytokine and free radicals as effector molecules in the destruction of pancreatic beta cells. *Curr.Top.Microbiol.Immunol.* 164:169-193, 1990

226. Hostens, K, Pavlovic, D, Zambre, Y, Ling, Z, Van Schravendijk, C, Eizirik, DL, Pipeleers, DG: Exposure of human islets to cytokines can result in disproportionately elevated proinsulin release. *J Clin.Invest.* 104:67-72, 1999

227. Steiner, L, Kroncke, K, Fehsel, K, Kolb-Bachofen, V: Endothelial cells as cytotoxic effector cells: cytokine-activated rat islet endothelial cells lyse syngeneic islet cells via nitric oxide. *Diabetologia* 40:150-155, 1997

228. Arnush, M, Scarim, AL, Heitmeier, MR, Kelly, CB, Corbett, JA: Potential role of resident islet macrophage activation in the initiation of autoimmune diabetes. *J. Immunol.* 160:2684-2691, 1998

229. Egan, RW, Paxton, J, Kuehl, FA, Jr.: Mechanism for irreversible self-deactivation of prostaglandin synthetase. *J.Biol.Chem.* 251:7329-7335, 1976

230. Landino, LM, Crews, BC, Timmons, MD, Morrow, JD, Marnett, LJ: Peroxynitrite, the coupling product of nitric oxide and superoxide, activates prostaglandin biosynthesis. *Proc.Natl.Acad.Sci.U.S.A.* 93:15069-15074, 1996

231. Hajjar, DP, Lander, HM, Pearce, FS, Upmacis, RK, Pomerantz, KB: Nitric oxide enhances prostaglandin-H synthase activity by a heme-independent mechanism: evidence implicating nitrosothiols. *J.Am.Chem.Soc.* 117:3340-336, 1995

232. Gaillard, T, Mulsch, A, Klein, H, Decker, K: Regulation by prostaglandin E2 of cytokine-elicited nitric oxide synthesis in rat liver macrophages. *Biol.Chem.Hoppe Seyler* 373:897-902, 1992

233. Aeberhard, EE, Henderson, SA, Arabolos, NS, Griscavage, JM, Castro, FE, Barrett, CT, Ignarro, LJ: Nonsteroidal anti-inflammatory drugs inhibit expression of the inducible nitric oxide synthase gene. *Biochem.Biophys.Res.Commun.* 208:1053-1059, 1995

234. Posadas, I, Terencio, MC, Guillen, I, Ferrandiz, ML, Coloma, J, Paya, M, Alcaraz, MJ: Co-regulation between cyclo-oxygenase-2 and inducible nitric oxide synthase expression in the time-course of murine inflammation. *Naunyn Schmiedebergs Arch.Pharmacol.* 361:98-106, 2000

235. Ganapathy, V, Gurlo, T, Jarstadmarken, HO, von Grafenstein, H: Regulation of TCR-induced IFN-γ release from islet-reactive non-obese diabetic $CD8^+$ T cells by prostaglandin E_2 receptor signaling. *Int.Immunol.* 12:851-860, 2000

236. Tabatabaie, T, Vasquez-Weldon, A, Moore, DR, Kotake, Y: Free Radicals and the Pathogenesis of Type 1 Diabetes: β-Cell Cytokine-

Mediated Free Radical Generation Via Cyclooxygenase-2. *Diabetes* 52:1994-1999, 2003

237. Kovanen, PE, Leonard, WJ: Inhibitors keep cytokines in check. *Curr.Biol.* 9:R899-R902, 1999

238. Karlsen, AE, Ronn, SG, Lindberg, K, Johannesen, J, Galsgaard, ED, Pociot, F, Nielsen, JH, Mandrup-Poulsen, T, Nerup, J, Billestrup, N: Suppressor of cytokine signaling 3 (SOCS-3) protects beta -cells against interleukin-1β - and interferon-γ -mediated toxicity. *Proc.Natl.Acad.Sci.U.S.A.* 98:12191-12196, 2001

239. Zambre, Y, Van Schravendijk, C, Ling, Z: Interleukin-1 β inhibits proinsulin conversion in rat beta-cells via a nitric oxide-dependent pathway. *Horm.Metab. Res.* 33:639-644, 2001

240. Roder, ME, Knip, M, Hartling, SG, Karjalainen, J, Åkerblom, HK, Binder, C: Disproportionately elevated proinsulin levels precede the onset of insulin-dependent diabetes mellitus in siblings with low first phase insulin responses. The Childhood Diabetes in Finland Study Group. *J.Clin.Endocrinol.Metab.* 79:1570-1575, 1994

241. Spinas, GA, Snorgaard, O, Hartling, SG, Oberholzer, M, Berger, W: Elevated proinsulin levels related to islet cell antibodies in first-degree relatives of IDDM patients. *Diabetes Care* 15:632-637, 1992

242. Cardozo, AK, Heimberg, H, Heremans, Y, Leeman, R, Kutlu, B, Kruhoffer, M, Orntoft, T, Eizirik, DL: A comprehensive analysis of cytokine-induced and nuclear factor-κB-dependent genes in primary rat pancreatic β-cells. *J.Biol.Chem.* 276:48879-48886, 2001

243. Jonas, JC, Laybutt, DR, Steil, GM, Trivedi, N, Pertusa, JG, Van de, CM, Weir, GC, Henquin, JC: High glucose stimulates early response gene c-Myc expression in rat pancreatic beta cells. *J.Biol.Chem.* 276:35375-35381, 2001

244. Kaneto, H, Sharma, A, Suzuma, K, Laybutt, DR, Xu, G, Bonner-Weir, S, Weir, GC: Induction of c-Myc expression suppresses insulin gene transcription by inhibiting NeuroD/BETA2-mediated transcriptional activation. *J.Biol.Chem.* 277:12998-13006, 2002

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