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OF MOLECULAR MEDICINE AND SURGERY
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CYTOKINE-INDUCED BETA-CELL APOPTOSIS AND ITS REGULATION BY SOCS-1 AND IMIDAZOLINE COMPOUNDS

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Karolinska Institutet

Stockholm 2014

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB, Karolinska Institute, SE-17177, Stockholm.

ISBN 978-91-7549-651-1

Cytokine-induced beta-cell apoptosis and its regulation by SOCS-1 and imidazoline compounds

THESIS FOR DOCTORAL DEGREE (Ph.D.)

by

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ABSTRACT

A selective destruction of pancreatic β-cells as a consequence of inflammation in the islets of Langerhans is a feature of type 1 diabetes. Pro-inflammatory cytokines secreted by T lymphocytes and macrophages infiltrating the pancreatic islets participate in the development of this autoimmune disease by acting directly on the β-cell. The aim of this thesis was to investigate mechanisms of β-cell dysfunction and death induced by the mixture of pro-inflammatory cytokines IL-1β, IFNγ and TNFα, i.e., under conditions modelling those during inflammation in type 1 diabetes. Furthermore, we aimed to study whether imidazoline compounds RX871024 and efaroxan can affect pancreatic β-cell death under these conditions and if so, to explore underlying molecular mechanisms.

Some imidazoline compounds can promote insulin secretion and have been discussed as potential therapeutic drugs in type 2 diabetes. Among those compounds are insulinotropic imidazolines RX871024 and efaroxan. It was previously shown that these imidazolines do not induce apoptosis in mouse pancreatic β-cells but even protect against IL-1β-induced primary β-cell apoptosis. The protective effect of RX871024 on IL-1β-induced β-cell apoptosis has been accompanied by inhibition of IL-1β-induced NO production. However, in a first study we have shown that the imidazoline compounds cannot protect pancreatic β-cells against death induced by a combination of pro-inflammatory cytokines IL-1β, IFNγ and TNFα, despite RX871024 decreases the cytokine-induced NO production both in islets and in β-cells. RX871024-induced decrease in p38 MAPK phosphorylation may explain the partial inhibitory effect of RX871024 on cytokine-induced NO production. Thus pancreatic β-cell death triggered by a mixture of pro-inflammatory cytokines IL-1β, IFNγ and TNFα, conditions resembling those that take place in type 1 diabetes, does not directly correlate with NO production and rather relies on other players which cannot be counteracted with agents such as imidazoline compounds.

Malignant insulinoma is an uncommon tumour, however, it has a poor prognosis. Chemotherapy to this tumour is not very effective. Therefore, search for effective and specific chemotherapeutical drugs for patients with malignant insulinomas is of utmost importance. Unlike primary β-cells where RX871024 was without any effect, the imidazoline compound selectively destructs insulinoma MIN6 cells and potentiates cytokine-induced insulinoma cell death. The cytotoxic effects of RX871024 does not include changes in NO production but involve increase in basal and cytokine-induced JNK activation associated with stimulation of initiator caspases-1, -8 and -9 and executor caspase-3. In contrast to primary mouse β-cells, there was no effect of cytokines or imidazolines on p38 activation in MIN6 cells.

It has been shown that expression of SOCS-1, an endogenous inhibitor of IFNγ-induced signalling, in pancreatic β-cells protects NOD mice against diabetes. In a third study we investigated how signaling via JAK/STAT pathway controls cytokine-induced β-cell death. SOCS-1 overexpression diminishes activation of both caspase-8 and -9 in primary mouse β-cells leading to inhibition of cytokine-induced β-cell death. This finding in association with the observation that SOCS-1 does not affect glucose stimulated insulin release and islet cell death in the absence of cytokines indicates the possibility to use an elevation of SOCS-1 expression in the treatment of type 1 diabetes.

In conclusion, results of this thesis implicate that pancreatic β-cell death induced by mixture of pro-inflammatory cytokines IL-1β, IFNγ and TNFα cannot be counteracted with agents such as imidazoline compounds, but can be suppressed by inhibition of IFNγ-induced signalling. We have also found that RX871024 exerts selective cytotoxic effect towards insulinoma cells.

LIST OF SCIENTIFIC PAPERS

I. **Zaitseva II**, Sharoyko V, Storling J, Efendic S, Guerin C, Mandrup-Poulsen T, Nicotera P, Berggren PO, and Zaitsev SV. RX871024 reduces NO production but does not protect against pancreatic beta-cell death induced by proinflammatory cytokines. *Biochem Biophys Res Commun*, 2006. 347(4): 1121-8.

II. **Zaitseva II**, Storling J, Mandrup-Poulsen T, Berggren PO, and Zaitsev SV. The imidazoline RX871024 induces death of proliferating insulin-secreting cells by activation of c-jun N-terminal kinase. *Cell Mol Life Sci*, 2008. 65(7-8): 1248-55.

III. **Zaitseva II**, Hultcrantz M, Sharoyko V, Flodstrom-Tullberg M, Zaitsev SV, and Berggren PO. Suppressor of cytokine signaling-1 inhibits caspase activation and protects from cytokine-induced beta cell death. *Cell Mol Life Sci*, 2009. 66(23): 3787-95.

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I. Zaitsev SV, Kohler M, **Loiko II***, Leibiger B, Leibiger I, Appelskog I, Kapelioukh I, and Berggren PO. Online monitoring of apoptosis in living pancreatic beta-cells. *Diabetologia*, 2002. 45(Supplement 2): A 149.

II. Kohler M, Zaitsev SV, **Zaitseva II**, Leibiger B, Leibiger IB, Turunen M, Kapelioukh IL, Bakkman L, Appelskog IB, de Monvel JB, Imreh G, and Berggren PO. On-line monitoring of apoptosis in insulin-secreting cells. *Diabetes*, 2003. 52(12): 2943-50.

III. Sharoyko VV, **Zaitseva II**, Varsanyi M, Portwood N, Leibiger B, Leibiger I, Berggren PO, Edendic S, and Zaitsev SV. Monomeric G-protein, Rhes, is not an imidazoline-regulated protein in pancreatic beta-cells. *Biochem Biophys Res Commun*, 2005. 338(3): 1455-1459.

IV. Sharoyko VV, **Zaitseva II**, Leibiger B, Edendic S, Berggren PO, and Zaitsev SV. Arachidonic acid signaling is involved in the mechanism of imidazoline-induced KATP channel-independent stimulation of insulin secretion. *Cell Mol Life Sci*, 2007. 64(22): 2985-93.

Loiko married Zaitseva

CONTENTS

LIST OF ABBREVIATIONS

AFC	7-Amino-4-trifluoromethyl coumarin
AIF	Apoptosis inducing factor
AIM	Absent in melanoma
ANT	Adenine nucleotide translocase
AP1	Activator protein 1
APAF	Apoptotic protease activating factor
ARE	AU-rich element
ASK	Apoptosis-stimulating kinase
aSMase	Acid sphingomyelinase
ATF	Activating transcription factor
BSA	Bovine serum albumin
CAD	Caspase activated DNase
CAPK	Ceramide-activated protein kinase
CDC	Cell division cycle
C/EBPβ	CCAAT/enhancer binding protein β
cFLIP	Cellular FLICE-like inhibitory protein
CHOP	C/EBP homologous protein
CINC	Cytokine-induced neutrophyl chemoattractant
COX	Cyclooxygenase
CREB	cAMP responsive element binding protein
CTSD	Cathepsin D
CXCL10	(C-X-C motif) ligand 10
DAG	Diacylglycerol

DAPK	Death-associated protein kinase
ECSIT	Evolutionary conserved signalling intermediate in Toll pathway
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FADD	Fas-associated death domain protein
FAN	Factor associated with neutral sphingomyelinase
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
FOXO	Forkhead box O
GCK	Germinal centre kinase
GPDH	Glyceraldehyde-3-phosphate dehydrogenase
HMGB1	High mobility group-box protein 1
hnRNP-A0	Heterogeneous nuclear ribonucleoprotein A0
Hsp	Heat shock protein
HuR	Hu antigen R
ICAM	Intercellular adhesion molecule
IFNGR	IFNγ receptor
IκB	Inhibitory protein κB
IKK	IκB-kinase
IL-1β	Interleukin-1β
IL-1RAcP	IL-1 - receptor accessory protein
IL-1RI	Type I IL-1 receptor
iNOS	Inducible nitric oxide synthase
IP10	Interferon-inducible protein 10

IRAK	IL-1 receptor associating kinase
IRF	IFN regulatory factor
IRS	Insulin receptor substrate
IFNγ	Interferon-γ
JAK	Janus activated kinase
JNK	c-Jun N-terminal kinase
KRB	Krebs-Ringer bicarbonate buffer
KSRP	KH-type splicing regulatory protein
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinases
MCP	Macrophage chemoattractant protein
MEKK	MAP kinase-Erk kinase kinase
MHC	Major histocompatibility antigens
MK	MAPK activated protein kinases
MKK	Mitogen-activated protein kinase kinases
MKP	MAPK phosphatase
MnSOD	Manganese superoxide dismutase
MPTP	Mitochondrial permeability transition pore
MSK	Mitogen- and stress-activated kinase
NFκB	Nuclear factor κB
NIK	NFκB-inducing kinase
NLR	Nucleotide oligomerization domain leucine-rich repeat-containing receptor
NO	Nitric oxide
NOD	Nonobese diabetic

nSMase	Neutral sphingomyelinase
PARP	Poly(ADP-ribose) polymerase
PBS	Phosphate-buffered saline
PDCD1L1	Programmed cell death 1 ligand 1
PDX	Pancreatic and duodenal homeobox
PI	Propidium iodide
PKA	Protein kinase A
PKB/AKT	Protein kinase B
PKC	Protein kinase C
PKD	Protein kinase D
PKG	Protein kinase G
PLC	Phospholipase C
PPARγ	Peroxisome proliferator-activated receptor gamma
RIP	Receptor-interacting protein
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gelelectrophoresis
SERCA	Sarcoplasmic/Endoplasmic Reticulum Calcium ATPase
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TAB	TAK1 binding protein
TAK	TGFβ-activated kinase
TBS	Tris buffered saline

TBST	TBS Tween
TLR	Toll-like receptor
TNFα	Tumor necrosis factor-α
TNFR1	TNF receptor 1
TRADD	TNFR1 associated death domain protein
TRAF	TNFR-associated factor
TRAIL	TNF related apoptosis inducing ligand
TRPM2	TNFα- and ROS-induced melastatin-like transient receptor potential 2
TUNEL	Terminal transferase-mediated dUTP nick end labelling
USF	Upstream stimulatory factor
VDAC	Voltage-dependent anion channel

1 BACKGROUND

A selective destruction of insulin-producing pancreatic β-cells as a consequence of inflammation in the islets of Langerhans is a feature of type 1 diabetes (1-3). Pro-inflammatory cytokines secreted by macrophages and T lymphocytes infiltrating the pancreatic islets may participate in the development of this autoimmune disease by acting directly on the β-cell (4-6).

The combination of pro-inflammatory cytokines IL-1β, IFNγ and TNFα induce either necrotic (swelling of the cell and its organelles leading to loss of plasma membrane integrity) or apoptotic (characterised by caspases activation, DNA fragmentation, and membrane blebbing) β-cell death (7-15). Cytokines trigger intracellular signalling pathways, but those that result in β-cell damage are not fully understood. Signal-transduction induced by cytokines in β-cells involves activation of MAPK, including ERK, p38 and JNK (16). MAPK activation contributes to β-cell destruction (17, 18), partly through participation in induction of NO production. The expression of iNOS, triggered by cytokines and concomitant increase in intracellular NO concentration, results in diminished glucose-stimulated insulin secretion (9) and is coupled to β-cell death (9, 13, 19) in cultured rodent islets. Abolishing NO production with iNOS inhibitors significantly decreases cytokine-induced pancreatic β-cell death (9, 15, 20). Nevertheless, NO-independent pathways contributing to β-cell death triggered by cytokines exist (11, 21-23). Many cytokine-induced signal transduction pathways converge at the level of caspase activation, and the importance of caspase-3 activation for execution of apoptosis in cytokine-treated islets and β-cell lines was shown by us and others (19, 24-26). An effector caspase-3 is usually activated by initiator caspases (27), such as caspase-8 and caspase-9. Caspase-8 is activated by TNFα in insulin-secreting cell lines (28, 29), and caspase-9 by the combination of IL-1β, IFNγ and TNFα in human pancreatic islets (30).

1.1 IL-1β, TNFα AND IFNγ-INDUCED SIGNALLING

IL-1β is a central mediator in inflammation and innate immune responses. This multifunctional pro-inflammatory cytokine also plays a role in type 1 diabetes. It is one of the substances responsible for the toxic effects of activated macrophages on pancreatic β-cells (31). In rodent islets, IL-1β alone is able to promote β-cell destruction (32) and to inhibit insulin release (33, 34). IL-1β signals through IL-1RI belonging to the TLR family (35). The signal transduction pathways putatively activated by IL-1β in β-cells are schematically shown in Fig. 1.

TNFα is a pleiotropic pro-inflammatory cytokine activating different signaling pathways. Most cell types, including primary β-cells, are resistant to TNFα-induced apoptosis (36). This cytokine binds to its cellular receptor TNFR1, a key mediator of TNFα signaling in the majority of cells including β-cells (37), which signaling under normal conditions induces activation rather than cell death (38). However, special circumstances can push the balance of TNFR1 signaling towards induction of apoptotic or necrotic cell death. Accordingly, TNFα induces apoptosis of primary β-cells only when added in combination with IFNγ (37). Overall, the process of TNFR1-induced cell death is tightly regulated and dependent on environmental and intracellular conditions (39, 40). A schematic overview of the signal transduction pathways putatively activated by TNFα in β-cells is presented in Fig. 1.

IFNγ is a pleiotropic cytokine involved in antiproliferative and antiviral responses, immune surveillance and tumour suppression (41). The signal transduction pathways putatively activated by IFNγ in β-cells are schematically presented in Fig. 1. IFNγ signals via a receptor consisting of two distinct subunits, namely IFNGR1 and IFNGR2 associated with JAK1 and JAK2. Upon binding of IFNγ to its receptor, JAK1 and JAK2 become phosphorylated and thus activated and phosphorylate IFN-receptor allowing STAT-1 to bind. The tyrosine Y701 phosphorylation of STAT-1 by activated JAKs is a crucial step in IFN-mediated signalling resulting in formation of STAT-1-STAT-1 homodimers

which translocate to the nucleus and initiate or suppress the transcription of IFNγ-regulated genes (42, 43). IFNγ indeed induces STAT-1 phosphorylation and sustained activation in pancreatic β-cells (4, 44). During the first wave of IFNγ-induced transcription executed by STAT-1 primary response genes are expressed (45). Many of the genes are transcription factors and among them is IRF1, a major mediator of the IFNγ signalling pathway, which participates in the next transcription wave of many secondary IFNγ-regulated genes (45). Surprisingly, in spite of STAT-1 activation is related to apoptosis induced by cytokines in pancreatic β-cells (46, 47), activation of IRF1 in primary purified pancreatic β-cells is not important for IFNγ-triggered elevation of NO production, increase in MHC class I expression and cytokine-induced apoptosis. However, IRF1 participates in cytokine-induced islet cell death probably by activating non-endocrine cells (e.g. macrophages) (48, 49). Nevertheless, the inhibition of IRF1 function is not meaningful for the protection against cytokine-induced β-cell death. Indeed, deletion of IRF1 leads to abrogation of glucose stimulated insulin secretion and elevation of iNOS and chemokine production, the latter results in more aggressive immune infiltration of grafted islets (49).

1.2 INTEGRATION OF PRO-INFLAMMATORY CYTOKINE SIGNALLING IN PANCREATIC β-CELLS

Under physiological conditions cells are not stimulated with one cytokine in isolation. Relevant for type 1 diabetes is that IFNγ signalling integrates with IL-1β- and TNFα-induced signalling pathways in triggering β-cell apoptosis. *In vitro*, IFNγ itself does not induce β-cell death (50), however, it potentiates the detrimental effects of IL-1β and TNFα on β-cell function and survival (7, 14, 50-53). The interplay of signal-transduction pathways putatively involved in the execution of cytokine-induced β-cell death is schematically presented in Fig. 1.

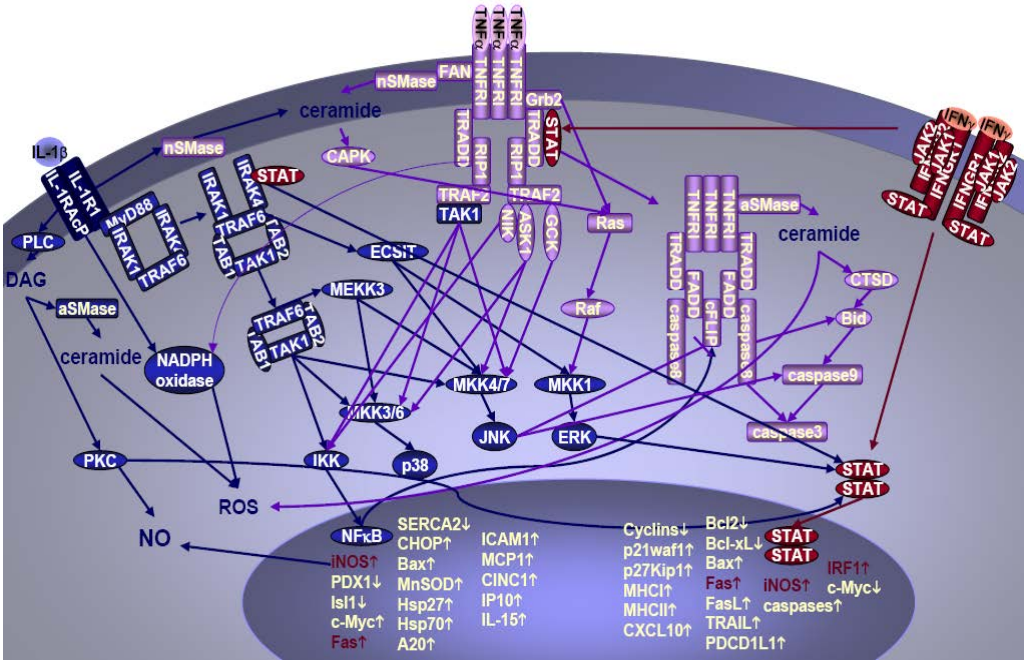

Figure 1. The different signal transduction pathways putatively involved in the execution of cytokine-induced β-cell death. The figure is based on Refs. (4, 16, 35-40, 42-45, 47, 48, 54-103).

Both IL-1β and TNFα stimulate ERK1/2 activation in pancreatic β-cells (16), which then can participate in STAT1 S727 phosphorylation and therefore in full activation of STAT1 (93). Further, IL-1β induces PKCδ (72) and Ca^{2+}/calmodulin-dependent protein kinase II activation in pancreatic β-cells (62) which can also phosphorylate STAT1 on S727 (93). Moreover, STAT-1 interacts with IRAK and undergoes IRAK-dependent phosphorylation of S727 following IL-1β treatment (98). Besides this, STAT-1 is a component of the TNFα receptor complex, and TRADD/STAT-1 interaction is induced by IFNγ (98-100). Similar to STAT-1, p65 subunit of NFκB requires phosphorylation and acetylation to generate a fully active NFκB complex (104). Phosphorylation of NFκB occurs after IκB degradation (104). The phosphorylation of p65can be executed among others by IKK and MSK1, the latter activated by ERK1/2 and p38 (104, 105). Phosphorylation of NFκB determines strength and duration of

the NFκB transcriptional response, regulates DNA binding and facilitates recruitment of transcriptional cofactors (104).

There is an interplay between STAT-1 or IRF1 and other transcription factors induced by IL-1β and TNFα, such as NFκB and c-Jun, the latter is phosphorylated and activated upon JNK activation. It is known that many genes contain binding sites for more than one transcription factor in their promoters and maximal transcription requires presence of all the signals (93, 106, 107). For example, an important gene containing binding sites for both STAT-1 and NFκB in its promoter is IRF1 (106). Co-stimulation with IL-1β indeed potentiates IFNγ-induced increase in IRF1 mRNA and protein content in the RINm5F β-cell line, however, this effect is absent in rat and human islets (108). Similarly, the cooperation between STAT-1, IRF1 and NFκB seems to be important for induction of iNOS expression in the RINm5F β-cell line and macrophages. Nevertheless, the cooperation is not observed in primary β-cells despite the presence of IFNγ-driven increase of IL-1β-induced NO production (106, 109). Accordingly IFNγ also applies other mechanisms to enhance NO production. In particular, IFNγ up-regulates argininosuccinate synthetase and GTP-cyclohydroxylase I (45). The former produces L-arginine, a substrate for iNOS while the latter supplies the tetrahydrobiopterin cofactor required for NO production (45). Another example of a gene regulated by transcription factors activated by different cytokines is tristetraprolin. The expression of this zinc finger binding protein, accelerating decay of a number of mRNAs, including iNOS mRNA, depends upon both STAT-1 and p38 MAPK activation (110). It should be noted, that while both IL-1β and TNFα induce NFκB activation in β-cells (111), blocking NFκB activation reduces NO production and β-cell apoptosis induced by a combination of IL-1β and IFNγ (112). However, the fact that iNOS expression is less does not lead to the protection of pancreatic β-cells against TNFα and IFNγ -induced cell death (113).

The up-regulation of expression of receptors and associated molecules is another level of crosstalk between IL-1β-, TNFα-, and IFNγ-induced signalling

pathways. MyD88 can participate in IRF1-induced gene transcription (114, 115) whereas IFNγ induces up-regulation of MyD88 and IRAK1 expression (106). Moreover, IFN up-regulates also TNFR1 expression (45). Expression of another component of TNFα signalling machinery, TRAF2, is elevated by IL-1β in a NFκB dependent manner in primary pancreatic β-cells (66). IL-1β can also affect IFNγ signaling by elevating NO production, which in turn increases expression of IFNGR1 and IFNGR2 (116).

Although IFNγ alone does not induce JNK and caspase activation, it successfully potentiates TNFα-induced JNK activation in the MIN6N8 β-cell line (81). The potentiation can be driven by IFNγ-induced inhibition of MKP1, the enzyme involved in dephosphorylation of JNK and other MAPKs (117), thus leading to sustained JNK activation and thereby promoting caspase-9 activation (39, 118). Further, IFNγ can facilitate IL-1β- and TNFα-induced pancreatic β-cell death by inducing IRF1-dependent increase in caspase-1 and caspase-8 expression (37, 101, 102) and by down-regulating the expression of genes involved in β-cell defence, in particular against endoplasmic reticulum stress (48, 103).

In conclusion, integration at multiple levels within IFNγ, IL-1β, and TNFα signal-transduction pathways serves to synergistic destruction of pancreatic β-cells, however precise mechanisms of IFNγ-driven potentiation of IL-1β and TNFα effects on pancreatic β-cell death are not known up to now.

1.3 SUPPRESSOR OF CYTOKINE SIGNALING-1

The JAK/STAT pathway is regulated by several families of proteins such as protein tyrosine phosphatases, SOCS and protein inhibitors of activated STAT (119, 120). Among them SOCS-1 is indispensable for the negative regulation of IFNγ-induced signaling (121-124). The SOCS-1 prevents activation of the JAK/STAT pathway, associating with phosphorylated JAK and one of the phosphotyrosine residues on the IFNγ receptor. The consequence of this interaction is inhibition of JAK and prevention of downstream STAT-1 activation (125, 126). Besides this, SOCS-1 has been shown to accelerate the

ubiquitination and degradation of phosphorylated JAK (127). Moreover, SOCS-1 has a role in signal transduction pathways activated by other cytokines. In particular SOCS-1 participates in TNFα-induced p38 and JNK activation and inhibits TNFα-induced apoptosis suppressing JAK activation (128-131). Accordingly, in β-cells SOCS-1 deficiency leads to hypersensitivity to TNFα-induced NO production and TNFα-induced cell death (50). SOCS-1 even interferes with TLR signalling (132-134), a receptor family that the IL-1β receptor belongs to, and participates in ubiquitination and proteolysis of NFκB subunit p65 (135).

Expression of endogenous SOCS-1 in pancreatic islet cells, almost undetectable under basal conditions (136), is enhanced by IFNγ treatment and during an autoimmune inflammatory process (137). However, even the expression level triggered by the cytokine is insufficient to terminate IFNγ-induced signal transduction (44). In addition SOCS proteins are generally unstable (138). Nevertheless, IFNγ-induced signal transduction in the β-cells can be abrogated by overexpression of SOCS-1 (44) and it was recently demonstrated that β-cell expression of SOCS-1 protects NOD mice from developing diabetes (4). The effect is not associated with reduced insulitis or changes in IFNγ-induced class I MHC expression and rather relies on direct protection of β-cells from inflammatory cytokines secreted by infiltrating immune cells correlating with decreased expression of CXCL10 (4, 97, 139).

1.4 MITOGEN-ACTIVATED PROTEIN KINASES

MAPK signal transduction pathways activated by a wide variety of stimuli transforms signals into phosphorylation events and thereby coordinate diverse cellular responses such as cellular metabolism, differentiation, migration, proliferation and survival (140, 141). The specificity of cellular reaction depends upon the kinetics of MAPKs activation, its subcellular localization, the scaffolds they interact with and the availability of substrates (141). The MAPK family consists of three major groups: ERK, JNK, and p38 MAPK (141).

1.4.1 ERK

In most cell types ERK is attributed to cell growth, proliferation and survival, however, its activation is also contributed to apoptosis. The decision on cell fate depends upon cell type and a combination of strength and duration of ERK activation, persistent low level of activation or transient strong burst of activity followed by a lower and sustained activation is required for proliferation, while sustained strong activation corresponds to senescence, apoptosis and differentiation (142, 143). Activated ERK1/2 phosphorylates more than 150 substrates including transcription factors, cytoskeletal proteins and many other types of proteins (105, 144-146). ERK-induced signalling is schematically represented in Fig. 2.

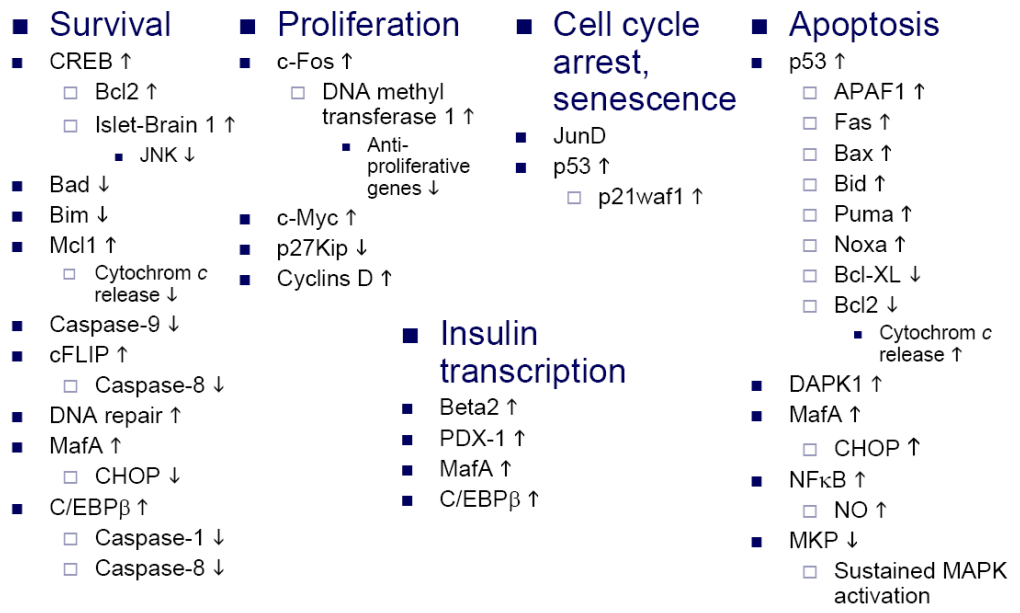

Figure 2. ERK-dependent targets grouped according to participation in different cellular processes. The consequent changes in activation or expression of other proteins or processes are shown in a smaller font. Arrows indicate ERK-dependent activation or inhibition of the targets (18, 142, 144-160).

Alternative consequences of ERK1/2 activation have also been shown in pancreatic β-cells. For example, PKA-dependent activation of ERK1/2 promotes

β-cell survival and protects against IFNγ and TNFα-induced apoptosis (161-163). Moreover, ERK1/2 is important for insulin gene transcription in response to elevated glucose and depolarization of β-cells (155). Transcription factors Beta2, PDX-1, and MafA responsible for insulin gene expression need to be phosphorylated and activated by ERK1/2 (155). However, ERK1/2 activation in response to IL-1β and IFNγ promotes apoptosis in primary rat β-cells (164). The ERK1/2 participates in IL-1β-induced NO production (152), increases NFκB activity presumably by phosphorylation of its p65 subunit (18) and is able to activate transcription factor C/EBPβ (153), which reduces insulin expression (154, 155) and together with NFκB initiates Fas expression (156). On the other hand C/EBPβ phosphorylated by ERK1/2 or p38 is able to bind and inhibit caspases-1 and -8 (148). Furthermore, both induction and suppression of CHOP transcription are ERK1/2-dependent in β-cells (157), thus ERK1/2 has a potential to affect ER stress.

1.4.2 p38

The p38 MAPK is involved mainly in the inflammatory and stress responses, cell differentiation, in particular in differentiation of pancreatic β-cells, growth inhibition, and apoptosis (165-170). In β-cells p38 participates in regulation of insulin expression by mediating phosphorylation of transcription factors C/EBPβ, USF1, CDX3 and E47 (148, 166, 171-175). Targets affected directly or indirectly by p38, which may be relevant for cytokine-induced β-cell dysfunction and destruction, are presented in Fig. 3.

Due to the important role which p38 plays in such biological processes as differentiation, cell cycle arrest and apoptosis induction, the kinase is discussed as a tumour suppressor. However, in tumour cells, granulocytes and excitable cells, including pancreatic β-cells, p38 contributes to DNA repair, cell cycle checkpoint and survival (168, 204-206). The discrepancy could rely on activation of different p38 isoforms (166) and prolongation of p38 activation, short-term p38 activation promotes β-cell survival, while long-term p38 activation leads to caspase activation in insulin-secreting cells (206). In line with

this IL-1β induces p38-dependent phosphorylation of CREB in insulin-secreting cells (192). Overexpression of the transcription factor in insulin-producing cells protects against cytokine-induced apoptosis by up-regulating expression of Islet-Brain 1 protein with subsequent inhibition of JNK and by promoting expression of anti-apoptotic Bcl2 protein (193, 194). However, phosphorylation of CREB as well as Bcl2 expression are decreased after long-term incubation of insulin-producing cells with cytokines (193).

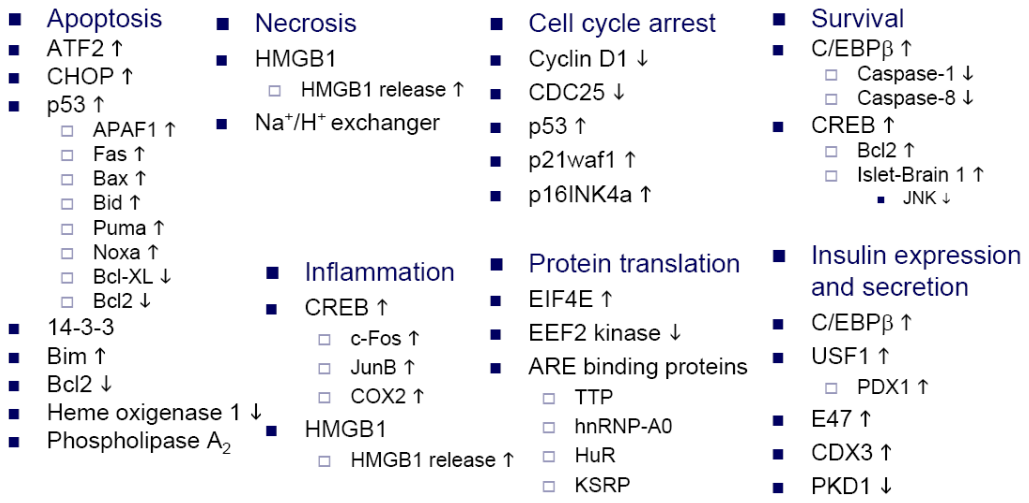

Figure 3. Proteins affected in a p38-dependent manner which are involved in cell survival, inflammation and insulin secretion. The consequent changes in activation or expression of other proteins or processes are shown in a smaller font with white squares. Arrows indicate p38-dependent activation or inhibition of the targets (105, 143, 148, 150, 152, 165-169, 171-203).

The p38 regulates gene expression by multiple mechanisms, including transcription, nuclear export, mRNA stability and translation (105, 166). In particular p38 increases stability of some mRNAs encoding inflammatory proteins, including iNOS, which otherwise are rapidly turned over (105, 143, 148, 166, 201). Accordingly, p38 activation in β-cells is necessary for IL-1β-induced NO production (152). The MAPK also regulates expression of COX2 supplying eicosanoids at sites of inflammation, including in the islets of Langerhans (105, 190, 195, 207). Moreover, p38 also regulate release of HMGB1 (168, 178, 185-187). HMGB1 can be released in the course of necrosis

from pancreatic β-cells in response to pro-inflammatory cytokines and participates in the development of insulitis and diabetes (183, 184). By phosphorylating CHOP and phospholipase A_2, p38 promotes ER stress, and p38-dependent up-regulation of CHOP participates in ER stress in insulin-producing cells (166, 203). Due to an important role in regulation of biosynthesis or release of pro-inflammatory cytokines and other inflammatory mediators, such as COX2, iNOS and HMGB1, p38 is a drug target for inflammation-associated diseases such as diabetes (105, 166, 207, 208).

1.4.3 JNK

The JNK pathway is an important regulator of cell migration, proliferation, survival, DNA repair, apoptosis and metabolism (143, 209). The consequence of JNK activation depends upon the scaffold the kinase interacts with and duration of activation: transient activation of JNK induces cell proliferation, whereas a sustained JNK activation (more than 1 h) mediates cell death (210). The pro-inflammatory cytokines IL-1β and TNFα indeed induce the prolonged activation of JNK in pancreatic islets (211). It was shown recently, that sustained activation of JNK, resulting from ROS-induced inactivation of MAPK phosphatases, contributes to β-cell death (212). In line with this, inhibition of JNK leads to PKB/AKT activation and protects human pancreatic islets against cytokine-induced death (213). Interestingly, activation of JNK was also implicated in generation of ROS in response to TNFα (214). The activated JNK is mainly retained in the cytoplasm in insulin-secreting cells (215).

JNK-induced signalling possibly involved in cytokine-induced β-cell dysfunction and destruction, are presented in Fig. 4. The data on participation of JNK in insulin secretion are intricate, at the one hand, JNK activation indirectly decreasing FOXO1 phosphorylation inhibits PDX-1 activity and therefore insulin expression (230-232), at the other hand, by inhibiting the glucocorticoid receptor, JNK may abrogate its negative effects on insulin release (218, 233, 234). Importantly, inhibition of JNK does not prevent cytokine-induced reduction of insulin release (235).

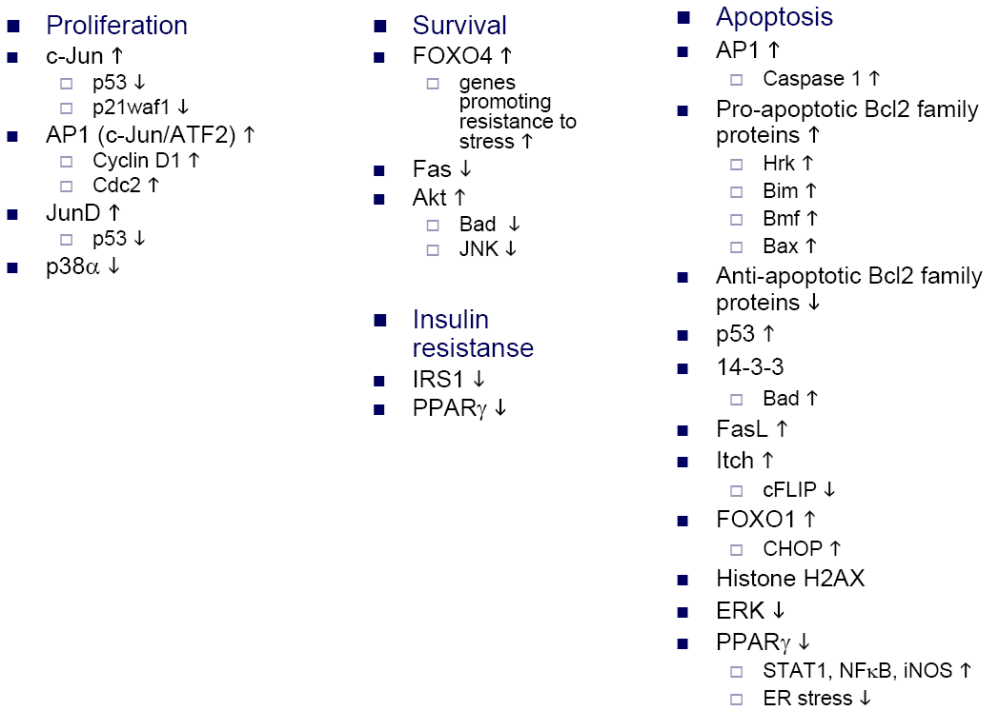

Figure 4. JNK-dependent targets participating in proliferation, survival, apoptosis and insulin resistance. The consequent changes in activation or expression of other proteins or processes are shown in a smaller font with white squares. Arrows indicate JNK-dependent activation or inhibition of the targets (39, 118, 143, 144, 147, 169, 209, 216-229).

Regulation of cell death by JNK is rather complex. Upon phosphorylation JNK activates components of the transcription factor AP1 which regulates inflammation, cell proliferation, death, survival and differentiation (144, 147, 216-218). AP1-mediated transcriptional activation was shown in cytokine-induced apoptosis of human pancreatic islets and in a β-cell line (220). In particular, JNK-dependent activation of AP1 is implicated in caspase-1 expression in human islets (221). Moreover, suppression of c-Jun, a component of AP1, results in inhibition of both caspase-3 activation and apoptosis in insulin-secreting cells (236). However, in insulinoma cells the pro-apoptotic effect of JNK might be independent on its effects on gene expression as the inhibition of JNK diminishes IL-1β-induced insulinoma cell apoptosis without affecting the transcription of major pro- and anti-apoptotic genes (235).

JNK activation can also promote cell death by phosphorylation and inhibition of anti-apoptotic Bcl2 family proteins, phosphorylation and activation of pro-apoptotic Bcl2 family protein Bax and BH3-only proteins Bim and Bmf as well as by induction of Bid cleavage (118, 216-219, 222). In addition JNK-dependent phosphorylation of the 14-3-3 protein releases Bad and allows mitochondrial translocation of pro-apoptotic Bcl2 family protein Bax (214, 218, 223). All together this leads to release of cytochrome *c* and Smac/Diablo from mitochondria with subsequent caspase-9 activation and cell apoptosis. JNK-induced phosphorylation of histone H2AX is required for caspase-driven apoptotic DNA fragmentation (217). JNK-dependent FOXO1 activation in insulin-secreting cells leads to induction of CHOP expression, ER stress and apoptosis (225). In turn, ER stress is shown to activate the JNK signalling (237, 238). In this context further potentiation of Ca^{2+} release from ER in the presence of Sarco(endo)plasmic reticulum Ca^{2+} ATPase inhibitor thapsigargin activates JNK and stimulates IL-1β activation of JNK in insulin-producing cells (62, 239). The ubiquitin ligase Itch, activated by JNK, promotes cFLIP degradation and thereby facilitating caspase-8 activation (39). Further, activation of JNK can elevate expression of Fas ligand in a c-Jun-dependent manner (222). Hence JNK activation can stimulate both intrinsic, mitochondria-dependent apoptosis and extrinsic, caspase-8-dependent apoptosis. Finally, the JNK/c-Jun pathway inhibits activation of p38 and ERK1/2, in particular in insulin-secreting cells (143, 240). Moreover, it was suggested that both activation of JNK and inhibition of ERK are required for the induction of apoptosis (241). Accordingly, inhibition of ERK augments IL-1β-induced death of insulin-producing cells (17).

The JNKs are encoded by three distinct genes, JNK1, JNK2 and JNK3 (105). Of them, JNK1 and 2 are ubiquitously expressed, while JNK3 is more restricted to the brain, heart and testis (143, 209, 223). All three isoforms are expressed in human islets, although JNK2 and JNK3 are the predominant. JNK3 seems to be cytoptrotective, while JNK1 and JNK2 are pro-apoptotic in insulin-producing cells (242). The difference relies on diverse effects on PKB/AKT, i.e. JNK3 activates it, whereas JNK1 and JNK2 inhibit it (243). JNK1 can control genes associated with suppression of apoptosis, for example A20 and PKB/AKT,

whereas JNK2 can regulate genes associated with tumour suppression, cell differentiation, apoptosis and growth arrest, for instance JunD and a cytotoxic proteinase granzyme C (217, 244). Both JNK1 and JNK2 are implicated in diabetes development, though by different pathways. JNK1 participates in insulin resistance in type 2 diabetes by phosphorylating and inhibiting IRS1 and PPARγ (218). JNK2, is important in the development of type 1 diabetes as deletion of the Jnk2 gene in NOD mice slows down the development of spontaneous diabetes pushing the balance of $CD4^+$ T cell differentiation towards a Th2 phenotype and increasing the resistance of β-cells to apoptosis *in vivo* (245). However, the absence of JNK2 does not protect β-cells against cytokine-induced apoptosis *in vitro*, most likely due to JNK1 activation (246). Nevertheless, total inhibition of JNK protects insulin-secreting cells against IL-1β-induced apoptosis (231, 235). The protection correlates with reduction in c-Jun phosphorylation (235). JNK1 is the major isoform responsible for c-Jun phosphorylation and stabilization (217). Correspondingly, JNK1-deficient islet cells are completely protected against cytokine-induced cell death (246). However, transplantation of neither JNK1-deficient nor JNK2-deficient islets into wild type animals with streptozotocin-induced diabetes protects them against diabetes, while transplantation of wild type islets into JNK1-deficient diabetic animals partially reverses diabetes and transplantation of wild type islets into JNK2-deficient diabetic animals does not (246). The functional and survival benefit of JNK1-deficient islets over JNK2-deficient islets was paralleled by decreased TNFα production by JNK1-deficient macrophages and elevated TNFα production by JNK2-deficient macrophages when co-cultured with wild type islets. This points out that JNK1 activation in macrophages that are in contact with islets is needed for diabetes development (246). Overall, activation of JNK regulates cell fate in a signal-specific and cell type-dependent manner (216).

1.5 NO

NO is a product of the conversion of L-arginine to L-citrulline by NOS (247). iNOS is expressed in response to inflammatory stimuli including cytokines

resulting in the continuous production of large amounts of NO (248, 249). Biological action of NO is diverse including function as a neurotransmitter, regulation of blood vessel dilatation as well as immune response. The mechanisms of NO action are attributed to RNS derived from NO (250-252). Different ROS and RNS have distinct chemical and biological properties and taken together are important regulators of apoptosis (253).

At low physiological concentrations (0.1 – 100 nM), NO regulates mitochondrial respiration and activates soluble guanylyl cyclase, thereby generally stimulates cell proliferation and inhibits apoptosis (249, 253, 254). Higher concentrations of NO block proliferation and in the absence of antioxidants induce apoptosis by oxidative/nitrosative stress or necrosis via energy depletion (252, 255, 256). Medium level of NO inhibits mitochondrial respiration with concomitant reduction in oxygen consumption, increase in cellular oxygen level and in superoxide (O_2^-) production (253, 257). The latter dismutates to hydrogen peroxide by MnSOD or reacts with NO to form peroxynitrite, depending on MnSOD and NO concentrations (253). NO or RNS can also inhibit decomposition of H_2O_2 further increasing its level (257). The pathways result in oxidative/nitrosative stress (252, 257). H_2O_2 produced in response to moderate NO level synergizes with NO to induce cell death and, as well as NO itself, participates in glutathione depletion, JNK and p38 MAPK induction and modulation of ERK1/2 activation (252, 253). In NOD islets glutathione depletion coincides with cytokine-induced high H_2O_2 production and reduction of cell viability (258). MnSOD overexpression partly inhibits cytokine-induced insulin-secreting cell death (259), suggesting importance of peroxynitrite for β-cell destruction. High concentration of NO (> 500 nM) facilitates formation of peroxynitrite and other NO derivatives (NO_2, N_2O_3) formed in the presence of high oxygen, elevated in response to inhibition of mitochondrial respiration (252, 257). Peroxynitrite is indeed produced due to increased generation of superoxide in cytokine-treated pancreatic islets and participates in destruction of human β-cells (260). The low level of antioxidant activity in pancreatic β-cells makes them susceptible for ROS-induced damage (241). Scavenging of NO and peroxynitrite production inhibits diabetes development in NOD mice (261).

Peroxynitrite irreversibly inhibits mitochondrial respiration, catalase and MnSOD leading to reduction in oxygen consumption, further increase in superoxide production and potentially to energy depletion (252, 257, 262). Beside this the RNS formed can cause DNA damage and lipid peroxidation (257). Treatment of β-cells with pro-inflammatory cytokines indeed induces disruption of mitochondrial membrane potential in a NO-dependent manner and lipid peroxidation with formation of the mutagenic and toxic products malondialdehyde and 4-hydroxynonenal (263-265). Stimulation of mitochondrial metabolism, inhibited by cytokines, or suppression of lipid peroxidation protects insulin-producing cells against cytokine-induced damage (265, 266).

Another deleterious consequence of NO is Ca^{2+} accumulation in mitochondria as a consequence of ER Ca^{2+} release (257, 267). This together with RNS-induced lipid degradation, inhibition of mitochondrial respiration and subsequent decrease in membrane potential favour increase in mitochondrial membrane permeability to small (up to 1.5 kDa) molecules as a result of opening of MPTP, a large multimeric complex spanning the outer and inner mitochondrial membranes (257, 267). This leads to cytochrome *c* release, caspase-9 activation and apoptosis (256, 257). Cytochrome *c* release further inhibits oxidative phosphorylation decreasing ATP production and increasing ROS formation (267, 268). The MPTP opening, possibly as a result of Ca^{2+} accumulation in mitochondria, cytochrome *c* release and caspase-9 activation are evident in cytokine-treated human pancreatic islets and insulin-secreting cells (30, 269).

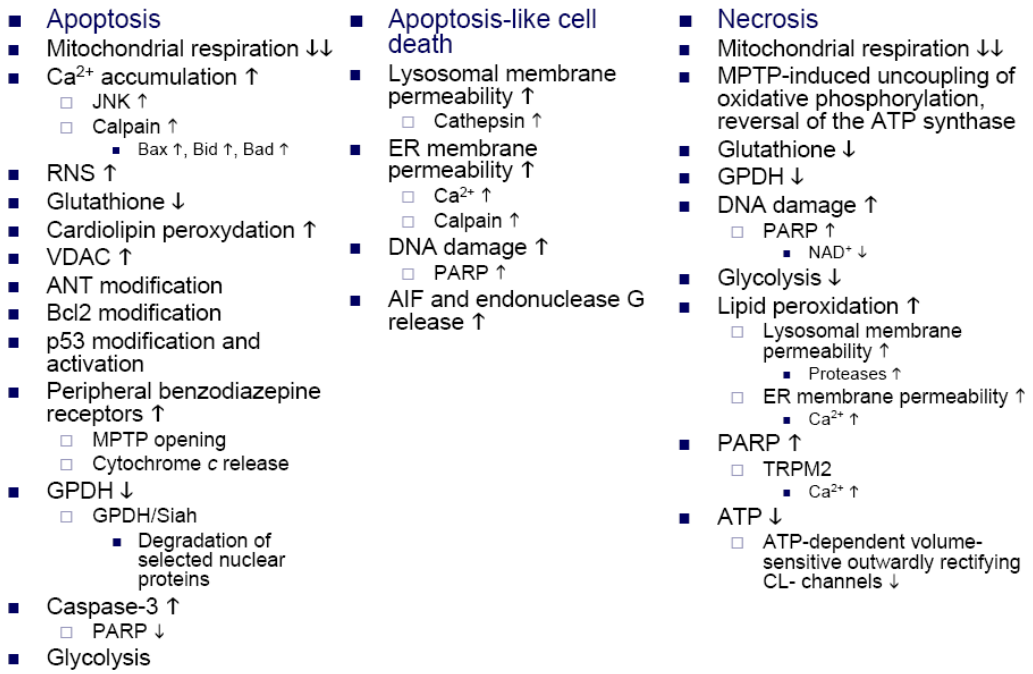

Figure 5. The effects of high NO concentration leading to different types of cell death. The consequent changes in activation or expression of other proteins or processes are shown in a smaller font. Arrows indicate NO-dependent activation or inhibition of the targets (15, 23, 30, 62, 89, 92, 226, 239, 241, 249, 250, 252, 253, 256-258, 260, 262-265, 267-291).

High NO concentration can evoke different modes of cell death (Fig. 5). A small decrease in ATP levels produced by NO interaction with mitochondria can be opposed by NO-dependently up-regulated glycolysis (273). This results in apoptosis, which is an energy consuming process (273). A large decrease in ATP along with insufficient glycolysis, resulting from RNS-induced glutathione depletion, inhibition of GPDH and depletion of adenine nucleotides and NAD^+, leads to extensive cellular damage and necrosis (252, 257, 271, 273). Pro-inflammatory cytokines induce a substantial drop in ATP content in insulinoma cells and trigger necrosis in pancreatic β-cells in NO-dependent manner (23, 274). Treatment of islets with cytokines for up to 24 hours induces repairable DNA damage (285), while prolonged incubation with cytokines triggers caspase activation, PARP cleavage and unrepairable DNA damage in β-cells (15, 285). Inhibition of caspase-3 elevates PARP-dependent necrosis in pancreatic islets

(286). Despite the fact that inhibition of PARP suppresses cytokine-induced insulinoma cell death, it does not protect rat islets against cytokine-induced apoptosis *in vitro* and against diabetes development *in vivo* (292-295).

Cytokine-induced NO can produce the depletion of ER Ca^{2+} and ER stress in pancreatic β-cells (239, 296-299). The depletion of ER Ca^{2+} may result from suppression of Ca^{2+} uptake from cytosol through SERCA inhibition by tyrosine nitration and from stimulation of Ca^{2+} release to cytosol through ryanodine receptor S-nitrosylation and activation (250). In addition, pro-inflammatory cytokines decrease SERCA2b expression in pancreatic β-cells (239). The increased concentration of cytosolic Ca^{2+} results in activation of calcium-dependent protein kinases and other enzymes (268). For example, NO- and ROS-induced elevated intracellular Ca^{2+} concentration contributes to JNK activation in insulin-secreting cells (62, 276-278). High Ca^{2+} concentration in ER is necessary for folding and disulfide bond formation of newly synthesized proteins, therefore, depletion of Ca^{2+} from ER disturbs ER function (250, 300). Furthermore, the function of ER depends on intracellular redox states and ER has a major role in the process of disulfide bond formation (250). NO- and ROS-dependent thiol oxidation decreases intracellular reducing capacity, while RNS-induced inhibitory S-nitrosylation of protein disulfide isomerise hinders disulphide bond formation in the proteins leading to accumulation of misfolded proteins in the ER (268). Accordingly, oxidative stress observed in pancreatic β-cells in response to pro-inflammatory cytokines can induce and exacerbate ER stress (250, 301).

In general the cytotoxic effects of NO excess differ depending on cell type and cellular ability to scavenge or to detoxify NO, in particular, on intracellular redox balance (255). The effects can include: 1) DNA damage, resulted from mutation of genes, DNA strand breaks and inhibition of DNA repair enzymes; 2) p53 activation due to its modification with the consequence of inhibition of p53 proteasome degradation and also in response to the DNA damage; 3) PARP activation as well as a consequence of DNA damage followed by NAD^+ and ATP depletion and subsequently necrosis; 4) inhibition of mitochondrial

functions; 5) increase in mitochondrial permeability; 6) activation of the ER stress pathway via disturbance of Ca^{2+} homeostasis; 7) MAPK stimulation possibly due to guanylyl cyclase/PKG/MEKK1 cascade activation, ER stress and/or elevated ROS production and concomitant inhibition of protein tyrosine phosphatases; 8) alteration in protein function through nitrosylation of SH group, amino acid nitration, binding with metals in heme and sulfide clusters; and 9) lipid peroxydation (241, 249, 250, 253, 291).

1.6 CASPASES

Caspases are a family of cysteine proteases that are constitutively expressed as inactive zymogens (procaspases) in resting cells and play an essential role in the regulation of immunity and apoptosis (302, 303). Procaspases become activated by proteolytic processing upon receiving of specific signals and cleave subsequently target proteins at defined aspartate residues (302, 304). The family is subdivided into two sub-families, namely inflammatory caspases and apoptotic caspases (302). The inflammatory caspases participate in maturation of pro-inflammatory cytokines and inflammatory responses, however, the activation of these caspases can also induce apoptosis (302, 305). Active apoptotic caspases cleave other procaspases and different cellular proteins in an amplification cascade resulting in cell death (271). The apoptotic caspases are further classified into initiator and effector caspases (302). Caspases-2, -8, -9 and -10 are initiator caspases (302, 304). Different pathways lead to apoptosis-associated caspase activation, and all of them culminate on recruitment of an initiator caspase to an activation scaffold, resulting in dimerization of the initiator caspase followed by proteolytic autoactivation of the enzyme (302, 305). Initiator caspases then activate the downstream effector caspases-3, -6, and -7 that in turn cleave hundreds of target proteins including inhibitor of CAD leading to CAD activation and inter-nucleosomal DNA fragmentation (302, 304).

In the extrinsic pathway, binding of ligands like TNFα, TRAIL and Fas to corresponding membrane receptors of TNF-receptor family can induce recruitment of death adaptor FADD (302, 303). The TNF-receptor recruits FADD in case of translocation of TRADD and RIP to the cytosol (Fig. 1) (39,

76, 83). FADD further interacts with procaspase-8, procaspase-10 and/or cFLIP (302). Recruitment of cFLIP to FADD blocks caspase-8 activation in this pro-apoptotic complex (39). The complex transduces the apoptotic signals, activating caspase-8 only in the case when NFκB is unable to promote up-regulation of the anti-apoptotic cFLIP or in the case when an ubiquitin ligase Itch activated by JNK promotes cFLIP degradation (39). Even inhibitor of apoptosis proteins can associate with the TNFR1 signalling complex through binding to TRAF2 and promote NFκB activation. In the same time caspase-8 activation is inhibited, thus making TNFR-induced caspase-8 activation insufficient for apoptotic induction in so called type II cells such as hepatocytes and β-cells (36, 86, 306, 307). Nevertheless, in the presence of IFNγ the pathway of caspase-8 activation is active in β-cell lines in response to stimulation with TNFα and can be inhibited by overexpression of cFLIP, leading to the reduction in caspase-8 activation and decrease in cell death without affecting β-cell NO production (28, 29, 308). In case when cFLIP is degraded or not up-regulated, the resulting assembly of procaspases-8 and -10 in close proximity to each other with favourable mutual orientation leads to their autoproteolytic activation and release to cytosol (302). Caspase-10 has similar functions to caspase-8, in particular it can cleave procaspase-3 as well as Bid with consequent activation of the intrinsic program (302, 309). However caspase-10 is absent in mouse (302, 309). Under oxidative stress conditions cleavage and activation of caspase-8 can also be fulfilled by the lysosomal proteins cathepsin L and D in the absence of extracellular receptor ligation (310). The activated caspase-8 is able to cleave procaspases-9, -10 and all effector ones, PARP, Bid, cFLIP and other proteins (302). The apoptotic signal in type II cells including β-cells, where amounts of activated caspase-8 are insufficient to generate adequate quantities of active caspase-3, the caspase-8-driven cleavage of Bid is nonetheless sufficient to activate the intrinsic pathway. Truncated Bid (tBid) translocates to the mitochondrial membrane where it promotes pro-apoptotic members of Bcl2 family to induce mitochondrial outer membrane permeabilization and release of cytochrome c with concomitant caspase-9 activation (36, 85-87, 304). Indeed, the mitochondrial migration of Bax, a pro-apoptotic member of the Bcl2 family,

in response to TNFα was shown in insulin-secreting cells (88). However, the loss of Bid only partly protects islet cells from apoptosis induced by IFNγ and TNFα (36). The absence of caspase-8 can protect against streptozotocin-induced diabetes, an *in vivo* model of type 1 diabetes (311). At the same time the long term deletion of caspase-8 leads to reduced CREB and PDX-1 expression, decreased CREB and PKB/AKT phosphorylation, and elevated caspase-3 activation and apoptosis, while cFLIP up-regulation elevates NFκB activity and PDX-1 expression (311, 312).

In the intrinsic program, activation of caspase-9 is triggered by release of cytochrome *c* into cytosol by damaged mitochondria (288, 303). APAF1, which exists as a monomer in the cytoplasm of unstimulated cells, oligomerizes in the presence of cytochrome *c* and dATP or ATP to form an apoptosome. The apoptosome contains seven APAF1 molecules as central scaffold proteins, each bound to one cytochrome *c* molecule and one caspase-9 dimer (305). The caspase-9 then become activated and in turn arouses activation of effector caspases-3 and -7 as well as PARP cleavage (302, 305). Caspase-9 is activated in cytokine-treated pancreatic islets underlining the importance of the mitochondrial pathway in β-cell death induced by pro-inflammatory cytokines (30, 313). However, the importance of the mitochondrial pathway and caspase-9 activation for cytokine-induced apoptosis in β-cells may not be decisive. For example, prevention of mitochondrial damage and cytochrome *c* release with help of increase in expression of anti-apoptotic Bcl2 family proteins or with help of inactivation of pro-apoptotic proteins Bax or Bak only confers partial protection against cytokine-induced β-cell death (36, 87, 263, 313-317). Moreover, despite inactivation of Bcl2 or Bcl-XL aggravates cytokine-induced primary β-cell death (318, 319), an overexpression of these anti-apoptotic proteins does not protect against autoimmune damage *in vivo* (320, 321). In line with this, the importance of NO, a well-known inducer of mitochondrial damage and cytochrome *c* release, for β-cell caspase activation and cell death is also controversial (322, 323). For instance, cytokine-induced apoptosis proceeds in

primary β-cells even in the absence of iNOS, and thus elevated NO production (23).

Caspase-1, or IL-1β-converting enzyme, is activated by a multiprotein complex named inflammasome (324). A sensor for cytosolic double-stranded DNA, AIM2, or members of NLR family structurally related to APAF1 are able to initiate inflammasome formation (324). NLR is a large family (23 members in humans and 34 members in mice) of cytosolic sensors for microbial molecules or endogenous products released from damaged or dying cells, such as nucleic acids, ATP, which is abundant locally in stressed tissue, and uric acid crystals (325). These endogenous danger-associated molecules released upon cell death are important in the aetiology of autoimmune diseases (325). In contrast to microbial products, many of the endogenous danger-associated molecules do not stimulate the corresponding receptor, NLRP3, directly, but rather activate it through elevation of ROS production and induction of lysosomal rupture (324-326). ROS-induced ERK1/2 activation as well as the release of lysosomal protease cathepsin B were suggested to be important for NLRP3 inflammasome activation (324). In fact, ROS production is elevated in cytokine-treated pancreatic β-cells and can entail the disruption of lysosomes (260, 279, 280). In insulin secreting cell lines, IL-1β-induced caspase-1 activation is preceded by induction of JNK1 and though this finding can reflect the fact that not only IRF1 but also JNK1 can up-regulate the expression of this caspase, this seems not to be the case, as the inhibition of JNK in these cells does not affect the expression of caspase-1 (221, 235, 236). The cytokine-induced IRF1-dependent up-regulation of caspase-1 expression does not depend upon NO production and therefore has been proposed to be involved in NO-independent β-cell apoptosis (102). However, NO can evoke activation of caspase-1 (255). On the other hand, NLRP3 expression is up-regulated by NFκB, a well-known inducer of NO production in β-cells (325). Consequently, cytokine-induced β-cell death driven by caspase-1 cannot be considered pure NO-independent. Upon stimulation, NLR or AIM2 oligomerizes and can subsequently recruit procaspase-1 (324). The formation of at least some inflammasomes can be regulated by Bcl2 family proteins (324, 327). The inflammasome then induces autocatalytic activation of

caspase-1 (324). Some NLR family proteins can also activate NFκB with the help of other adaptor molecules (324, 328). The mature caspase-1 cleaves and activates the pro-inflammatory cytokines IL-1β and IL-18 and inactivates IL-33 (324). Caspase-1 is also able to induce inflammatory cell death called pyroptosis with the features of both apoptosis and necrosis (324). Pyroptosis involves caspase-1-dependent DNA fragmentation and pore formation in the plasma membrane resulting in osmotic cellular lysis and release of pro-inflammatory cellular content (324, 325). The activated caspase-1 inhibits glycolysis by proteolysis of the key enzymes and cleaves procaspases-1, -3, -4 and -7, PARP and lamins (the proteins of nuclear lamina) (302, 324). Activation of caspase-1 has been shown to promote death of insulin-producing cells (236).

Activated effector caspases cleave other caspases, resulting in a positive feedback amplification loop (329). In particular, caspase-3 processes and activates caspases-2, -6 and -9, whereas caspase-6 in its turn activates caspases-8 and -10 (329). Furthermore, effector caspases cleave components of cytoskeleton, focal adhesion sites as well as cell-cell adherent junction, nuclear, ribosomal and Golgi proteins, proteins participating in DNA metabolism and repair, transcription factors, translation initiation factors, cell cycle and cell proliferative proteins, protein kinases and others (302, 304). The proteolysis results in rounding up of the apoptotic cell, its detachment from neighbouring cells, loss of contacts with the extracellular matrix, membrane blebbing, condensation and fragmentation of the nucleus, pronounced fragmentation of the Golgi, ER and mitochondrial networks, and the formation of membrane-bound apoptotic bodies (304).

On the whole the cytokine-induced β-cell death is executed by a lot of cooperating and possibly substituting pathways which are not fully understood. In particular, the exact mechanisms of NO-induced β-cell death are not known as well as the relative importance of cytokine-induced NO production in β-cell death, compared to the other pathways leading to β-cell destruction. For

example, inhibition of effector caspases in insulin-producing cell line results in abolishing of cytokine-induced cell death despite significant elevation of NO production (330). Signal transduction pathways initiated by MAPK in the course of cytokine-induced β-cell apoptosis are also incompletely understood. The comparative role of individual initiator caspases is almost not studied at all. Moreover, the relative impact of apoptosis and necrosis in cytokine-induced β-cell death needs further clarification, as well as the possibility of using the caspase inhibition approach for diabetes prevention and treatment. Despite caspase-3 activation and apoptosis induction was shown to be important for cytokine-induced β-cell destruction (19, 24-26), others argue that IL-1β-induced rat β-cell death is rather the result of necrosis than apoptosis (183). Furthermore, blockage of caspases can often lead to nonapoptotic cell death resulting from the relief of caspase-mediated inhibition of necrosis and autophagy (307, 331). The increase in necrosis in response to caspase inactivation was shown in particular in pancreatic β-cells (332) and can be especially important in the presence of high NO concentration. A lot of work has been performed with insulin-secreting cell lines to understand the mechanisms of cytokine-induced β-cell death. However, there are enough examples showing that the cytokine-induced signalling may be quite different in cell lines compared to primary β-cells. For instance, IFNγ-induced IRF1 expression is further elevated by IL-1β in the RINm5F β-cell line, however, not in rat and human islets (108). Similarly, transcription factors STAT-1, IRF1 and NFκB seem to cooperate in the augmentation of iNOS expression in RINm5F cells. Nevertheless, the cooperation is not observed in primary β-cells despite the presence of IFNγ-driven elevation of IL-1β-induced NO production (106, 109).

1.7 IMIDAZOLINE COMPOUNDS RX871024 AND EFAROXAN AND CYTOKINE-INDUCED PANCREATIC β-CELL DEATH

Imidazoline compounds represent a large group of chemical substances possessing an imidazoline moiety within their structure (Fig. 6). Some imidazoline compounds are known to promote insulin secretion and, therefore, have been discussed as potential therapeutic drugs in type 2 diabetes (333).

Among those compounds are classical insulinotropic imidazolines RX871024 and efaroxan (Fig. 6).

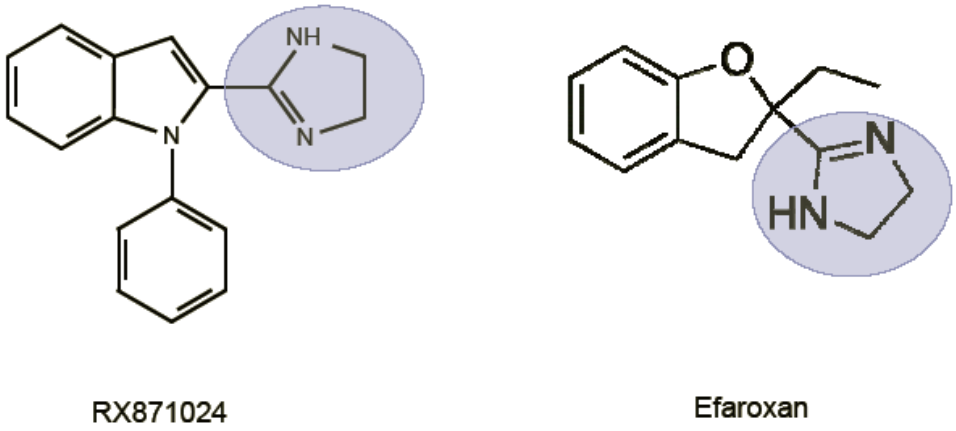

Figure 6. Chemical structures of imidazoline compounds RX871024 and efaroxan. The imidazoline group is in the blue circle.

Although the mechanisms of insulinotropic activity of RX871024 and efaroxan have been intensively studied, they are still not fully clarified. It has been discovered previously, in our group, that RX871024 stimulates insulin release in pancreatic β-cells by both blocking K_{ATP}-channels and by directly affecting the β-cell exocytotic machinery, the latter effect involving PKA and PKC (333-338). Similar type of insulinotropic activity has also been shown for efaroxan (339). In addition, we have demonstrated that RX871024 induces Ca^{2+} mobilization from the endoplasmic reticulum Ca^{2+} stores (340). In contrast efaroxan does not show such type of activity (341).

Concerning the effects of RX871024 and efaroxan on cytokine-induced pancreatic β-cell death, it has been demonstrated in our group, that both imidazolines do not induce apoptosis in mouse pancreatic β-cells but on the contrary even protect against IL-1β-induced primary β-cell apoptosis (19). The protective effect of RX871024 on IL-1β-induced β-cell apoptosis has been accompanied by inhibition of IL-1β-induced expression of iNOS and NO

production. An inhibitory effect of RX871024 on endogenous NO production has been reproduced in rat pancreatic islet β-cells by others (342). In addition, the protective effect of efaroxan on IL-1β-induced β-cell apoptosis has also been confirmed in rat pancreatic islet β-cells (343).

2 AIMS

The overall objective of this thesis was to investigate mechanisms of β-cell dysfunction and death induced by the mixture of pro-inflammatory cytokines IL-1β, IFNγ and TNFα, i.e., under conditions modelling those during inflammation in type 1 diabetes. Furthermore, we aimed to study whether insulinotropic imidazoline compounds RX871024 and efaroxan can affect pancreatic β-cell death under these conditions and if so, to explore underlying molecular mechanisms.

The specific aims were:

- To investigate whether the imidazoline compounds can affect primary pancreatic β-cell death in the presence of IL-1β, IFNγ and TNFα and explore the role of NO and MAPK under these conditions.
- To study the effects of the imidazoline compounds on death of the insulinoma cells, in the presence or absence of IL-1β, IFNγ and TNFα and to evaluate the role of NO, MAPK and caspase activation in the imidazoline effects.
- To investigate the effects of SOCS-1 overexpression on insulin release, cytokine-induced NO production, caspase activation and islet cell death in the presence of IL-1β, IFNγ and TNFα.

3 MATERIALS AND METHODS

3.1 MATERIALS

Materials used in the experiments reported in this work are described in detail in papers (I-III).

3.2 MOUSE MODELS

Obese (*ob/ob*) mice were obtained from a local colony (Karolinska Institutet).

C57BL/6 (here denoted B6) and SOCS-1-Tg mice on the B6 background (97, 344), originally obtained from The Scripps Research Institute (La Jolla, CA), were bred and maintained in a specific pathogen free environment at Karolinska Institutet. Heterozygote SOCS-1-Tg B6 mice were bred with non-transgenic B6 mice and the littermates were genotyped by PCR analysis of tail DNA (344).

3.3 ISOLATION OF PANCREATIC ISLETS AND ISLET CELLS

Islets of Langerhans from two to six months old B6 mice or SOCS-1-Tg B6 mice and ten to twelve months old *ob/ob* mice were isolated by collagenase digestion and hand-picked as previously described (345). The islets were cultured in RPMI-1640 medium supplemented with 11 mM glucose, 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (standard medium) with or without a mixture of IL-1β and TNFα (IL-1β, 25 U/ml; TNFα, 100 U/ml) or mixture of IL-1β, TNFα and IFNγ (IL-1β, 25 U/ml; IFNγ, 100 U/ml; TNFα, 100 U/ml), at 37^{o}C for 40 h.

The *ob/ob* islets containing 95 % β-cells were dispersed into small β-cell clusters in Ca^{2+}- and Mg^{2+}-deficient medium as previously described (346). The β-cell clusters were plated on glass coverslips and cultured for 40 h at 37^{o}C in 2 ml standard medium with or without the mixture of cytokines IL-1β, 25 U/ml; IFNγ, 100 U/ml and TNFα, 100 U/ml, hereafter denoted cytokine combination 1, or the cytokine combination 2 (380 U/ml IL-1β, 100 U/ml IFNγ and 100 U/ml TNFα), which differs from the cytokine combination 1 by higher concentration

of IL-1β, or with a mixture of one of these cytokine combinations with 50 μM of imidazoline compound.

In cell-survival experiments, for determination of NO production and for measurements of caspase activity, islets were disrupted into a suspension of single cells with dispase followed by centrifugation in BSA as previously described (347, 348). Cell preparations were plated in microtiter plates and cultured in standard medium with or without one of the cytokine combinations in the presence or absence of an imidazoline compound at 37°C for 40 h.

3.4 CELL CULTURE

The β-cell line MIN6 (passages 32 – 42) was cultured in DMEM containing 25 mM glucose supplemented with 10% (vol/vol) fetal calf serum (FCS), 50 μM β-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin. MIN6 cells were exposed to 50 μM of imidazolines with or without the cytokine combination 1 at 37°C for 20 h.

3.5 FUNCTIONAL ASSAYS

3.5.1 Measurements of insulin release

To investigate whether RX871024 and efaroxan are able to stimulate insulin release under culture conditions, *ob/ob* islets were incubated in standard medium supplemented with 3.3 or 11 mM glucose with or without cytokine combination 1 in the presence or absence of an imidazoline compound at 37°C for 1 h.

To study the effect of SOCS-1 overexpression on cytokine-induced inhibition of glucose-stimulated insulin secretion B6 islets and SOCS-1-Tg islets were cultured with or without cytokine combination 1 at 37°C for 40 h. After that the islets were pre-incubated in KRB containing 115 mM NaCl, 4.7 mM KCl, 2.6 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 20 mM $NaHCO_3$, 16 mM HEPES, 2 mg/ml BSA, pH 7.4 with 3.3 mM glucose at 37°C for 1 h. Groups of 3 islets were then incubated in 0.3 ml KRB supplemented with 3.3 mM or 16.7 mM glucose at 37°C for 1 h.

Following incubation, medium was removed and stored at -20^{o}C until insulin content was analyzed by radioimmunoassay using rat insulin as a standard (Novo Nordisk, Denmark).

3.5.2 Nitrite determination

β-Cell nitrite production was determined with Griess reaction. Culture medium was withdrawn and centrifuged for two minutes at 1,500 g and 100 μl samples of supernatant were transferred to a 96-well plate and mixed with 50 μl of Griess reagent, Alexis Corporation (Carlsbad, CA, USA). The reaction was carried out for 15 minutes at room temperature. Nitrite production was determined by 540 nm absorbance with reference at 620 nm in a 96-well plate reader. For calibration of data, a standard curve for $NaNO_2$ in culture medium was established in each assay. The results were expressed as μM of NO_2^- per μg of total protein.

3.5.3 In vitro kinase assay

MIN6 cells or isolated *ob/ob* islets were exposed to cytokine combination 1 in the presence or absence of imidazoline compounds for 1h and cell extracts were subjected to *in vitro* kinase assay using the substrates GST-Elk-1, GST-c-jun and Hsp25. It has been previously shown that GST-Elk-1 is a substrate for ERK, that GST-c-jun is a substrate for JNK, and that Hsp25 is a substrate for the p38-activated kinase MK2 in β-cells (62, 152). Phosphotransferase activities toward GST-c-jun, GST-Elk-1 and Hsp25 were measured by a whole cell lysate *in vitro* kinase assay essentially as described (152), except that 2 μg of GST-c-jun [1-79] was used instead of ATF2. Phosphorylated substrates were visualized by autoradiography and quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA, USA).

3.5.4 Immunoblotting

Isolated *ob/ob* islets were exposed to cytokine combination 1 in the presence or absence of imidazoline compounds for 1h and islet extracts were subjected to immunoblotting. Equal amounts of protein in whole cell lysates were mixed with

4× SDS sample buffer (Invitrogen), boiled for 10 min, and subjected to 10% SDS-PAGE. Proteins were electrotransferred to nitrocellulose filter membranes. Blocking of nonspecific protein binding was done by incubating the filter membrane in blocking buffer (1× TBS, pH 7.6, 0.1% Tween 20, and 5% non-fat dried milk) for 1 h. Following washing in TBST (1× TBS, pH 7.6 and 0.1% Tween 20), filter membranes were incubated with the respective antibodies diluted in either TBST containing 5% BSA or in blocking buffer. Filter membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies. Immune complexes were detected by chemiluminescence using LumiGLO (Cell Signaling), and light emission was captured digitally using FUJI LAS3000. For immunodetection of the three MAPKs, two nitrocellulose filter membranes were prepared identically in parallel. One membrane was probed with anti-Thr183/Tyr185-phosphorylated JNK1/2 and anti-Thr180/Tyr182-phosphorylated p38 antibodies, and the other membrane was probed with anti-Thr202/Tyr204-phosphorylated ERK1/2 and anti-actin antibodies.

3.6 ASSESSMENT OF CELL DEATH

3.6.1 Measurement of β-cell viability

Detection of β-cell viability was performed using CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega (USA), according to the manufacturer's instructions. The method is based on spectrophotometric detection of a colored formazan product converted from an MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] by NADPH or NADH in metabolically active cells. Subsequent to incubation with the test substances cells were washed three times with fresh culture medium and then incubated in fresh medium for 2 hours. Assays were performed by adding 20 µl of the CellTiter 96® AQueous One Solution Reagent to culture wells (100 µl culture medium/well), incubating for 1-4 h, and then recording absorbance at 490 nm with a 96-well plate reader.

3.6.2 Assessment of islet cell apoptosis

The TUNEL technique was used to detect DNA strand breaks in situ as previously described (346). After 40 h incubation with cytokines, in the presence or absence of imidazolines, B6 islets and SOCS-1-Tg islets or *ob/ob* β-cells were washed with PBS and fixed in 80 % methanol at 4^{o}C. After rinsing with PBS, cells or islets were covered with 0.1 ml of reaction mixture for the TUNEL enzymatic reaction and were incubated at 37^{o}C for 1 h. The reaction was stopped by adding a buffer containing 300 mM NaCl and 30 mM sodium citrate. B6 islets and SOCS-1-Tg islets or *ob/ob* β-cells were double-stained with FITC and PI, and fixed on glass slides with 80% glycerol in PBS. Fluorescence was monitored with Leica TCS-NT laser scanning confocal microscope (Leica Microsystems GmbH, Germany) or Carl Zeiss Laser Scanning System LSM 5 PASCAL (Carl Zeiss GmbH, Germany), with excitation from the 488-nm line of an argon/krypton laser. Fluorescence emission was detected with a band-pass filter (Chroma Technology, Rockingham, VT, USA) centered at 530 nm for FITC and above 590 nm for PI. Several confocal images were used for counting the number of apoptotic cells in each islet or slide (observation). Percentage of *ob/ob* β-cell apoptosis under control conditions in each experiment (7-14%) was taken as 100%.

3.6.3 Assessment of MIN6 cell death

Assessment of MIN6 cell death was performed using CytoTox 96® Non-Radioactive Cytotoxicity Assay, Promega (Madison, WI, USA), according to the manufacturer's specifications. The method is based on quantitative measurements of LDH upon cell lysis. Absorbance was recorded at 490 nm with a 96 well plate reader. Cell death was assessed as percentage of LDH released into the culture medium in relation to the total LDH content.

3.6.4 Caspase activity measurements

Measurements of caspase activity was performed using fluorometric Caspase-3 and Caspase-8 Activity Assays, Oncogene (Darmstadt, Germany), and Caspase-1 and Caspase-9 Fluorometric Assays, R&D Systems (Minneapolis, MN, USA),

according to the supplier's instructions. The assays are based on the cleavage of a caspase-specific substrate labelled with a fluorescent molecule, AFC. Reaction was monitored by a blue to green shift in fluorescence upon cleavage of the AFC fluorophore. In brief, 50 μl of cell lysate was mixed with 50 μl of reaction buffer and caspase fluorogenic substrate. After incubation at 37^{o}C for 2 h, readings were performed using a fluorescence plate reader, with excitation and emission wavelengths of 400 nm and 505 nm, respectively, and a longpass filter of 430 nm. Results are expressed as a percentage of control.

3.7 PRESENTATION OF RESULTS

Data analysis was performed using Sigma Plot 2001 for Windows (Jandel Corp., USA) or Statistica for Windows (v. 5.0, StatSoft, Inc., USA). The statistical significance between means was estimated by Student's t-test or with analysis of variance test (ANOVA).

4 RESULTS AND DISCUSSION

4.1 THE EFFECTS OF IMIDAZOLINE COMPOUNDS ON PRIMARY PANCREATIC β-CELLS EXPOSED TO IL-1β, IFNγ AND TNFα (PAPER I)

4.1.1 Insulinotropic imidazoline compounds do not affect pancreatic β-cell death induced by a combination of pro-inflammatory cytokines

Previous studies from our group have demonstrated that imidazoline compounds RX871024 and efaroxan protect against IL-1β-induced apoptosis of pancreatic β-cells (19). Therefore, we investigated whether these imidazoline compounds can protect against pancreatic β-cell death, under conditions modelling those during inflammation in type 1 diabetes; i.e., in the presence of a combination of pro-inflammatory cytokines containing IL-1β, IFNγ and TNFα. To this end, β-cells isolated by the same procedure and from the same type of mice (ten to twelve months old ob/ob mice) as in the previous study (19) were incubated in standard medium for 40 h with or without the cytokine combination 1 in the presence or absence of RX871024 or efaroxan. We found that there were no significant effects of any of the imidazoline compounds on the reduced viability or apoptosis induced by the cytokine mixture.

Most likely the absence of effects of RX871024 or efaroxan on β-cell survival could be explained by the fact that cytokine mixture affect signal-transduction pathways proximal to the interaction site for the imidazolines and by this way hinder imidazolines to exert cytoprotective action. In order to give imidazolines possibility to trigger signal-transduction before cytokines start to produce cytotoxic effects, β-cells were pre-incubated in standard medium with the imidazolines for 30 min before exposure to the cytokine combination 1. Before performing these experiments, the ability of RX871024 and efaroxan to stimulate insulin release in the very same medium that was used for investigation of β-cell death, i.e. complete culture medium RPMI 1640 containing not only 11 mM glucose but amino acids and 10% FCS, was studied. Despite the absence of

effects on β-cell viability, both tested imidazolines potently stimulated insulin secretion meaning that the imidazolines successfully triggered at least signal-transduction towards elevation of glucose-stimulated insulin release. RX871024 also stimulated insulin release from *ob/ob* mouse islets during 1 hour incubation in the presence of cytokine combination 1. Thus RX871024-induced signal-transduction leading to increased insulin release is not blocked by the cytokines. However, both RX871024 and efaroxan neither affected cytokine-induced β-cell apoptosis nor β-cell viability under these conditions. The study described above was carried out on freshly isolated islets. Under conditions when islets were cultured for 24 h before the experiments, to recover after the isolation procedure, and then pre-incubated with the imidazolines, there was still no significant effect of RX871024 or efaroxan on β-cell apoptosis induced by cytokine combination 1. In the previous investigation (19), the inhibition of IL-1β-induced pancreatic β-cell apoptosis by these imidazoline compounds was observed at a cytokine concentration of 380 U/ml. Taking this into account, in another set of experiments β-cell death was induced with the cytokine combination 2 containing 380 U/ml IL-1β, 100 U/ml IFNγ and 100 U/ml TNFα. However, independent of the duration of β-cell death induction, or the presence of pre-incubation with RX871024 or efaroxan before the addition of the cytokine combination, there was no protective effect of the imidazolines on cytokine-induced β-cell death.

Taken together, these results indicate that imidazoline compounds cannot protect pancreatic β-cells against death induced by a combination of pro-inflammatory cytokines IL-1β, IFNγ and TNFα, conditions modelling those that take place in type 1 diabetes.

4.1.2 RX871024 but not efaroxan reduces NO formation and p38 activation in the pancreatic β-cells induced by cytokines

In the previous study (19) RX871024 protected from IL-1β-induced β-cell apoptosis through inhibition of NO synthesis triggered by the cytokine. To investigate whether imidazolines affect NO formation induced by a mixture of

IL-1β, IFNγ and TNFα, *ob/ob* islets were incubated in standard medium for 40 h in the presence or absence of cytokine combination 1, with or without addition of imidazoline compounds RX871024 or efaroxan. In agreement with previous findings RX871024, but not efaroxan, caused a 45% reduction in NO formation in the presence of the pro-inflammatory cytokines.

We next evaluated the effect of imidazolines on NO production under the same conditions as apoptotic measurements were done. To this end *ob/ob* mouse β-cells were incubated in standard medium for 40 h in the presence or absence of cytokine combination 1 or 2 with or without addition of RX871024 or efaroxan. We found that either cytokine combination induced production of the same amount of NO in *ob/ob* mouse β-cells. In line with our findings that RX871024 inhibits NO production triggered by both 380 U/ml IL-1β and cytokine combination 1 in islets, the imidazoline decreased also NO formation stimulated by cytokine combination 2, while efaroxan had no effect. Hence, RX871024 inhibited NO production in *ob/ob* mouse islets and dispersed *ob/ob* mouse β-cells also in the presence of all three pro-inflammatory cytokines. However, this RX871024-dependent decrease in NO production was not correlated with any effect of the imidazoline compound on β-cell death induced by cytokines. Our results lead to the conclusion that mechanism of cytokine-induced pancreatic β-cell death does not directly depend on NO formation. A cytokine mixture containing IL-1β, IFNγ and TNFα evokes *ob/ob* mouse β-cell death which may be less dependent on NO, as compared to the conditions when IL-1β is used alone. A possible explanation is activation of an extrinsic apoptotic pathway by a combination of TNFα and IFNγ leading to caspase 8 activation. The apoptotic signal is then amplified through the mitochondrial apoptotic pathway and thereby caspase 9 activation. Another probable mechanism is an IFNγ driven potentiation of TNFα-induced JNK activation in β-cells (81), which can then facilitate mitochondrial membrane permeabilization and caspase 9 activation. Cytokine-induced apoptosis of human pancreatic β-cells was also shown to be NO-independent (22). In this way *ob/ob* mouse β-cell death induced by a

combination of IL-1β, IFNγ and TNFα resembles human pancreatic β-cells death.

Taking into consideration that MAPKs are important mediators of cytokine effects on iNOS expression and induction of apoptosis in β-cells (17, 152, 192, 349, 350), we examined if RX871024 affects cytokine-induced signal transduction at the level of MAPKs. Cytokine combination 1 induced a marked activation of JNK1/2 and p38 MAPK, however, not ERK1/2 in *ob/ob* mouse islets, as assessed by both *in vitro* kinase assay using MAPK substrates and immunoblotting of phosphorylated activated MAPK. RX871024 neither alone nor in combination with cytokines influenced ERK1/2 as well as JNK1/2 activation. This is in agreement with the absence of a protective effect of the imidazoline on β-cell death induced by the pro-inflammatory cytokines. However, despite RX871024 did not affect basal or cytokine-induced *in vitro* kinase activity towards Hsp25 it diminished cytokine-induced phosphorylation of p38. Thus MK2-mediated phosphorylation of Hsp25 may not exclusively depend on p38 in *ob/ob* mouse islets. RX871024-induced decrease in p38 MAPK phosphorylation in the presence of cytokines may explain the partial inhibitory effect of RX871024 on cytokine-induced NO production, as p38 may participate in iNOS expression (152).

Thus, our observations indicate that pancreatic β-cell death triggered by a mixture of pro-inflammatory cytokines IL-1β, IFNγ and TNFα, conditions resembling those that take place in type 1 diabetes, does not directly correlate with NO production and rather relies on other players like JNK which cannot be counteracted with agents such as imidazoline compounds.

4.2 RX871024-INDUCES DEATH OF HIGHLY PROLIFERATING INSULIN-SECRETING CELLS BY ELEVATION IN JNK ACTIVITY (PAPER II)

Malignant insulinoma is an uncommon tumour. However, it has a poor prognosis and involves hyperinsulinemia and consequently hypoglycemia leading to neuroglycopenia and catecholamine response (351). Chemotherapy to this

tumour is not very effective (352, 353). Therefore, search for effective and specific chemotherapeutical drugs for patients with malignant insulinomas is of utmost importance. Activation of JNK and caspase 3 was reported to cause antitumorigenic effect on insulinoma cells (354).

We have previously shown that the imidazoline compound RX871024 does not evoke apoptosis in primary pancreatic β-cells (Paper I) and even protects primary β-cells against IL-1β-induced apoptosis (19). Although, the protective effect of RX871024 on primary β-cell apoptosis disappeared when a combination of pro-inflammatory cytokines (i.e. IL-1β, TNFα and INFγ) was used, this imidazoline did not increase cytotoxicity under these conditions (Paper I). Therefore, the main question addressed in this study was whether imidazoline compounds RX871024 and efaroxan shown to be nontoxic towards primary β-cells, can specifically influence insulinoma cell death alone or in the presence of a combination of the pro-inflammatory cytokines IL-1β, IFNγ and TNFα, which are known inducers of JNK and caspase 3 activation in insulin-secreting cells, leading to β-cell death (1, 3). To address these issues, we have investigated the effects of RX871024 on cell death using the mouse pancreatic β-cell insulinoma MIN6. As was expected, pro-inflammatory cytokines induced MIN6 cell death. Unlike the effect of RX871024 on primary β-cells (19, 355), this imidazoline induced cell death in the absence of cytokines and substantially increased cytokine-induced MIN6 cell death. In contrast, efaroxan did not affect MIN6 cell death in either the absence or the presence of cytokines. These data indicate that RX871024 potentially can induce death of the insulinoma cells.

Taking into consideration that RX871024 protects against IL-1β-induced apoptosis in mouse β-cells by inhibiting NO production induced by the cytokine (19), we investigated the mechanisms of RX871024 cytotoxicity to the insulinoma cell line first by examining how the imidazoline affects MIN6 cell NO formation. However, in contrast to our data using primary β-cells (Paper I), neither RX871024 nor efaroxan affected NO production in the insulinoma cells regardless of the presence of cytokines. Therefore the cytotoxic effect of RX871024 on MIN6 cells cannot be explained by changes in NO production.

Inhibition of cytokine-induced NO production triggered by RX871024 in primary β-cells was correlated with decrease in cytokine-induced p38 MAPK phosphorylation. p38 participates in regulation of iNOS expression and NO formation (152). We therefore investigated if RX871024 or efaroxan affects cytokine-induced signal transduction at the level of MAPKs in highly proliferating insulin-secreting cells. In contrast to primary mouse β-cells (Paper I), there was no effect of cytokines or imidazolines on p38 activation in MIN6 cells. The lack of cytokine-induced p38 activation has been observed with another mouse insulinoma cell line, namely βTC3 cells. Hence, it is likely that p38 is less sensitive to activation by cytokines in highly proliferating insulin-producing cells than in primary β-cells. Moreover, insensitivity of p38 to imidazolines can explain inefficiency of the substances on cytokine-induced NO production in the insulinoma cells. In line with our finding in primary β-cells (Paper I) cytokines significantly increased JNK activity in MIN6 cells. However, unlike our previous results in primary β-cells (Paper I), RX871024 alone caused a modest, but significant increase in JNK activation in the insulinoma cells, whereas efaroxan inhibited basal JNK activity. In combination with cytokines, RX871024 but not efaroxan, exerted potentiating effect on cytokine-stimulated JNK enzymatic activity. The stimulating effect of RX871024 on JNK activity was in good correlation with the effect of the imidazoline on MIN6 cell death. These data lead to the conclusion that RX871024 is specifically toxic for the insulinoma cells by inducing JNK activation, as the substance was without effect on either basal or cytokine-induced JNK activation in primary β-cells, as well as it did not induce primary β-cell apoptosis (Paper I). Surprisingly, cytokines significantly suppressed the enzymatic activity of ERK (Paper II, Fig. 2A) in MIN6 cells. The suppression could be explained by the presence of IFNγ in the cytokine mixture.

As was discussed above caspases play an important role in cell death. To understand their role in the imidazoline effects on the insulinoma cell death, caspase activation in MIN6 cells was evaluated. In accordance with the stimulatory effect on MIN6 cell death, RX871024 alone as well as cytokines

induced caspase-3 activation. RX871024 also further increased cytokine-induced caspase-3 activation, being added in combination with pro-inflammatory cytokines to MIN6 cells. On the contrary, efaroxan did not affect either basal or cytokine-induced caspase-3 activation in MIN6 cells. As RX871024-triggered caspase-3 activation was even stronger than that stimulated by cytokines (known inducer of apoptosis in insulin-producing cells), we suggest that the imidazoline induces apoptosis. Nevertheless, the presence of primary or secondary necrosis cannot be excluded because the elevation of LDH activity into culture medium was also observed.

It is well known that effector caspase-3 is activated by initiator caspases (1, 27). The combination of TNFα and IFNγ activates caspase-8 in β-cell lines (28, 29). Activation of JNK by pro-inflammatory cytokines may trigger the intrinsic apoptotic pathway, leading to release of cytochrome *c* from mitochondria (356) and thereby to caspase 9 activation. Elevation of NO production and JNK activation can potentially lead to caspase-1 activation (236, 255). Therefore, we next investigated activation of initiator caspases-1, -8 and -9 in the insulinoma cell death. Pro-inflammatory cytokines activated caspases-1, -8 and especially -9, which was associated with JNK activation. The data indicate that activation of the intrinsic (mitochondrial) pathway induced by JNK and culminating in strong caspase-9 activation is an important player in MIN6 cell destruction. Nevertheless, activation of caspase-1, which may as well rely on JNK activation (236), can also be significant for progression of inflammation and β-cell destruction, as the caspase participates in IL-1β production and induction of pyroptosis, a form of cell death with the features of both necrosis and apoptosis (324, 325). Accordingly, the pattern of elevation of LDH activity into culture medium well correlates with the one of caspase-1 activation. Despite clear absence of elevation in NO production, RX871024 alone triggered caspase-1 activation and to a larger extent activation of caspases-8 and -9 in MIN6 cells. The imidazoline also further elevated cytokine-induced activation of caspase-1, -8 and -9 in the insulinoma cells. The activation of initiator caspases by RX871024 is in good agreement with the induction of effector caspase-3 under the same conditions and with JNK activation triggered by the imidazoline.

Therefore we anticipated that JNK can be pointed out as a trigger of caspase activation in the insulinoma cells. Efaroxan did not influence activation of initiator caspases in MIN6 cells regardless the absence or presence of cytokines. The results suggest that RX871024 induces death of the insulinoma cells through activation of initiator caspases-1, -8 and -9 followed by effector caspase-3.

Interestingly, in contrast to primary β-cells where RX871024 was without any effect, the imidazoline compound selectively destructs highly proliferating insulin-secreting cells and potentiates cytokine-induced cell death in insulinoma, which may have important clinical implications.

4.3 SOCS-1 INHIBITS CASPASE ACTIVATION AND PROTECTS FROM CYTOKINE-INDUCED NO-INDEPENDENT β-CELL DEATH (PAPER III)

It has been shown that expression of SOCS-1, an endogenous inhibitor of IFNγ-induced signalling, in pancreatic β-cells protects NOD mice against diabetes (4). Moreover, the absence of SOCS-1 hypersensitizes β-cells to TNFα-induced NO production and death (50). Nevertheless the issue how signaling via the JAK/STAT pathway controls β-cell dysfunction and death induced by IL-1β or TNFα is not fully understood. To address this question we have studied whether islet cell dysfunction and death induced by a mixture of pro-inflammatory cytokines (IL-1β, IFNγ and TNFα) is affected by β-cell expression of SOCS-1. One of the deleterious effects of pro-inflammatory cytokines on islet cell function is the decrease in glucose-induced insulin secretion, which is dependent upon the IFNγ-driven signal-transduction pathway (357, 358). Taken this into consideration, we first examined whether β-cell expression of SOCS-1 affects cytokine-induced reduction of glucose-stimulated insulin release. SOCS-1 overexpression in β-cells did not influence glucose-induced insulin release in the absence of cytokines. In line with previous findings (9), B6 islets incubated with cytokine combination 1 showed inhibited glucose-induced insulin secretion. Similarly, cytokine combination 1 impaired glucose response in islets from SOCS-1-Tg B6 mice, suggesting that overexpression of SOCS-1 in β-cells does not prevent impairment of glucose-stimulated insulin release induced by pro-

inflammatory cytokines. In murine islets reduction of insulin secretion in the presence of cytokines was attributed to an increased NO production (7, 9, 358). Therefore the effect of SOCS-1 overexpression on cytokine-induced NO formation was studied. The obtained results show that cytokine combination 1 drastically elevated NO formation in B6 mouse islet cells, while SOCS-1 did not protect from cytokine-induced increase in NO production.

It has been established that IFNγ substantially elevates IL-1β-induced NO formation in pancreatic islets (7). The fact that there is no difference in NO production in B6 and SOCS-1-Tg B6 mouse islet cells treated with cytokine combination 1 raised the question whether IFNγ-triggered signalling pathway(s) leading to increase in NO formation are active in B6 and SOCS-1-Tg B6 mouse islet cells under our experimental conditions. So, we investigated whether NO formation can be elevated by the mixture of IL-1β and TNFα and whether IFNγ have any effect on NO production induced by the mixture of IL-1β and TNFα in B6 mice and SOCS-1-Tg B6 mouse islet cells. We found that in the absence of IFNγ the mixture of IL-1β and TNFα, at concentrations used, did not elevate NO production in either B6 mouse islet cells or in SOCS-1-Tg B6 mouse islet cells. On the other hand, an addition of IFNγ to the mixture of IL-1β and TNFα brought about a strong elevation in NO production in B6 as well as in SOCS-1-Tg B6 mouse islet cells. These data suggest that, IFNγ stimulates signal-transduction pathway(s) leading to a rise in NO formation both in B6 and in SOCS-1-Tg B6 mouse islet cells.

Our results showing that overexpression of SOCS-1 does not influence NO production indicates that the pathways which SOCS-1 interfere with are not important for cytokine-induced NO formation. This observation was unexpected, as IFNγ stimulated IL-1β-induced islet cell NO production ((7) and Paper III, Fig. 3). We therefore suggest that SOCS-1 does not affect the signal-transduction triggered by IL-1β and the pathway(s) activated by IFNγ which increase IL-1β-driven NO formation. Similarly, the signalling pathways that are activated by the mixture of IL-1β, TNFα and IFNγ leading to the reduction in glucose-stimulated insulin release, may not be affected by SOCS-1. The results of our investigation

demonstrate that deterioration of glucose-induced insulin release by cytokine combination 1 is paralleled by an elevation in NO formation. Taking this as well as previously published data on dependence of glucose-stimulated insulin release upon NO in murine islets (7, 9, 358) into consideration, we surmise that the reduction in glucose-stimulated insulin release stimulated by cytokines is a consequence of elevated NO formation induced by IFNγ-activated signal-transduction pathway(s) other than the JAK/STAT pathway.

SOCS-1 may have a protective effect against β-cell death, as NOD mice harbouring β-cells expressing SOCS-1 have significantly decreased incidence of diabetes (4). Cytokines, as discussed above, are potent inducers of β-cell death. Therefore we investigated whether SOCS-1 expression protects islet cells against death induced by cytokine combination 1. The data obtained demonstrate that overexpression of SOCS-1 does not influence the level of islet cell death in the absence of cytokines. As expected, cytokine combination 1 distinctly elevated apoptotic cell death in B6 islets. However, in SOCS-1-Tg B6 islets the level of cytokine-induced apoptosis was substantially diminished. These findings indicate that β-cells expressing SOCS-1 are markedly protected against cytokine-induced cell death, which is not attributed to changes in NO formation. In fact, NO formation is increased both in the SOCS-1-Tg B6 and in B6 mouse islet cells treated with cytokine combination 1 to the same extent. Cell death under these conditions is however less in the SOCS-1-Tg B6 islets compared to the B6 islets.

In SOCS-1-Tg B6 mouse islet cells NO production induced by the cytokine combination containing IFNγ is substantially increased compared to that induced by the mixture of IL-1β and TNFα. Taking this into consideration, we next investigated whether the presence of IFNγ in the cytokine combination influences also the amount of cell death in SOCS-1-Tg B6 islets. In accordance with the absence of NO formation, the mixture of two cytokines (IL-1β and TNFα) did not elevate cell death in SOCS-1-Tg B6 islets. While the addition of IFNγ to the mixture increased SOCS-1-Tg B6 islet cell death to the level which

is approximately half of that in B6 islets under the identical conditions, despite the same amount of NO production as in B6 islets.

As was pointed out above, activation of caspase-3 may be a decisive event in the induction of apoptosis in β-cells. Previous studies by us and others demonstrate that caspase-3 activation is induced by cytokines in pancreatic β-cells (25, 359), and that activation of this effector caspase is indispensable for the induction of β-cell apoptosis (25, 29). SOCS-1 deficiency elevates caspase activation triggered by TNFα in pancreatic β-cells (50). To further promote our understanding of the mechanisms involved in the pro-survival effect of SOCS-1, we then examined caspase-3 activation induced by cytokine combination 1 in islet cells from B6 and SOCS-1-Tg B6 mice. In line with the observed increase in B6 mouse islet cell apoptosis, cytokines elevated caspase-3 activation in these cells. On the contrary, in SOCS-1 overexpressing islet cells caspase-3 was not activated significantly by cytokine combination 1. The data obtained imply that the protective effect of SOCS-1 overexpression in β-cells at least partly depends on the inhibition of cytokine-induced caspase-3 activation.

The effector caspase-3 is activated by initiator caspases (27), including caspase-8 and caspase-9, which activation was previously demonstrated to be cytokine-induced in β-cell lines or primary β-cells (29, 30) (Paper II). To evaluate which of the initiator caspases can be inhibited by SOCS-1, we investigated the effects of SOCS-1 overexpression on caspase-8 and caspase-9 activation in response to treatment with cytokine combination 1. However, we failed to find any caspase-8 activation in primary B6 islet cells incubated with cytokines. The absence of caspase-8 activation by pro-inflammatory cytokines in primary β-cell is in contrast to our finding in insulinoma cells (Paper II). The fact that caspase-8, known to be activated after ligation of the TNF receptor (360), is not induced by a mixture of IL-1β, TNFα and IFNγ in primary mouse islet cells supports our suggestion that cytokine-induced primary β-cell death and death in β-cell lines may have a different mechanistic explanation. On the other hand, cytokine combination 1 induced caspase-9 activation in islet cells from B6 mice. Thus, the intrinsic mitochondrial apoptotic pathway resulting in caspase-9 activation,

followed by an enhanced caspase-3 activity, probably plays an important role in the execution of primary islet cell death induced by pro-inflammatory cytokines. In agreement with the absence of caspase-3 activation in SOCS-1-Tg B6 islet cells, neither caspase-8 nor caspase-9 was activated in response to cytokine stimulation. On the contrary, caspase-8 activity was decreased in SOCS-1 overexpressing β-cells after treatment with cytokine combination 1. These results suggest that SOCS-1 may interfere with both extrinsic and intrinsic apoptotic pathways in primary β-cells. SOCS-1 interferes with the extrinsic pathway activating caspase-8 possibly by blocking interaction between STAT-1 and TNF receptor (29, 50, 100) or by suppression of STAT-1-dependent IFNγ-induced elevation of caspase-8 expression (101). The former pathway initiated by the TNF receptor can stimulate both caspase-8 and JNK (360). Previous studies have demonstrated that SOCS-1 prevents TNFα-induced apoptosis partly by interfering with TNFα-induced activation of JNK (129-131). Therefore it is likely that SOCS-1, by interfering with the TNFα pathway, hinders the TNFα-induced potentiation of IL-1β-triggered JNK activation and in this way decreases caspase-9 activation. If that is the case, then it is possible that SOCS-1 suppresses the activation of both tested initiator caspases by its interference with the pathway(s) by which IFNγ controls the TNFα signaling.

In conclusion the results of this study suggest that IFNγ induces signal-transduction in B6 mouse islet cells and that SOCS-1 overexpression in β-cells does not defend pancreatic islet cells against reduction of glucose-stimulated insulin release or hinder islet cell NO formation stimulated by a combination of IL-1β, TNFα and IFNγ. Thus, protection from type 1 diabetes in NOD mice provided by SOCS-1 overexpression in β-cells may at least partly be accounted for by a decreased potential of pro-inflammatory cytokines to trigger caspase activation and as a consequence islet cell death. Consequently, it renders a robust protection against islet cell death induced by a mixture of IL-1β, TNFα and IFNγ. This effect is achieved by a suppression of the pathways resulting in caspase-9 and subsequently caspase-3 activation. However, a suppression of solely the JAK/STAT signalling pathway may not be enough to block all the

negative biological effects of IFNγ in β-cells. Nevertheless, the decreased potential of pro-inflammatory cytokines to trigger cell death in SOCS-1-Tg islets suggests that blockage of IFNγ-triggered signalling pathway(s) is promising for treatment of type 1 diabetes. It is also opens for possibilities of transplanting pancreatic islets with induced expression of SOCS-1 to type 1 diabetes patients.

4.4 CONCLUSIONS AND GENERAL REMARKS

β-cell death in the course of type 1 diabetes may be induced by pro-inflammatory cytokines IL-1β, TNFα and IFNγ and is executed by a network of cooperating and possibly substituting pathways which are not fully understood. The present work furthers our understanding of the mechanisms underlying cytokine-induced β-cell death.

Pro-inflammatory cytokines can induce both NO-dependent and NO-independent β-cell death. The death of *ob/ob* mouse β-cells induced by a mixture of cytokines (i.e. IL-1β, TNFα and IFNγ) is less NO-dependent than that induced by IL-1β alone and may serve as a model to study human NO-independent β-cell death. NO-independent cell death can rely on caspase activation. Our data demonstrate that pro-inflammatory cytokines IL-1β, TNFα and IFNγ activate caspase-1 in insulin-producing cells in the absence of viral or bacterial infection thereby having a potential to exacerbate inflammation inducing further production of IL-1β and elevating cell death by pyroptosis. The activation of caspase-1 presumably relies upon JNK induction. The cytokines also induce activation of caspase-8 and to a greater extent caspase-9 in highly proliferating insulin-secreting cells. However, the pro-inflammatory cytokines failed to activate extrinsic apoptotic pathway in primary islet cells. Instead, our data suggest that JNK-mediated induction of the intrinsic (mitochondrial) pathway, culminating in caspase-9 activation, is an important player in β-cell destruction. Thus activation of JNK is essential for cytokine-induced β-cell destruction.

RX871024 diminishes cytokine-induced NO production in primary β-cells by inhibiting p38 activation elevated by pro-inflammatory cytokines. However, in highly proliferating insulin-secreting cells p38 is less susceptible to activation by cytokines or inhibition by imidazolines. The insensitivity of p38 to the influence of RX871024 results in the absence of protective effects of the imidazoline on cytokine-induced NO production in highly proliferating insulin-secreting cells. Instead, contrary to primary β-cells where RX871024 does not affect JNK activation and cytokine-induced cell death, the imidazoline elevates basal as well as cytokine-induced activity of JNK and thereby death of highly proliferating insulin-producing cells. This is associated with stimulation of initiator caspases-1, -8 and -9 and consequently effector caspase-3, indicating that both the extrinsic and the intrinsic apoptotic pathways are involved in RX871024-dependent death of insulinoma cells. Thus, RX871024 may represent a specific antitumorigenic substance against insulinomas.

On the whole imidazoline RX871024 cannot counteract NO-independent β-cell apoptosis which, however, can be prevented by SOCS-1 overexpression.

SOCS-1 diminishes activation of both caspase-8 and -9 in primary β-cells possibly by interfering with TNFα-driven signal-transduction and the pathway(s) used by IFNγ to regulate TNFα signalling. Suppression of these signalling pathways with SOCS-1 diminishes cytokine-induced β-cell death. This finding in association with the notice that SOCS-1 does not affect glucose stimulated insulin release and islet cell death in the absence of cytokines indicates the possibility to use an elevation of SOCS-1 expression in the treatment of type 1 diabetes. Nevertheless, to prevent all the biological activities of IFNγ in β-cells it is not sufficient to suppress the JAK/STAT pathway alone. In particular, suppression of the JAK/STAT pathway with SOCS-1 does not protect pancreatic islet cells against IFNγ-dependent potentiation in NO production and impairment in glucose-stimulated insulin secretion. The elevation in NO formation driven by an IFNγ-dependent but JAK/STAT-independent pathway can actually lead to reduction in glucose-stimulated insulin secretion.

In summary, this thesis supports the notion that pro-inflammatory cytokines IL-1β, TNFα and IFNγ can induce NO-dependent and NO-independent β-cell death. The NO-independent β-cell death may rely on JNK-mediated caspase activation and cannot be counteracted by such substances like RX871024, but can be prevented by SOCS-1.

5 ACKNOWLEDGEMENTS

This work has been performed at the Rolf Luft Center for Diabetes Research and Endocrinology, Department of Molecular Medicine and Surgery, Karolinska Institute. I would like to express my gratitude to all colleagues at the Rolf Luft Center and at the Department. In particular I would like to thank:

Per-Olof Berggren, my supervisor, for giving me the possibility to perform my thesis work at Karolinska Institutet, for financial support and for all help with revisions of manuscripts and my thesis.

Rolf Luft for the creation of the Rolf Luft Center.

Kerstin Brismar for valuable advises and interesting discussions in the field of diabetes.

Suad Efendic for support of the imidazoline project.

Katarina Breitholtz for enormous help with organisation of the thesis defence and administrative assistance.

Monica Isaksson Strand, Yvonne Strömberg, Annika Lindgren and Hanne-Lore Rotter for help with getting primary material and for keeping the lab running.

Ann-Britt Wikström, Therese Kindåker, Christina Bremer, Kerstin Florell, Britt-Marie Witasp, Elinor Petersson, Karolina Hettinger and Jan-Erik Kaare - a lot of thanks for administrative help and assistance.

Martin Köhler for successful solving of my various technical problems especially with confocal microscopy and my personal lock.

Ioulia Appelskog and Iouri Kapelioukh for sharing your molecular biology experience with me.

Tilo Moede for introducing me to the field of confocal microscopy.

Andrea Dicker, Erwin Ilegems, Lars Selander, Midhat Abdulreda, Stefan Jacob and Tomas Alanentalo for introducing me into new fields, teaching me advanced techniques and warm collaboration.

All my co-authers from other labs: **Malin Flodström-Tullberg, Monica Hultcrantz, Thomas Mandrup-Poulsen, Joachim Störling, Pierluigi Nicotera, Christopher Guerin, Vladimir Sharoyko** - for participating in our research.

Barbara Leibiger for providing me with MIN6 cells and excellent order in cell culture room.

Lisa Juntti-Berggren for discussions about medical side of our research.

Stella Jacobson for assistance with the genotyping of the mice.

All present and former colleagues: **Jia Yu, Shao-Nian Yang, Yue Shi, Lina Yu, Luo-Sheng Li, Ingo Leibiger, Rayner Rodrigues-Diaz, Essam Refai, Karin Åvall, Subu Rajasekaran, Meike Paschen, Elisabetta Dare, Jaeyoon Kim, Yan Xiong, Pim van Krieken, Teresa Pereira, Galyna Bryzgalova, Neil Portwood, Chris Barker, Rebecka Holmberg, Nancy Wenna, Jenny Johansson, Slavena Mandic, Sabine Uhles, Daniel Nyqvist, Roberta Fiume.**

I wish to express my gratitude to my **father** for everything and especially for his conviction that the thesis work should be finished, to my beloved **husband** for introducing me into the field of cytokine-induced β-cell death, for all support, for many useful discussions and creative suggestions and to my **children** for showing me my own negative features and by this providing the need of being better.

This work was funded by the Juvenile Diabetes Foundation International grant N5-2001-34 (SVZ), the Swedish Foundation for Strategic Research, the European Foundation for the Study of Diabetes, EuroDia (FP6-518153), the Swedish Research Council, the Novo Nordisk Foundation, the Swedish Diabetes Association, the Berth von Kantzow's Foundation, the Knut and Alice

Wallenberg Foundation, Skandia Insurance Company Ltd, the Family Erling-Persson Foundation, Stichting af Jochnick Foundation, ERC 338936-Betalmage, Diabetes Wellness Foundation, Strategic Research Program in Diabetes at the Karolinska Institutet, STINT Korea-Sweden Research Cooperation, Wenner-Gren Foundations, Karolinska Institutet, Novo Nordisk A/S, SynSignal 613879, Beta Train 289932 and Immedia 247472.

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